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Gas-chromatographic determination of methylthiohydantoin amino acid as N(O)-butyldimethylsilyl derivatives in amino acid sequencing with methylisothiocyanate

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Abstract : For effective determination of methylthiohydantoin amino acids(MTHs) by gas liquid chromatography in the protein sequencing, derivatization with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide(MTBSTFA), a new silylating reagents, was attempted instead of trimethylsilyl(TMSi) derivatives by N,O-*bis*(trimethylsilyl)trifluoroacetamide(BSTFA) used up to the present and N(O)-butyldimethylsilyl MTHs derivatized by MTBSTFA were analysed on HP-1 capillary column. Twenty one protein amino acids except cystine were indentified. Especially arginine that did not detected with TMSi derivative on packed column until now was resolved by derivatization with MTBSTFA. N(O)-butyldimethylsilyl MTHs showed multiple peaks by MTBSTFA were proline, isoleucine, glycine and tyrosine and hydroxyproline especially showed several extraneous peaks more than two. Calibration curves of N(O)-butyldimethysilyl MTHs of amino acids in the range of 2.5 nmol~7.5 nmole showed good linearity, however, those of lysine, histidine and arginine showed linearity in the range of 5.0 nmole~15.0 nmole. Correlation coefficients and regression coefficients of all calibration curves were highly significant($p<0.001$)(Received February 25, 1992, accepted April 17, 1992).

Edman degradation of proteins or peptides with phenylisothiocyanate(PITC)[1] or methylisothicyanate[2,3](MITC) has become a routine method for determination of amino acid sequence, although a highly absorptive chromophoric reagent for protein sequene analysis, 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate(DABITC) was proposed by Chang *et al.*[4]

Unfortunately, a number of amino acids derivatized by PITC or MITC in each cycle of Edman degradation are not cleaved.[3] So, after a few cycles phenylthiohydration or methylthiohydantoin amino acids(PTHs or MTHs) that should have been determined in prior cycle are found in the reaction products at the next cycle.

Quantitative determination and reference to all prior cycles in the sequence as well as qualitative identification are important key factor in determining the resolving cycles of the releasing PTH or MTH derivatives.[5] Qualitative identification of PTHs or MTHs by thin-layer chromatography was often complicated by background and did not provide the security of quantitative determination.[3]

Several problems still remain to be solved, however, gas liquid chromatography(GLC) has been used as a simple routine method for qualitative identification and quantitative determination of thiohydantoins because of its high sensitivity and usefulness for quantifying of amino acid derivatives compared with other techniques.[6,7] Complete resolutions of all PTHs or MTHs have not been achieved with a single stationary phase and single co-

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lumn system in the packed column.[8]

MTHs have two advantages over the PTHs. First, the preparation of MTHs by Edman degradation is easy and rapid. Second, because of higher volatility compared with PTHs, they have more desirable features for GLC analysis.[9] But all MTHs can not be directly separated by GLC without adequate derivatization.

GLC analysis of PTHs and MTHs have been carried out with only trimethylsilyl(TMSi) derivatives and generally packed column.[8−10] But TMSi MTH of arginine has not been detected because it was nonvolatile.[6,8,10,11] TMSi MTH of serine was unstable[7] and cysteine and arginine were analysed as S-methylcysteine and ornithine, respectively.[6] Separation of TMSi MTH of histidine still has some problems.

Because of incomplete derivatization, degradation and byproducts from coupling mixture during Edman degradation and derivatization for the GLC analysis, the separation of derivatized thiohydantonis on the packed column is less reliable compared with capillary column. Futhermore, the analysis on the capillary column is more usefull in the combination with mass spectrometry. GLC analysis of PTHs on the fused silica capillary column was reported,[7] but MTHs analysis on the capillary column has not been attempted till now. Inspite of several adventages of GLC analysis on the capillary column, i.e., sensitivity, versatility and combination with mass spectrometry, in recently analyses by high performance liquid chromatography have mainly been studied.[12−14]

N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide(MTBSTFA) is a new silylating reagent which is expected to be successful in converting all protein amino acids into suitable derivatives for GLC.[15] But derivatization of MTH amino acids by MTBSTFA for GLC analysis has not been attempted.

In this study, the quantitative and qualitative analysis by GLC of N(O)-butyldimethylsilyl MTHs derivatized by MTBSTFA on the capillary column are examined and compared with TMSi derivatives.

Materials and Methods

Materials

Methylisothiocyanate, purchased from the Aldrich Chemical Co.(Milwaukee, WI, U.S.A.), was distilled (b.p. 116-119 ℃) under nitrogen gas, and stored in the small screw capped vials(1 m*l*) at −40 ℃ under nitrogen gas. Once opened this reagent was not used for no more than one week. N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide(MTBSTFA) and N,O-bis(trimethylsilyl)trifluoroacetamide(BSTFA) were purchased from the Aldrich. All other solvents except MTBSTFA and BSTFA were distilled before use. The MTHs of alanine, valine, leucine, isoleucine, glycine, phenylalanine, methionine, tyrosine, aspartic acid, glutamic acid, asparagine, glutamine, proline, tryptophan, lysine(ε-methylthiocarbamyl), histidine, arginine, and hydroxyproline used as standard were obtained from Sigma Chemical Co.(St Louis, MO, U.S.A.) or synthesized with the MTHs of cysteine, serine and threonine by Edman method[1] with modification as following.

Synthesis of methylthiohydantoins

The reaction was carried out in a vial of 5 m*l* with rubber septum stopper which was immersed in a water bath at 40 ℃. Ten milligrams of DL-amino acid from Sigma was dissolved in 2 m*l* of pyridine-water(1 : 1) containing 3%(w/v) bromothymol blue. By addition of triethylamine the color of mixture was adjusted to the standard color solution of pH 9.0. Two injection needles of stainless steel were pierced into the vial through the septum. One needle connected with nitrogen gas was put into the solution and the other was connected to the vaccum pump. Nitrogen gas was bubbled into solution for 1 min and simultaneously evacuated with vaccum pump, and then 50 μ*l* methylisothiocyanate was injected into the solution through the septum with microinjection syringe. Nitrogen gas was continually bubbled into the reaction mixture through the reaction and the vial was immersed in a 40 ℃ water bath. The pH was maintained at 9.0 by addition of triethylamine from microsyringe.

The completion of reaction was identified by the unchange of color in the reacting solution. After the reaction excess pyridine and methylisothiocyanate were removed by extraction of several times with benzene. The aqueous solution of the methylthiocarbamyl(MTC) intermediate was completely dried by a lypophilizing process. Then 2 m*l* of 1 N HCl was added, the vial was closed with a teflon-lined screw cap and the MTC intermediate converted to the MTH derivative by heating for 10 min at 80 ℃. All MTH derivatives except MTH of histidine and MTH of arginine were obtained from 1 N HCl repeated extraction of several times with ethyl acetate. Extraction solution was dried with nitrogen gas at 80 ℃ and then dissolved to known volumn of ethyl acetate. MTHs solutions of cysteine, serine and threonine were used as standard solution, and the others were used as comparision with the MTHs standard from Sigma.

Each standard solutions(1 mg/m*l*) of the methylthiohydantoins from Sigma were made up in ethyl acetate with following exceptions; MTH of glutamine was dissolved in 1 : 1 pyridine/ethyl acetate; MTH of aspartic acid in 2 : 1 pyridine/ethyl acetate; MTH of glutamic acid in 1 : 2 pyridine/ethyl acetate; MTH of histidine in pyridine. Standard solutions were stored under nitrogen in the desiccator at −60 ℃.

Silylation procedure

MTHs standard solution(15 μ*l*～50 μ*l*) was transferred to a 3 m*l* glass reaction vessel with a conical bottom and screw cap. The solution was completely dried with nitrogen gas at 80 ℃ oil bath. For the N(O)-*tert.*-butyldimethylsilyl derivatization, 25 μ*l* of internal standard solution(0.25 mg pyrene/m*l* of pyridine), 15 μ*l* of MTBSTFA and 2 μ*l* triethylamine were added and then tightly capped. The vessel was heated at 80 ℃ for 15 min. After cooling to room temperature, 1 μ*l* of derivatized solution was injected to GLC.

For TMS derivatization, 40 μ*l* of the silylation solution consisted of 1 : 3(v/v) of BSTFA and acetonitrile containing 0.5 mg pyrene per milliliter was added. The other procedures were the same as N(O)-*tert.*-butyldimethylsilyl derivatization procedure mentioned above.

Gas-liquid chromatogrphy

A Hitachi gas chromatography, model 163 with split mode capillary adaptor was used. The analysis on N(O)-butyldimethylsilyl MTHs and TMSi MTHs carried out with HP-1 fused silica capillary column (Hewlett Packard, cross linked methyl silicone gum, 25 m×0.32 mm×0.17 μm thickness) and with flame ionization detector.

Results and Discussion

GLC identification of N(O)-butyldimethylsilyl MTH amino acids and TMSi MTH amino acids

Fig. 1 showed the gas chromatogram obtained on the HP-1 capillary column of the N(O)-butyldimethylsilyl MTH derivatives of 20 amino acids except hydroxyproline. Hydroxyproline was excepted in the standard mixture because it was resolved more than four peaks. Alanine and glycine$_1$, leucine and isoleucine, methionine and proline$_2$, phenylalanine, threonine and glycine$_2$, asparagine and glutamic acid derivatives were incompletely resolved. N(O)-

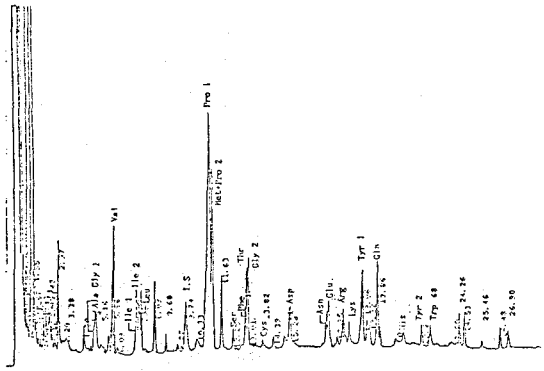

Fig. 1. Gas chromatogram of 20 N(O)-butyldimethylsilyl MTH amino acids on HP-1 capillary column. Injection amount : 2.5 nmole, exception Asp, Glu : 2.0 nmole, His, Arg, Lys, Tyr, Gly, Val, Ala : 5.0 nmole, Gln, Pro : 7.5 nmol. Carrier gas flow : H_2 5 m*l*/min. Temperature programming : 120 ℃～300 ℃. Split : 5 : 1, Make up gas : N_2 40 m*l*/min.

butyldimethylsilyl MTH amino acids showed only small numbers of impure peaks compared with TMSi MTH amino acids(Fig. 1, 3). MTH of arginine and histidine showed good resolution with N(O)-butyldimethylsilyl derivatives, but histidine was unstable after 2 weeks storage with standard solution (Fig. 4). Arginine showed single peak at injection amount of 5.0 nmole, but at higher level showed extraneous peaks considering with impurity of stadard(Fig. 5). N(O)-butyldimethylsilyl MTH amino acids showed the multiple peaks were hydroxyproline, isoleucine, proline, glycine and tyrosine(Fig. 2). Isoleucine, proline, glycine and tyrosine showed two peaks but hydroxyproline was resolved with several peaks even though it was stable.

Fig. 3 showed the gas chromatogram obtained on the HP-1 capillary column of the TMSi MTH derivatives of 18 amino acids. TMSi MTH derivatives showed many impure peaks so it couldn't be used in the quantitative determination. But TMSi MTHs were more volatile compared with N(O)-butyldimethyldimethylsilyl MTHs. TMSi MTHs of arginine, histidine and aspargine were not detected on the

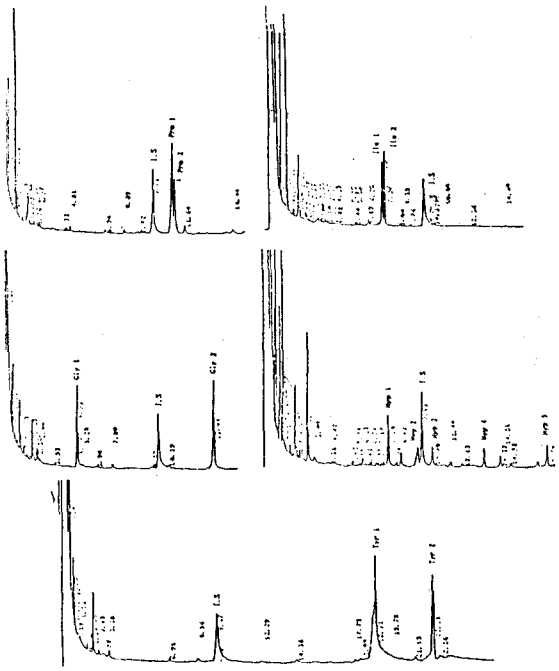

Fig. 2. Gas chromatograms of N(O)-butyldimethylsilyl MTH amino acids showed multiple peaks on HP-1 capillary column.
Injection amount : 5.0 nmole, Carrier gas flow : H_2 5 ml/min, Temperature programming : 120 ℃~300 ℃, 5 ℃/min. Split 5 : 1. Make up gas : N_2 40 ml/min.

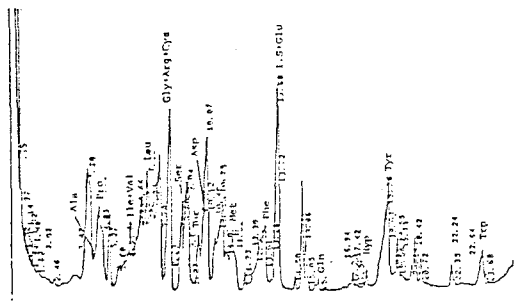

Fig. 3. Gas chromatogram of TMS-derivative of MTH amino acids on HP-1 capiliary column.
Injection amount : 2.5 nmole, Carrier gas flow : H_2 5 ml/min. Temperature programming : 100 ℃~245 ℃. 5 ℃/min. Split 5 : 1, Make up gas; N_2 40 ml/min.

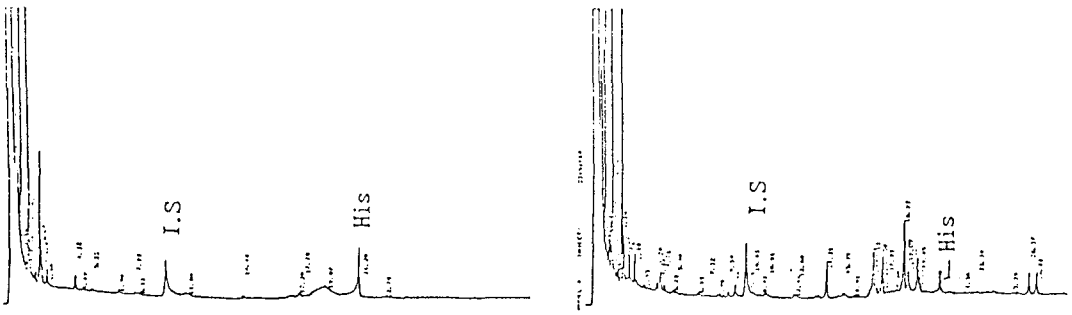

Fig. 4. Gas chromatograms of N(O)-butyldimethylsilyl MTH of His in 2 weeks(left) and after 2 weeks(right) storage with standard solution.
Injection amount : 5.0 nmole

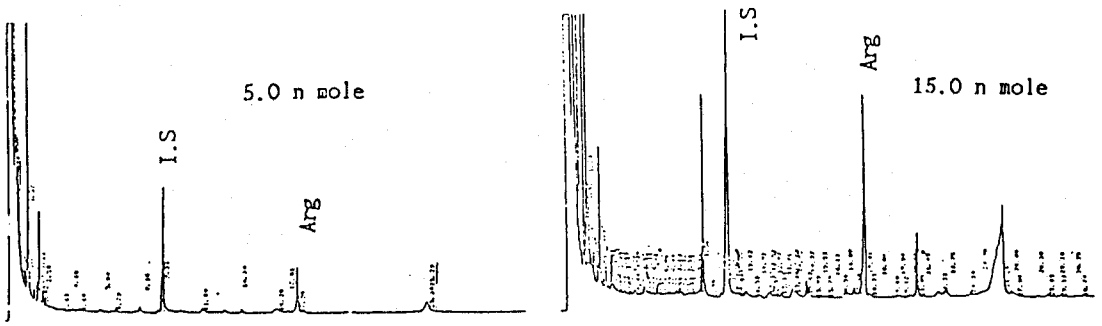

Fig. 5. Gas chromatograms of N(O)-butyldimethylsilyl MTH of Arg at injection amounts of 5.0 nmole and 15.0 nmole.

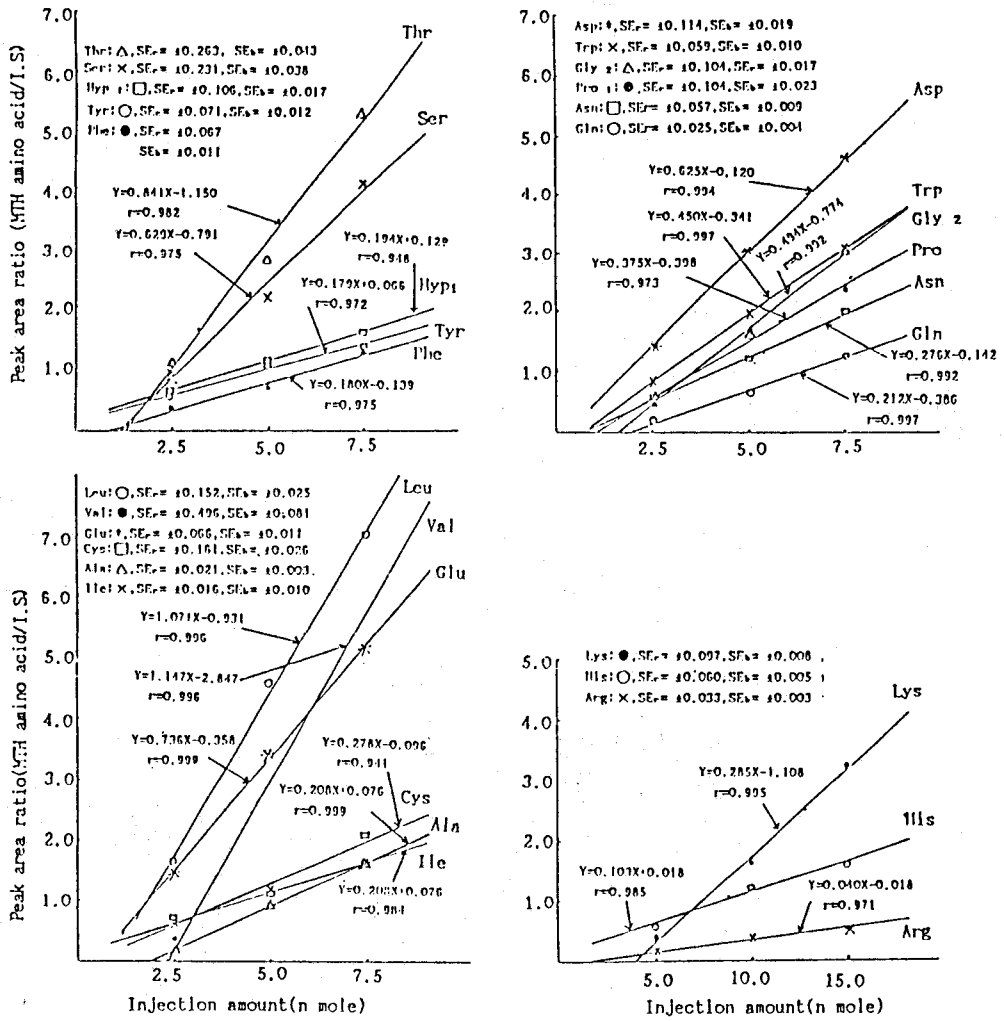

Fig. 6. Calibration curves for N(O)-butyldimethylsilyl MTH amino acids in the range 2.5 nmole~7.5 nmole and 5.0 nmole~15.0 nmole on HP-1 capillary column.
Internal standard : pyrene, 0.75 nmole, A spot is the average of three determinations. SE_r : Standard error of regression, SE_b : Standard error of slope.

HP-1 capillary column. Pisano and Bronzert[8] reported the blended phase packed column, DC-560, SP-400, OV-210 and OV-225. They separated all TMSi MTHs except arginine on the stationary phase, OV-225. Lamkin et al.[11] accomplished GC separation of TMSi MTHs of 19 amino acids except arginine, but separation of unresolved pair(asparagine and phenylalanine) needed an additional procedure. Using the flame photometric sulfur detector, some extraneous multiple peaks observed with the flame ionization detector were eliminated.

Eyem and Sjöqist[6] also reported the separation of 19 of 20 TMSi MTHs on the short(4.5 m) glass capillary column. They could be identified histidine on same column by starting the analysis at a higher temperature and cysteine and arginine were analysed as S-methylcysteine and ornithine, respectively.

In this study, arginine and cysteine were directly derivatized with MTBSTFA and detected. The only disadvantage was some extraneous multiple peaks of hydroxyproline. But TMSi MTH of hydroxyproline also showed many multiple peaks.

Fig. 6 showed the calibration curves for N(O)-butyldimethylsilyl MTH amino acids ranged from 2.5 nmol to 7.5 nmole and lysine, histidine and arginine is in the range of 5.0 nmole~15.0 nmole. Lysine, histidine and arginine were not detected at 2.5 nmole but detected at level more than 5.0 nmole. Calibration curves showed good linearity in the measured range but did not pass through the origin because probably at lower levels than measured amounts the detector couldn't respond. Correlation coefficients and regression coefficients of all calibration curves were highly significant(<0.001).

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Methylisothiocyanate를 이용한 아미노산 배열결정시 N(O)-butyldimethylsilyl 유도체로서의 methylthiohydantoin 아미노산의 기체 크로마토그래피에 의한 분석

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초록 : Methylisothiocyanate에 의한 단백질의 아미노산 배열 결정시 순차적으로 분리되어 나오는 methylthiohydantion 아미노산을 기체 크로마토그라피로 효과적으로 정성 및 정량하기 위하여 새로운 silylating reagent인 N-methyl-N-(*tert.*-butyldimethylsily)trifluoroacetamide를 사용하여 N-*tert.*-butyldimethylsily MTH 유도체로 silylation 한 후 HP-1 capillary column으로 분석하였다. Cystine을 제외한 21개의 단백질 구성 아미노산을 동정할 수 있었고 지금까지 packed column에서 TMS 유도체로 동정할 수 없었던 arginine도 분리 동정되었다. 2개 이상의 peak를 나타낸 것으로는 hydroxyproline, proline, isoleucine, glycine 및 tyrosine이었고 이중 hydroxyproline은 많은 수의 peak들로 분리되었다. Lysine, histidine 및 arginine은 주입량 5.0 nmole～15.0 nmole의 범위에서 나머지는 2.5 nmole～7.5 nmole의 범위에서 상관관계를 측정한 결과 고도의 직선 상관관계를 나타내었다($p<0.001$). TMS 유도체에 의한 분석은 많은 불순 peak들 때문에 정량분석에 이용할 수 없었다.