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# GLC–Mass Spectrometry of Several Important Anticancer Drugs I: Pertrimethylsilylation and *O*-Methoxime Formation

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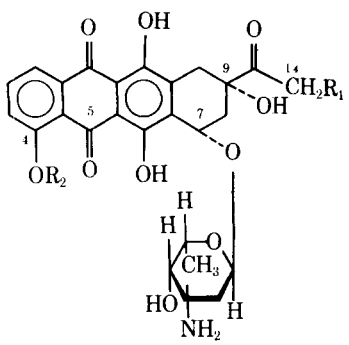
**Abstract** □ Procedures are reported for the formation of pertrimethylsilyl and pertrimethylsilyl methoxime derivatives of the aglycones of doxorubicin, daunorubicin, carminomycin, chromomycin $A_3$, and mithramycin. The mass spectra are consistent with the formation of these derivatives. Fragmentation patterns highly specific for these derivatives are proposed, and the potential application for the identification of metabolites of these compounds is discussed.

**Keyphrases** □ Doxorubicin—GLC–mass spectral analysis, bulk drug □ Daunorubicin—GLC–mass spectral analysis, bulk drug □ GLC–mass spectrometry—analyses, doxorubicin, daunorubicin, and other antineoplastic agents, bulk drug □ Antineoplastic agents, various—GLC–mass spectral analyses in bulk drug

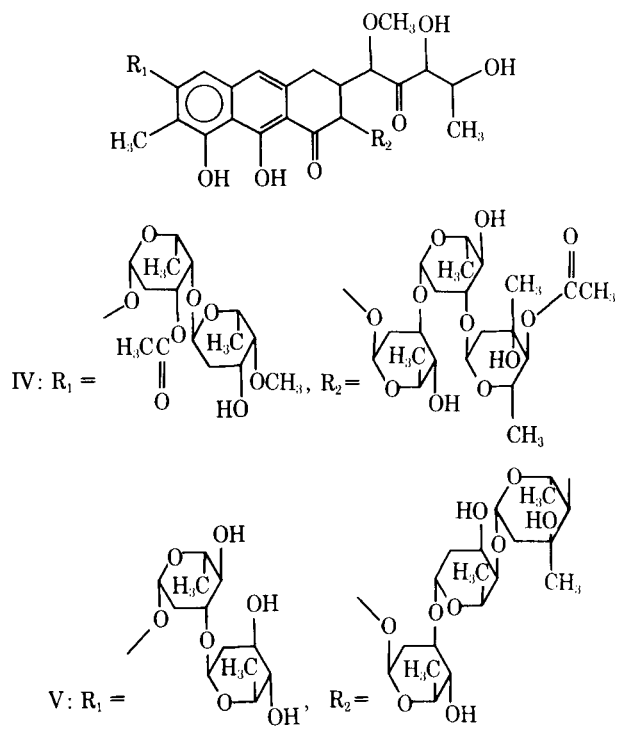
Doxorubicin[1,2] (I), daunorubicin[1,3] (II), carminomycin[1,4] (III), chromomycin $A_3$[1,5] (IV), and mithramycin[1,6] (V) are naturally occurring antibiotics (1–6) which possess significant antineoplastic activities. The first three are structurally related, and the last two differ only in the sugar moieties (6). Both I and V have been marketed in the United States. Of these five drugs, I is the most important clinically and has proven to be extremely effective against various tumors (7–9).



I: $R_1$ = H, $R_2$ = $CH_3$
II: $R_1$ = OH, $R_2$ = $CH_3$
III: $R_1$ = $R_2$ = H

The disposition of I and II in animals and humans has been studied in this and other laboratories (1–14). Of major concern has been the development of sensitive, specific analytical procedures for I in physiological fluids and the identification of its metabolites.

GLC–mass spectrometry is one of the most effective instrumental methods for the separation and identification of compounds extracted from biological fluids. The relative retention times and the supplemental mass fragmentographic data provide for the positive characterization of unknown compounds. No reports have yet been published



IV: $R_1$ = , $R_2$ =

V: $R_1$ = , $R_2$ =

[1] Supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.
[2] Adriamycin hydrochloride, NSC-123127, Adria Laboratories.
[3] Daunomycin, rubidomycin, NSC-82151.
[4] NSC-180024.
[5] NSC-58514, Toyomycin, Takeda Chemical Industries, Osaka, Japan.
[6] NSC-143020, Mithracin, Pfizer.

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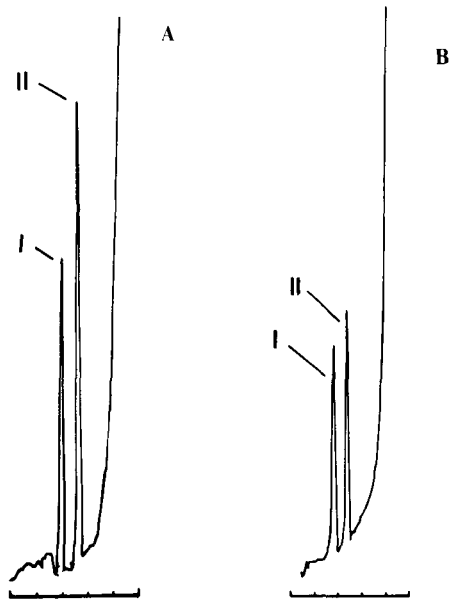


**Figure 1**—*Chromatograms. A: Peak I = pertrimethylsilyl I aglycone, and peak II = pertrimethylsilyl II aglycone. B: Peak I = pertrimethylsilyl I aglycone methoxime, and peak II = pertrimethylsilyl II aglycone methoxime.*

describing the GLC–mass spectral analysis of I, probably because of the inability to prepare sufficiently volatile and stable derivatives that can be subjected to GLC.

Therefore, the present study was initiated to investigate conditions that would allow the GLC analysis of I and its metabolites and II–V. This report describes two derivatization procedures for the GLC–mass spectral analysis of the aglycones of these anticancer agents. One method used a direct silylation of these aglycones; in the other method, the keto group on the side chain was converted to the methoxime derivative prior to silylation. Mass spectral fragmentations of these compounds are proposed, and the potential application of these methods for the identification of their metabolites is discussed.

## EXPERIMENTAL

**Instrumental Conditions**—A magnetic sector mass spectrometer[7] interfaced with a gas chromatograph was used. The GLC conditions were as follows.

A coiled glass column (92.3 cm × 2 mm i.d.) was packed with 3% OV-101 on 100–120-mesh Gas Chrom Q[8]. The flow rate of the carrier gas (helium) was 22 ml/min. Temperatures were: column, 260°; flash heater, 260°; and molecular separator and restricter, 210°. The operating conditions of the mass spectrometer were: electron energy, 70 ev; ion source temperature, 200°; ionization current, 60 μamp; and accelerating voltage, 3 kv.

Excess reagent was vented to the force-vacuum pump by an all-glass venting system (15). Use of this device considerably reduced contamination problems in the ion source caused by excess silylating agent.

**Chemicals and Reagents**—Compounds I–V were used without further purification. These compounds were greater than 95% pure as determined by TLC[9]. The aglycones were obtained by heating the parent compounds in 0.1 *N* HCl for 10 min at 95°. This solution was then cooled, and the aglycones were extracted with ethyl acetate. The ethyl acetate was removed by evaporation using dry nitrogen. Methoxylamine hydrochloride[8] and the silylation reagent[10] composed of bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane–trimethylsilylimidazole (3:3:2) were obtained commercially. The pyridine[10] was silylation grade.

[7] Varian model CH-7, Springfield, N.J.
[8] Applied Science Laboratories, State College, Pa.
[9] Silica gel GH with chloroform–methanol–acetic acid (80:20:4).
[10] Powersil, Pierce Chemical Co., Rockford, Ill.

**Table I—Retention Times of Anthracycline Aglycone Derivatives on 3% OV-101 at 260°**

| Pertrimethylsilyl Aglycone Derivatives | Retention Time, min |
|---|---|
| I | 5.6 |
| I methoxime | 5.6 |
| II | 4.6 |
| II methoxime | 4.6 |
| III | 5.5 |
| III methoxime | 5.5 |
| IV/V | 6.5 |
| IV/V methoxime | 7.2 |

**Preparation of Pertrimethylsilyl and Pertrimethylsilyl Methoxime Derivatives**—Approximately 100 μg of each aglycone was reacted with 50 μl of the silylation mixture. After a few minutes, 1–2-μl samples were injected into the gas chromatograph–mass spectrometer. *O*-Methoximes were prepared according to Fales and Luukkainen (16).

The aglycone (200 μg) was reacted with about 5 mg of methoxylamine hydrochloride and 100 μl of pyridine for 45 min at 75°. The pyridine was evaporated using a nitrogen stream. The methoxime derivative was isolated by adding 1 ml of water and extracting with 1 ml of ethyl acetate. The ethyl acetate was evaporated under nitrogen. Silylation was then carried out as already described.

## RESULTS AND DISCUSSION

**GLC Characteristics**—Since the total ion chromatograms of the derivatives following GLC–mass spectrometry were essentially the same, only the chromatograms obtained from the pertrimethylsilyl and pertrimethylsilyl methoxime derivatives of I and II aglycones are illustrated (Fig. 1). The retention times of all derivatives are given in Table I.

All derivatives except two chromatographed as single well-resolved peaks with excellent GLC properties. The pertrimethylsilyl methoxime of the IV/V aglycone reaction mixture contained two peaks. The first peak was the pertrimethylsilyl derivative and the second was the pertrimethylsilyl methoxime derivative, indicating incomplete methoxime formation under the experimental conditions.

The stability of the derivatives during chromatography was dependent on the column condition. Commercially prepared packings containing 3% OV-1, 3% SE-30, 1% JXR, or 3% OV-101 were evaluated for derivative stability. The 3% OV-101 was superior. This particular packing has been in constant use for over 3 months without showing any deterioration.

**Derivatization Procedure**—All compounds studied contained polar polyhydroxyl groups, making it virtually impossible for them to be chromatographed directly. It was necessary to prepare derivatives with the necessary properties for GLC.

Trifluoroacetic anhydride, pentafluoropropionic anhydride, and acetic anhydride were evaluated as possible derivatization agents. However, none formed volatile derivatives with the I or II aglycone that could be detected following GLC.

In recent years, silyl derivatives have been used for the GLC analyses of extremely polar compounds. Not only does the greatly increased volatility of the silyl derivative lend itself to GLC separation, but the silyl

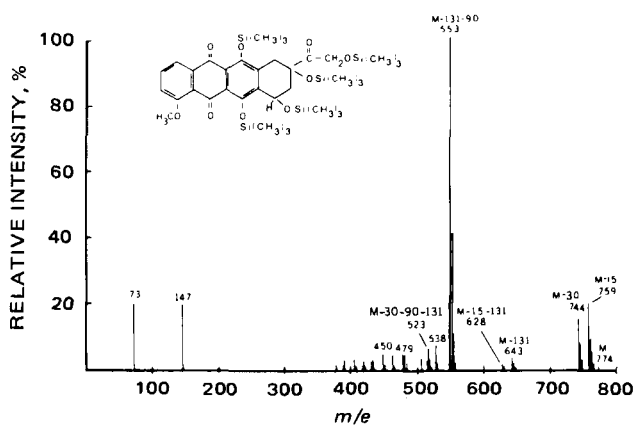


**Figure 2**—*GLC–mass spectrum of the pertrimethylsilyl I aglycone.*

**Table II—Mass Spectral Fragmentations [a] of Pertrimethylsilyl Derivatives of Anthracycline Aglycones**

| Common Fragmentation | *m/e* (Percent Relative Intensity) I | II | III |
|---|---|---|---|
| M | 774 (1) | 686 (1) | 744 (0) |
| M − 15 | 759 (20) | 671 (37) | 729 (40) |
| M − 30 | 744 (15) | 656 (45) | 714 (90) |
| M − side chain | 643 (5) | 643 (2) | 701 (0) |
| M − 15 − side chain | 628 (5) | 628 (3) | 686 (20) |
| M − 90 − side chain | 553 (100) | 553 (80) | 611 (100) |
| M − 15 − 90 − side chain | 538 (10) | 538 (20) | 596 (25) |
| M − 30 − 90 − side chain | 523 (10) | 523 (20) | 581 (50) |
| $(CH_3)_2SiO^+Si(CH_3)_3$ | 147 (20) | 147 (10) | 147 (41) |

[a] Only common major fragments are presented.

group appears to direct fragmentation patterns in many instances (17).

Several reagents were evaluated for the extent of pertrimethylsilylation with the I aglycone as the standard. Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane gave a small total ion peak during GLC–mass spectrometry only after the reaction mixture had been heated at 75° for at least 1 hr. With bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane (1:1), a large peak was obtained under the same reaction conditions. Since this approach seemed promising, combinations of silylating agents with stronger catalysts were then considered.

A strong, commercially available silylating agent comprised of bis(trimethylsilyl)trifluoroacetamide – trimethylchlorosilane–trimethylsilylimidazole (3:3:2) formed a fully silylated derivative with the I aglycone within a few minutes at room temperature. This reagent was first reported in 1967 for the derivatization of steroids (18), but few reports have described its use. The reagent has several important advantages. First, the use of a solvent is unnecessary since the reagent acts as its own solvent. Second, the derivatization procedure is complete within a few minutes. Third, derivatization leads to a persilylated derivative. The ability of this reagent to silylate all of the hydroxyl groups is extremely critical to evaluating the course of I metabolism by GLC–mass spectrometry, for example, the generation of hydroxyl groups *via* metabolic reactions. Numerous unsuccessful attempts have been made to chromatograph pertrimethylsilyl derivatives of I and II.

The structures of I and II aglycones can be considered somewhat similar to those steroids possessing the 17α,21-dihydroxy-20-keto side chain. The preferred method for the GLC analysis of these compounds is to convert the keto group to the methoxime derivative using methoxylamine, followed by silylation of the remaining hydroxyl group (19). This procedure yields stable volatile derivatives for aglycones of I, II, III, and IV/V with excellent GLC characteristics. The side-chain derivatization also yields useful information concerning the metabolic change of the keto group.

The extent of the I aglycone methoxime formation was evaluated by reacting this derivative with the silylation mixture and monitoring the disappearance of the characteristic *m/e* 553 fragment of the trimethylsilyl derivative. After a reaction time of 45 min at 75°, the *m/e* fragment was negligible. Under these conditions, the methoxime derivatization of IV and V aglycones proceeded to about 70% completion.

The stability of the trimethylsilyl derivative of the I aglycone was

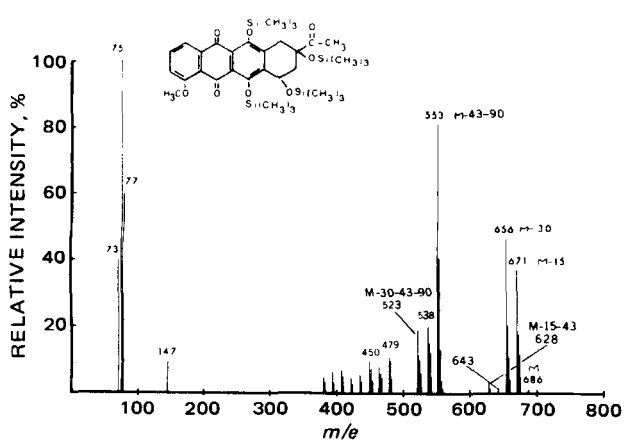


**Figure 3**—*GLC–mass spectrum of the pertrimethylsilyl II aglycone.*

**Table III—Mass Spectral Fragmentations [a] of Pertrimethylsilyl Methoxime Derivatives of Anthracycline Aglycones**

| Common Fragmentation | *m/e* (Percent Relative Intensity) I | II | III |
|---|---|---|---|
| M | 803 (2) | 715 (7) | 773 (10) |
| M − 15 | 788 (100) | 700 (45) | 758 (45) |
| M − 30 | 773 (20) | 685 (15) | 743 (21) |
| M − 15 − 90 | 698 (40) | 616 (90) | 668 (75) |
| M − 30 − 90 | 683 (55) | 595 (100) | 653 (70) |
| M − 15 − 90 − 31 | 667 (15) | 579 (42) | 637 (36) |
| M − 30 − 90 − 31 | 652 (7) | 564 (25) | 622 (22) |

[a] Only common major fragments are presented.

studied by injecting aliquots from the same reaction sample during 24 hr. The reaction with the silylation mixture was complete after 10 min. Allowing the reaction to proceed for up to 1 hr did not significantly increase the total ion response. After 1 hr, a small additional peak, which chromatographed immediately after the pertrimethylsilyl I aglycone, was observed. The height of this peak increased with time. After 24 hr, it equaled that of pertrimethylsilyl I aglycone.

Mass spectral evaluation of this peak indicated that its highest *m/e* fragment was 72 units higher than the highest fragment in the pertrimethylsilyl I aglycone mass spectrum. This component was tentatively identified as the enol pertrimethylsilyl derivative. The experiment was repeated with the pertrimethylsilyl methoxime I aglycone derivative, and no additional component was formed. The derivative remained stable during this period with no signs of degradation.

**Mass Spectrometric Characteristics**—The mass spectra of silyl ethers of I–V aglycones are shown in Figs. 2–5. Both IV and V aglycones showed identical mass spectra, as expected on the basis of their identical structures.

The mass spectra of these silylated derivatives were characterized by cleavages of methyl radicals (M − 15 and M − 15 − 15), typical of pertrimethylsilyl derivatives (17). With the exception of the IV aglycone, the pertrimethylsilyl derivatives did not show significant amounts of molecular ions. Cleavage of the entire side chain and elimination of silanol appeared to be the favorable processes for the anthracyclines. The latter process is consistent with the fragmentation of pertrimethylsilyl derivatives of polyols. The fragments at *m/e* 147 commonly found for these trimethylsilyl anthracyclines probably represent $(CH_3)_2SiO^+Si(CH_3)_3$, also similar to pertrimethylsilyl derivatives of polyols (20). The common fragmentation patterns of these pertrimethylsilyl derivatives are summarized in Table II.

The mass spectrum of pertrimethylsilyl methoxime derivatives of I aglycone is shown in Fig. 6. Major fragments of pertrimethylsilyl methoxime derivatives of I–III aglycones[11] are summarized in Table III. The spectra indicated that, in all cases, persilylated derivatives were formed and the fragmentation patterns were consistent with the proposed structures. The mass spectrum of pertrimethylsilyl methoxime IV/V aglycones[11] gave a molecular ion at *m/e* 910 (15%) and major fragments at *m/e* 895 (M − 15), 589 (M − side chain), 500 (M − 89 − side chain), and

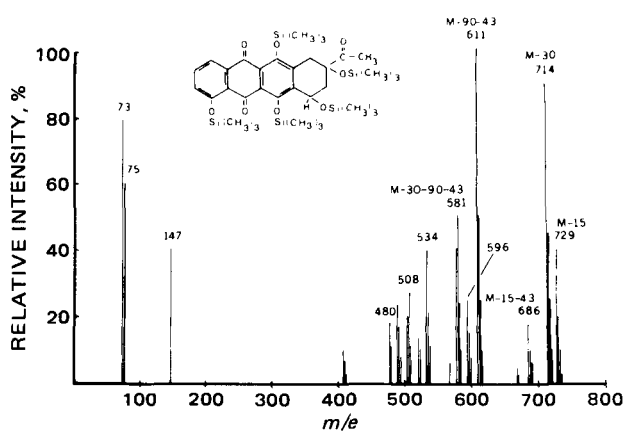


**Figure 4**—*GLC–mass spectrum of the pertrimethylsilyl III aglycone.*

[11] Mass spectra of these compounds are available from the authors upon request.

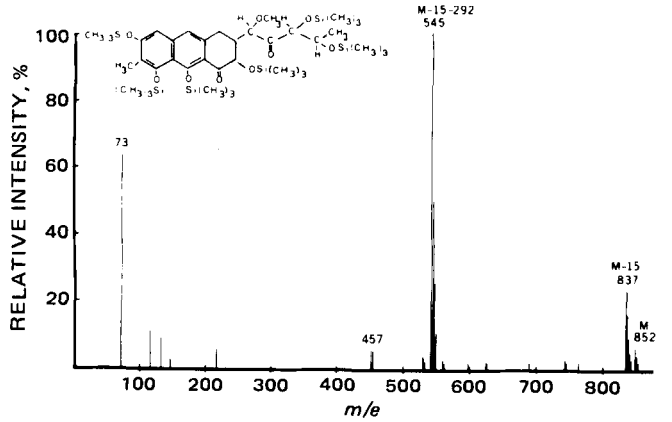


**Figure 5**—*GLC-mass spectrum of the pertrimethylsilyl IV/V aglycones.*

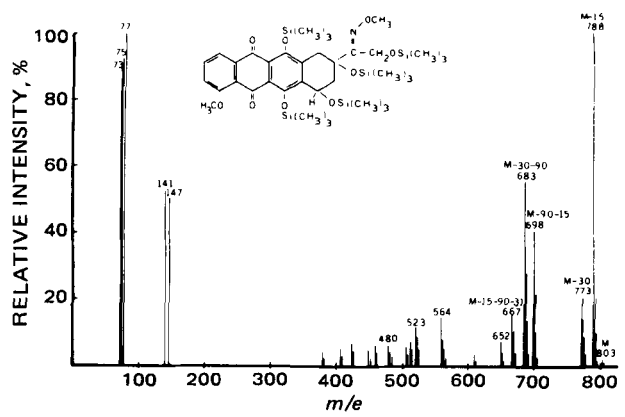


**Figure 6**—*GLC-mass spectrum of pertrimethylsilyl I aglycone methoxime.*

73 (base peak), all consistent with a bis(*O*-methoxime) pertrimethylsilylated structure.

The most characteristic and, perhaps, diagnostic ion of the silyl ether derivative of anthracycline results from the loss of the side chain at C-9 with simultaneous elimination of silanol. Thus, in the mass spectra of the silyl ethers of I and II aglycones, the ion at *m/e* 553 (Figs. 2–4) is clearly the most dominant. The silyl ether of the III aglycone showed a similar cleavage at C-9 with its corresponding base peak at *m/e* 611. The increase in *m/e* is consistent with the formation of a stable silyl derivative at C-4. The silyl ether of the IV aglycone showed the base at *m/e* 545 formed by the elimination of the side chain at C-3 and a methyl radical. This result indicates that side-chain cleavage is a very favorable process for these silylated compounds regardless of the differences in the side chain. Changes in the ring did not appear to alter this cleavage.

Thus, the fragmentation process is relevant to identifying the metabolite of I. Demethylation of I at C-4, leading to the formation of a phenolic hydroxyl group, was reported (14). The base peak in the spectrum of the pertrimethylsilyl III aglycone is found at *m/e* 611, which is consistent with a stable silyl derivative at C-4. Therefore, the corresponding pertrimethylsilyl metabolite formed from I also should show a similar base peak at *m/e* 611. Moreover, the presence or loss of hydroxyl groups in the ring structure should be reflected in easily identifiable masses from *m/e* 553.

The mass spectra of the methoxime silyl ether derivatives of I–III aglycones are characterized by the presence of a molecular ion and by a series of peaks at M − 15 ($CH_3$), M − 31 ($OCH_3$), M − 15 − 90 ($CH_3$ − trimethylsilanol), and M − 31 − 90 ($OCH_3$ − trimethylsilanol). Several peaks at lower *m/e* values, representing losses of $CH_3$ units, are not of diagnostic importance. In contrast to the pertrimethylsilyl ethers of anthracycline aglycones, the methoxime derivatives stabilize the side chain to such an extent that no cleavage is apparent at C-9.

Metabolic reduction of the carbonyl side chains of I and II have been reported to yield the hydroxyl derivatives. Following the fragmentation trend discussed, both of the pertrimethylsilyl derivatives of I and II aglycones should yield a base peak at *m/e* 553. The derivatives obtained by a combination of treatments with the methoxyamine and the above-mentioned silylating agent, giving rise to the pertrimethylsilyl methoxime derivative, should yield identical mass spectra compared to those treated with the silylating agent alone since the lack of the carbonyl group on the side chain should negate the methoxime formation.

A similar approach can be used to probe into the metabolism of IV/V.

The combination of these two derivatization procedures should prove valuable in the identification of the various metabolites of I. Formation of the silyl derivative establishes the extent of metabolism on the ring portion as well as the side chain. Formation of the pertrimethylsilyl methoxime derivative offers corroboration of the identity of the compound.

Investigations are now in progress using the procedures described here to evaluate the metabolism of I in rabbits and in humans. The ease of formation and excellent chromatographic properties of the derivatives, together with their characteristic mass spectra, should make these derivatization procedures valuable for the study of the metabolism of I–V.

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