TRIMETHYLSILYLATION OF AMINO ACIDS
Effect of Solvents on Derivatization Using BSTFA
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INTRODUCTION

Since the introduction of the trimethylsilyl (TMS) derivatives of the amino acids by Rühlmann and Giesecke (1), efforts have been made by several groups of researchers in attempts to use these derivatives for the quantitative gas-liquid chromatographic analysis of the 20 protein amino acids. The primary interest in the TMS derivative is the one step derivatization procedure; whereas almost all other derivatives are formed by two or more reaction steps. Rühlmann and Giesecke used hexamethyldisilazane and trimethylchlorosilane to obtain derivatives for most of the protein amino acids. Smith et al. (2,3) made a study of the optimum silylation conditions for leucine, serine, and aspartic acid. They concluded that trimethylsilyldiethylamine with some kind of catalyst was the best silylation reagent. Trimethylsilyldimethylamine recently has been claimed to be

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Experimental data taken in part from master's research, University of Missouri
more volatile and was recommended for these reasons (4). N-tri-
methylsilyl N-methylacetamide has been recommended by Birkofer
and Donike (5). Klebe, Finkbeiner, and White (6) used Bis-(trimethyl-
silyl)acetamide (BSA) to obtain sharp single peaks for all the pro-
tein amino acids except arginine, which showed indications of decompo-
sition on the column. However, they were unable to separate the
derivatives of glycine and alanine from the Bis-(trimethylsilyl)
acetamide on an SE-30 column. The introduction of Bis-(trimethyl-
silyl)trifluoroacetamide (BSTFA) by Stalling, Gehrke, and Zumwalt
(7) has solved the problem of separation of the trimethylsilyl
derivatives of glycine and alanine from the reagents and reaction
products. Then, Gehrke, Nakamoto, and Zumwalt (8,9) published a
comprehensive method for the GLC analysis of all 20 protein amino
acids as their trimethylsilyl derivatives using BSTFA as the
silylating agent, but they reported that problems still existed in
the analysis of biological fluids such as urine.

Bergström, Gürtler and Blomstrand (10) recently reported on
the trimethylsilylation of amino acids. These workers used BSTFA
with and without solvent at 125°C for 15 minutes to obtain chroma-
tographic peaks for 18 amino acids. They also reported on the mass
spectra of the two derivatives of glycine and lysine. The results
of the mass spectra are in agreement with the structural assignment
given by Gehrke and coworkers (8,9).

Gehrke and coworkers report that urine samples, containing
large amounts of glycine presented difficulties in obtaining a single
peak for glycine. Both the di-trimethylsilyl (GLY₂) derivative and
the tri-trimethylsilyl (GLY₃, ca. 10%) derivatives were obtained
when the samples were derivatized at 135°C for both 10 and 15 min. Because of the large quantity of glycine in the urine samples, the GLY₃ peak interfered with the resolution of TMS isoleucine and TMS proline. In preliminary work on the TMS derivative of glycine by the present authors, it was observed that only the first peak for glycine (GLY₂) was obtained when using methylene chloride as a solvent instead of acetonitrile. This study was undertaken to investigate the effect of different solvents on silylation and reports on improvements in the chromatographic separation, thus permitting the analysis of biological fluids such as urine.

EXPERIMENTAL

Reagents and Materials

Acetonitrile, hexane, and chloroform were obtained from Mallinkrodt Chemical Works, St. Louis, Missouri, and were of "nanograde" purity. Methylene chloride was obtained from Mallinkrodt Chemical Works, St. Louis, Missouri, and was Analytical Reagent grade. Pyridine, 1,2-dichloroethane, dimethylformamide, tetrahydrofuran, and triethylamine were obtained from Distillation Products Industries, Rochester 3, New York. All solvents were dried over calcium chloride and redistilled before use.

The BSTFA was obtained from Regis Chemical Company, Chicago, Illinois.

The OV-7, OV-11, and OV-22 liquid phases and solid support, Supelcoport, 100/120 mesh, were obtained from Supelco, Inc., Bellefonte, Pennsylvania.
The amino acids were obtained from Mann Research Laboratories, New York, New York, and were "Mann Assayed."

**Equipment**

A Micro Tek Model MT-220 gas chromatograph with a four column oven bath, two dual channel electrometers and four flame ionization detectors were used in this study. A Varian Model 30 recorder was used for the chart presentation.

**Chromatographic Column**

The chromatography column was a mixed phase consisting of 3 w/w% OV-22 and 6 w/w% OV-7 on 100/120 mesh Supelcoport in a 2 m x 4 mm I.D. glass column. Also used was 10% OV-11 on Supelcoport in a glass column 6 m x 2 mm I.D.

**Derivatization Method**

Two ml of a stock solution containing 0.1 mg/ml of each amino acid was pipetted into a Corning No. 9826 1.6 cm x 7.5 cm reaction tube and dried under a stream of dry filtered nitrogen at 75°C. One (1.0) ml of methylene chloride was added and evaporated under nitrogen to azeotrope any remaining water. Then, one (1.0) ml of BSTFA and one (1.0) ml of acetonitrile were added to the tube. The sample tube was closed with a Teflon lined cap and heated at different temperatures and times in a constant temperature oil bath. For the solvent study, the only change made was to substitute the appropriate solvent for acetonitrile. Also, in some experiments, 1% trimethylchlorosilane in BSTFA was substituted for the BSTFA.
RESULTS

Table I presents a summary of the solvents used in this research and their results. No quantitation was attempted using these solvents. In these experiments, essentially the same results were obtained on a qualitative basis as reported by Gehrke et al. (7) as illustrated in Figures 1 and 2. Gehrke and coworkers reported two peaks for glycine and one peak for arginine using acetonitrile as solvent. In this study using methylene chloride, hexane, chloroform, and 1,2 dichloroethane, it was observed that glycine produced only one peak, as illustrated in Figures 3 and 4; whereas two peaks were formed in acetonitrile, dimethylformamide, pyridine, triethylamine, and tetrahydrofuran. The number of peaks for glycine derivatized in those non-polar solvents was not affected by the time or temperature as times from 15 minutes to 6 hours, and temperatures from 50°C to 150°C were used. Also, arginine under the same experimental conditions is either not derivatized, or decomposed, as no peak was obtained. In these non-polar solvents, one notes two peaks for glutamic acid, mostly LYS₃ instead of LYS₄, no peak for arginine, and nearly all GLY₂.

Chambaz et al. (11,12,13) has recently reported that the use of 1% trimethylchlorosilane has a catalytic effect on the silylation of steroids using Bis-(trimethylsilyl)acetamide. Regis Chemical Company (14) has suggested the use of 1% trimethylchlorosilane with BSTFA. Figures 5 and 6 show the effect of the use of 1% trimethylchlorosilane with BSTFA in methylene chloride. The use of 1% trimethylchlorosilane as a catalyst results in a more powerful silylating agent as there is a larger amount of GLY₃, LYS₄, and an arginine peak on silylation at 135°C for four hours (Figure 6). As shown in Figure 5, silylation under these conditions for 15 minutes is incomplete. No differences were noted with
1% trimethylchlorosilane as catalyst in a polar solvent, acetonitrile, from the use of BSTFA in acetonitrile.

It appears that the derivatization of glycine, arginine, lysine, and glutamic acid involves the polarity of the solvent, with polar solvents giving two peaks for glycine and one peak for arginine. In non-polar solvents only the first chromatographic peak for glycine and no peaks for arginine were obtained. The explanation for not obtaining a peak for arginine is probably due to solubility of arginine in the solvent, or a difference in the silylating strength of BSTFA in various polarity solvents.

There is a need to expand the earlier work of Gehrke and coworkers especially in the area of chromatographic resolution. The results of preliminary studies are given in Figure 7. This chromatogram shows excellent resolution for 19 amino acids. Further work is in progress to investigate the chromatography on this column, a 6 mm x 2 mm I.D. 10 w/w% OV-11 on 100/120 mesh Supelcoport.

In the analysis of biological fluids containing large amounts of glycine, it may be necessary to derivatize the sample with two different solvents to avoid interference of the GLY₃ peak with isoleucine and proline.
### TABLE 1

SOLVENT EFFECTS ON TMS DERIVATIZATION OF PROTEIN AMINO ACIDS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No. of Glycine Peaks</th>
<th>No. of Arginine Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Pyridine</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>10% Acetonitrile in Methylene Chloride</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5% Acetonitrile in Methylene Chloride</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hexane</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Methylene Chloride</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chloroform</td>
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<td>0</td>
</tr>
<tr>
<td>1,2-Dichloroethane</td>
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</tr>
</tbody>
</table>
GLC CHROMATOGRAM OF TMS AMINO ACIDS

TMS AMINO ACIDS. SAMPLE: 0.2 mg OF EACH AMINO ACID IN 2.0 ml CH₃CN/BSTFA 1:1 v/v. CLOSED TUBE SILYLATION, 135°C-15 MIN., 5 μl INJECTED. COLUMN: MIXED LIQUID PHASE 6.0 w/w% OV-7 AND 3.0 w/w% OV-22 ON 100/120 MESH SUPELCOPORT, 2 m x 4 mm I.D. GLASS. I.T. 80°C-5 MIN. HOLD, THEN 5°C/MIN.

FIGURE 1
GLC CHROMATOGRAM OF TMS AMINO ACIDS

TMS AMINO ACIDS. SAMPLE: 0.2 mg OF EACH AMINO ACID IN 2.0 ml CH₃CN/BSTFA 1:1 v/v.
CLOSED TUBE Silylation, 135°C-4 HR, 5 µl INJECTED. COLUMN: MIXED LIQUID PHASE 6.0 w/w% OV-7 AND 3.0 w/w% OV-22 ON 100/120 MESH SUPERCOPORT, 2 m x 4 mm I.D. GLASS.
I.T. 80°C-5 MIN. HOLD, THEN 5°C/MIN.

FIGURE 2
GLC CHROMATOGRAM OF TMS AMINO ACIDS

TMS AMINO ACIDS. SAMPLE: 0.2 mg OF EACH AMINO ACID IN 2.0 ml CH₂Cl₂/BSTFA 1:1 v/v. CLOSED TUBE SLYLATION, 135°C-15 MIN 5 µl INJECTED. COLUMN: MIXED LIQUID PHASE 6.0 w/w% OV-7 AND 3.0 w/w% OV-22 ON 100/120 MESH SUPELCOPORT, 2 m x 4 mm I.D. GLASS. I.T. 80°C-5 MIN. HOLD, THEN 5°C/MIN.

FIGURE 3
GLC CHROMATOGRAM OF TMS AMINO ACIDS

TMS AMINO ACIDS. SAMPLE: 0.2 mg OF EACH AMINO ACID IN 2.0 ml CH₂Cl₂/BSTFA 1:1 v/v.
CLOSED TUBE Silylation, 135°C-4 HRS., 5 µl INJECTED. COLUMN: MIXED LIQUID PHASE 6.0
w/w% OV-7 AND 3.0 w/w% OV-22 ON 100/120 MESH SUPELCO PORT, 2 m x 4 mm I.D. GLASS.
I.T. 80°C-5 MIN. HOLD, THEN 5°C/MIN. EACH PEAK çα 500 ng.

FIGURE 4
GLC CHROMATOGRAM OF TMS AMINO ACIDS

TMS AMINO ACIDS. SAMPLE: 0.2 mg OF EACH AMINO ACID IN 2.0 ml CH$_2$Cl$_2$/1% TMCS IN BSTFA 1:1 v/v. CLOSED TUBE SILYLATION, 135°C-15 MIN., 5 µl INJECTED. COLUMN: MIXED LIQUID PHASE 6.0 w/w% OV-7 AND 3.0 w/w% OV-22 ON 100/120 MESH SUPELCOPORT, 2 m x 4 mm I.D. GLASS. I.T. 80°C-5 MIN. HOLD, THEN 5°C/MIN.

FIGURE 5
GLC CHROMATOGRAM OF TMS AMINO ACIDS

TMS AMINO ACIDS. SAMPLE: 0.2 mg OF EACH AMINO ACID IN 2.0 ml CH$_2$Cl$_2$/1% TMCS IN BSTFA 1:1 v/v. CLOSED TUBE SILYLATION, 135°C-4 HRS., 5 µl INJECTED. COLUMN: MIXED LIQUID PHASE 6.0 w/w% OV-7 AND 3.0 w/w% OV-22 ON 100/120 MESH SUPELCOPORT, 2 m x 4 mm I.D. GLASS. I.T. 80°C-5 MIN. HOLD, THEN 5°C/MIN.

FIGURE 6
CHROMATOGRAM OF TMS PROTEIN AMINO ACIDS

TMS AMINO ACIDS. SAMPLE: 2.0 mg in 1.0 ml. 1.0 μg each amino acid injected. BSTFA/CH3CN 1:1 v/v, 150°C for 2.5 hrs. COLUMN: 10% OV-11 on Supelcoport 100/120 mesh.
6m x 2mm I.D. CONDITIONS: Injector 275°C, Detector 300°C, Initial Temperature 110°C, 2°C/min for 22 min, 5°C/min to 285°C, Carrier Gas N2 20ml/min. I.S. Phenanthrene

FIGURE 7
SUMMARY AND CONCLUSIONS

The number of chromatographic peaks for the TMS derivatives of glycine and arginine are determined by the polarity of the solvent. With hexane, methylene chloride, chloroform, and 1,2-dichloroethane, one peak is obtained for glycine (GLY₂), and two peaks (GLY₂ and GLY₃) in six other more polar solvents. Arginine gives no peak in the four less polar solvents studied and one peak in the other six more polar solvents.

Preliminary work is reported on chromatography using a 6 m x 2 mm I.D. glass column of 10 w/w% OV-11 on 100/120 mesh Supelcoport of which good resolution was obtained for the TMS derivatives of 19 of the protein amino acids. More detailed chromatographic studies will be the subject of a separate paper.
REFERENCES