GLC–Mass Spectrometry of Several Important Anticancer Drugs II: Doxorubicin and Daunorubicin Aglycone Analogs

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Received November 28, 1977, from the *School of Pharmacy, University of Southern California, Los Angeles, CA 90033.* Accepted for publication March 31, 1978.

Abstract □ By using previously developed pertrimethylsilylation and methoxime formation procedures, the GLC–mass spectra of pertrimethylsilyl and pertrimethylsilyl methoxime derivatives of 12 doxorubicin and daunorubicin glycone analogs, mostly synthesized in this laboratory, were studied. The fragmentation pathways were readily interpretable, and the patterns were highly specific for corresponding structural changes, thus supporting the previous contention that this method has potential for the identification of doxorubicin and daunorubicin metabolites. Four aglycone metabolites were identified, on the basis of their GLC–mass spectra, in the hydrolysate of the 1-butanol extract of bile obtained from a doxorubicin-treated rabbit.

Keyphrases □ Doxorubicin glycone analogs, various—GLC–mass spectral analyses, prepared samples □ Daunorubicin glycone analogs, various—GLC–mass spectral analyses, prepared samples □ GLC–mass spectrometry—analyses, various doxorubicin and daunorubicin glycone analogs, prepared samples □ Antineoplastic agents—doxorubicin and daunorubicin, various glycone analogs, GLC–mass spectral analyses, prepared samples □ Metabolites, aglycone—GLC–mass spectral identification in bile of doxorubicin-treated rabbit

The anthracycline antitumor agents doxorubicin[1,2] (I*a*) and daunorubicin[2,3] (II*a*) undergo extensive metabolism (1–11). Isolation of their metabolites involved usual extraction of the material from biological fluids followed by extensive chromatographic separations. Identification methods were based on spectroscopic and mass spectral data obtained from these isolated components. In all cases, these components had to be pure.

GLC–mass spectrometry often offers advantages in the separation of properly derivatized substances. This method simplifies purification procedures and yields good mass spectral data. Previously (12), stable volatile pertrimethylsilyl and pertrimethylsilyl methoxime derivatives were obtained for doxorubicinone (I*b*), daunorubicinone (II*b*), carminomycinone (III*b*), and mithramycinone/chromomycinone, and these derivatives exhibited excellent GLC properties and readily interpretable mass fragments. This paper reports the application of this method to study the fragmentation patterns of the derivatives of I*a* and II*a*. Several of these derivatives have been reported as metabolites of these agents.

EXPERIMENTAL

Instrumental Conditions—A magnetic sector mass spectrometer interfaced with a gas chromatograph[4] was used to obtain the mass spectra under the previously described conditions (12).

Chemicals and Reagents—Compounds I*a*, II*a*, carminomycin[5,6] (III*a*), daunomycinol[5] (IX*a*), 7-deoxydaunomycinone[3] (VI), and doxorubicin carboxylic acid[7] (XIV*a*) were found to be greater than 95% pure by TLC[8]. The aglycones of these compounds were obtained as described previously (12). Silylating mixtures composed of trimethylsilylimidazole–bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane[9] (3:3:2) and methoxyamine hydrochloride[10] were obtained commercially. Pyridine was silylation grade. Aluminum chloride, sodium dithionite, sodium bicarbonate, disodium ethylenediaminetetraacetate, benzene, ethyl acetate, and methylene dichloride were all analytical grade and were used without further purification.

Compound III*b* was obtained by hydrolysis of III*a* or was synthesized by the procedure of Kende *et al.* (13), starting from II*b*. 4-Demethyldoxorubicinone (IV) was synthesized by a procedure identical to that used for III*b* (13). Red crystals, obtained after crystallization in ethyl acetate, gave a melting point of 237–240°. The electron-impact mass spectrum did not yield a substantial parent ion; the GLC–mass spectrum of the pertrimethylsilyl derivative (Table I) was consistent with the assigned structure.

Compound VI was synthesized by the procedure of Smith *et al.* (14). The product obtained was identical in all respects (UV and mass spectra and melting point) with an authentic sample[5]. 7-Deoxydoxorubicinone (V) was synthesized by an identical procedure. After crystallization from dichloromethane, the red crystals gave a melting point of 246–249°. The high-resolution electron-impact mass spectrum[11] gave a prominent peak at *m/e* 338.076, corresponding to M − 60 [HC(=O)CH_2OH] ($C_{19}H_{14}O_6$). The GLC–mass spectrum (Table I) was consistent with the expected structure.

Similarly, 7-deoxycarminomycinone (4-demethyl-7-deoxydaunorubicinone, VIII), was synthesized according to the same procedure. After crystallization from dichloromethane, the red crystals gave a parent ion at *m/e* 368.090, corresponding to $C_{20}H_{16}O_7$, under high-resolution electron-impact mass spectrometry.

Doxorubicinol (X*a*) was isolated from I*a*-treated rabbit bile according to the modified procedure of Takanashi and Bachur (11). The bile was extracted extensively with 1-butanol. The residue obtained after evaporation of the solvent was chromatographed on an XAD resin[10] and eluted first with water and then with methanol. The red band corresponding to X*a* eluted off the column by methanol was collected. The red residue obtained after removal of the solvent *in vacuo* was purified on semipreparative TLC plates[12] with chloroform–methanol–water (80:40:4) as the eluting solvent. The isolated band was chromatographically identical with reported X*a* (11). The fluorescence spectrum was identical with that of I*a*.

The aglycones of IX*a* and X*a*, *i.e.*, IX*b* and X*b*, respectively, were obtained by acid hydrolysis as reported previously (12).

Alternatively, X*a* was synthesized by reduction of I*a* *via* sodium cyanoborohydride in an aqueous solution; the pH was controlled at 3.5[13] The isolated product was identical in all respects (TLC and UV and GLC–mass spectra of its aglycone) with the product of metabolic origin[14].

4-Demethyl-7-deoxydoxorubicinone (VII) was synthesized by demethylation of previously synthesized V by the procedure described for the synthesis of IV. After crystallization from dichloromethane, the red

[1] Adriamycin, NSC-123127.
[2] Supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutic Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.
[3] Daunomycin, rubidomycin, NSC-82151.
[4] Varian model CH-7, Springfield, N.J.
[5] Gift of Dr. F. Arcamone, Farmatalia, Milan, Italy.
[6] NSC-180024.
[7] Gift of Dr. David Henry, Stanford Research Institute, Palo Alto, Calif.
[8] Silica gel GH with chloroform–methanol–acetic acid (80:20:4).
[9] Powersil, Pierce Chemical Co., Rockford, Ill.
[10] Applied Science Laboratories, State College, Pa.
[11] DuPont mass spectrometer model 21-492B, at the California Institute of Technology, Pasadena, Calif.
[12] Gelman ITLC, SA type, Ann Arbor, Mich.
[13] Dr. David Henry, Stanford Research Institute, provided the reduction procedure.
[14] No identical stereochemistry is implied.

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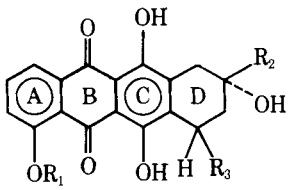
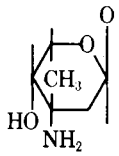
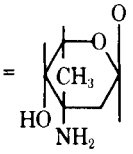


Ia: $R_1 = CH_3$, $R_2 = C(=O)CH_2OH$, R_3 =



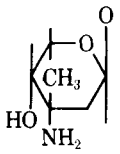
Ib: $R_1 = CH_3$, $R_2 = C(=O)CH_2OH$, $R_3 = OH$

IIa: $R_1 = CH_3$, $R_2 = C(=O)CH_3$, R_3 =



IIb: $R_1 = CH_3$, $R_2 = C(=O)CH_3$, $R_3 = OH$

IIIa: $R_1 = H$, $R_2 = C(=O)CH_3$, R_3 =



IIIb: $R_1 = H$, $R_2 = C(=O)CH_3$, $R_3 = OH$

IV: $R_1 = H$, $R_2 = C(=O)CH_2OH$, $R_3 = OH$

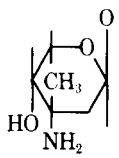
V: $R_1 = CH_3$ $R_2 = C(=O)CH_2OH$, $R_3 = H$

VI: $R_1 = CH_3$, $R_2 = C(=O)CH_3$, $R_3 = H$

VII: $R_1 = R_3 = H$, $R_2 = C(=O)CH_2OH$

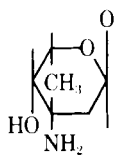
VIII: $R_1 = R_3 = H$, $R_2 = C(=O)CH_3$

IXa: $R_1 = CH_3$, $R_2 = CH(OH)CH_3$, R_3 =



IXb: $R_1 = CH_3$, $R_2 = CH(OH)CH_3$, $R_3 = OH$

Xa: $R_1 = CH_3$, $R_2 = CH(OH)CH_2OH$, R_3 =



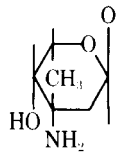
Xb: $R_1 = CH_3$, $R_2 = CH(OH)CH_2OH$, $R_3 = OH$

XI: $R_1 = CH_3$, $R_2 = CH(OH)CH_2OH$, $R_3 = H$

XII: $R_1 = H$, $R_2 = CH(OH)CH_2OH$, $R_3 = OH$

XIII: $R_1 = R_3 = H$, $R_2 = CH(OH)CH_2OH$

XIVa: $R_1 = CH_3$, $R_2 = COOH$, R_3 =



XIVb: $R_1 = CH_3$, $R_2 = COOH$, $R_3 = OH$

XV: $R_1 = H$, $R_2 = COOH$, $R_3 = OH$

crystals gave a melting point of 167°. The high-resolution electron-impact mass spectrum gave a major fragment at *m/e* 324.063, corresponding to M − 60 [HC(=O)CH_2OH] ($C_{18}H_{12}O_6$), which is consistent with the expected structure.

Similarly, 4-demethyldoxorubicinol aglycone (XII) and 4-demethyldoxorubicinone carboxylic acid (XV) were prepared. The mass spectra of the respective pertrimethylsilylated derivatives gave fragments (Table II) consistent with their expected structures.

7-Deoxydoxorubicinol aglycone (XI) was synthesized in a manner similar to that used for V. 4-Demethyl-7-deoxydoxorubicinol aglycone (XIII) was synthesized by a procedure similar to that used for VII. The GLC–mass spectral data (Table II) of their respective pertrimethylsilyl derivatives were consistent with their expected structures.

Pertrimethylsilyl derivatives of these aglycones were prepared using the previously published procedure (12). *O*-Methoxime derivatives and *O*-methoxime silyl derivatives also were obtained (12).

Table I—Mass Spectral Analysis [a] of Pertrimethylsilyl Derivatives of Ia and IIa Analogs

Presumed Fragmentation	*m/e* (Percent Relative Intensity) Trimethylsilyl IIIb	Trimethylsilyl IV	Trimethylsilyl VI	Trimethylsilyl V
M	744 (0)	832 (0)	598 (0)	686 (0)
M − 15	729 (40)	817 (10)	583 (15)	671 (8)
M − 30	714 (90)	802 (20)	568 (100)	656 (25)
M − 15 − side chain	686 (20)	686 (10)	540 (10)	540 (16)
M − 90 − side chain	611 (100)	611 (100)	—	—
M − 15 − 90 − side chain	596 (25)	596 (20)	—	—
M − 30 − 90 − side chain	581 (50)	581 (30)	—	—

[a] Only common major fragments are presented.

Table II—Mass Spectral Analysis [a] of Pertrimethylsilyl Derivatives of I*a* Analogs

Presumed Fragmentation	*m/e* (Percent Relative Intensity) Trimethylsilyl XIV*b*	Trimethylsilyl XV	Trimethylsilyl XII	Trimethylsilyl XI	Trimethylsilyl XIII
M	760 (0)	818 (0)	906 (0)	760 (0)	818 (0)
M − 15	745 (55)	803 (45)	891 (2)	745 (12)	803 (16)
M − 30	730 (100)	788 (85)	876 (1.5)	730 (45)	768 (31)
M − 15 − 90	655 (11)	713 (5)	—	655 (10)	713 (4)
M − 30 − 90	640 (20)	698 (9)	786 (2)	640 (8)	698 (5)
M − side chain	—	—	—	555 (8)	613 (8)
M − 15 − side chain	—	—	686 (3)	540 (20)	598 (17)
M − 30 − side chain	—	670 (4)	—	525 (15)	—
M − 90 − side chain	553 (17)	611 (16)	611 (17)	—	—
M − 15 − 90 − side chain	—	596 (16)	—	—	—
M − 30 − 90 − side chain	523 (26)	581 (20)	581 (7)	—	—
M − 30 − 320 (retro-Diels-Alder)	—	—	—	—	468 (5)

[a] Only common major fragments are presented.

RESULTS AND DISCUSSION

The mass spectra of pertrimethylsilyl derivatives of I*b* and II*b* were reported previously (12). The major common fragments are shown in Table III. As indicated, these two compounds underwent quantitatively and qualitatively similar fragmentations.

Common major mass fragments of the pertrimethylsilyl derivatives[15] of II*b* analogs (III*b*, VI, VIII, and IX*b*) are listed in pairs with the pertrimethylsilyl derivatives of the corresponding analogs of I*b* in Tables I and IV. Similar data for their corresponding pertrimethylsilyl methoxime derivatives[15] are shown in Tables V and VI. In all cases, mass fragmentations were consistent with the proposed structures.

As previously indicated (12), one major mass fragment of trimethyl silyl I*b* and trimethylsilyl II*b*, *m/e* 553, resulting from silanol and side-chain cleavages (Table III), appears to possess diagnostic value capable of reflecting changes on the anthracycline rings. This notion was tested for trimethylsilyl III*b*, where the fragment at *m/e* 611 was the base peak (12) consistent with silanol and side-chain cleavages. A mass unit gain of 58 was observed, representing the addition of a trimethylsilyl group on the demethylated ring A relative to the parent compound, as expected.

Similar fragmentation would be expected for trimethylsilyl IV. Major mass fragmentation of trimethylsilyl IV included cleavages of methyl radicals, the side chain, silanol, and their combinations (Table I). The base peak was at *m/e* 611 (M − silanol − side chain), identical to trimethylsilyl III*b*, as expected. Other fragments exhibited quantitative and qualitative similarity to trimethylsilyl III*b*.

One prominent metabolic pathway of II*a* is a reductive cleavage product at the glycosidic linkage of the D ring (11). By using this approach, the structural change at the D ring could be similarly revealed. The mass spectral data of VI (Table I) showed the usual fragmentations expected for silylated derivatives and the base peak was, indeed, at *m/e* 568 (M − 30). However, unlike other pertrimethylsilyl aglycones, elimination of silanol did not appear to be a favorable process, because of the lack of a hydroxy group at the 7-position. Instead, methyl and side-chain cleavages gave a substantial fragment at *m/e* 540.

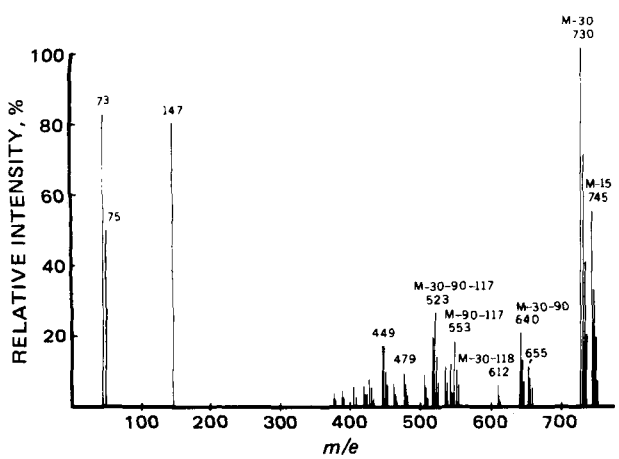


Figure 1—*GLC–mass spectrum of pertrimethylsilyl derivative of XIV*a.

Similar fragmentations were expected for trimethylsilyl V. Its fragmentation data (Table I) exhibited a substantial mass fragment at *m/e* 540 and an overall qualitative resemblance to fragments of trimethylsilyl VI.

The keto side chains of I*b* and II*b* form stable *O*-methoximes with methoxyamine. When this procedure was coupled with persilylation, a volatile derivative with good GLC characteristics was obtained (12). The methoxime appeared to stabilize that side chain with respect to cleavage. Little or no cleavage occurred between the carbonyl side chain and ring D. Thus, a comparison of the mass fragments of the pertrimethylsilyl derivative to those of pertrimethylsilyl methoxime could possibly reveal structural changes of the side chain. The methoxime derivatives of trimethylsilyl III*b* and trimethylsilyl IV (Table V) gave typical fragmentations of silylated compounds without significant side-chain cleavage. However, the lack of an $OSi(CH_3)_3$ group at the 7-position, as in the pertrimethylsilyl methoxime derivatives of V and VI, appeared to decrease the stabilizing effect on the side chain. Consequently, a substantial fragment at *m/e* 524 (M − 30 − side chain) was still observed for these derivatives (Table V).

Minor structural change such as side-chain carbonyl reduction would not be expected to result in a significant alteration in the fragmentation. Thus, trimethylsilyl IX*b* and X*b* gave quantitatively and qualitatively similar fragmentations to each other as well as to their respective parent compounds (Table IV). The mass fragment at *m/e* 553, representing side-chain and silanol elimination, was still one prominent peak of these derivatives. Identical spectra were obtained on their pertrimethylsilyl derivatives after pretreatment with methoxyamine, consistent with a lack of the carbonyl side chain.

A more drastic alteration of the side chain was examined for the corresponding change in fragmentation. Compound XIV*a*, which could conceivably be generated metabolically by oxidation from either I*a* or X*a*, was selected. The mass spectrum of the pertrimethylsilyl derivative of its aglycone, XIV*b*, gave a base peak at *m/e* 730 (M − 30) and two significantly abundant fragments at *m/e* 553 and 523 (Table II and Fig. 1), representing the M − 90 − side chain and M − 30 − side chain, re-

Table III—Mass Spectral Analysis [a] of Pertrimethylsilyl I*b* and II*b*

Presumed Fragmentation	*m/e* (Percent Relative Intensity) Trimethylsilyl I*b*	Trimethylsilyl II*b*
M	774 (1)	686 (1)
M − 15	759 (20)	671 (37)
M − 30	744 (15)	656 (45)
M − side chain	643 (5)	643 (2)
M − 15 − side chain	628 (5)	628 (3)
M − 90 − side chain	553 (100)	553 (80)
M − 15 − 90 − side chain	538 (10)	538 (20)
M − 30 − 90 − side chain	523 (10)	523 (20)
$(CH_3)_2Si\overset{+}{O}Si(CH_3)_3$	147 (20)	147 (10)

[a] Only major fragments are presented.

[15] Mass spectra are available from the authors upon request.

Table IV—Mass Spectral Analysis [a] of Pertrimethylsilyl Derivatives of Ia and IIa Analogs

Presumed Fragmentation	*m/e* (Percent Relative Intensity) Trimethylsilyl VIII	Trimethylsilyl VII	Trimethylsilyl IX*b*	Trimethylsilyl X*b*
M	656 (0)	744 (0)	760 (0)	840 (0)
M − 15	641 (37)	729 (5)	745 (25)	833 (8)
M − 30	626 (100)	714 (9)	730 (12)	818 (8)
M − 15 − side chain	598 (10)	598 (5)	628 (3)	628 (6)
M − 30 − neutral side chain	582 (13)	582 (5)	—	—
M − 30 − 72 − side chain	510 (15)	510 (7)	—	—
M − 90 − side chain	—	—	553 (100)	553 (50)
M − 30 − 90 − side chain	—	—	523 (10)	523 (12)

[a] Only common major fragments are presented.

Table V—Mass Spectral Analysis [a] of Pertrimethylsilyl Methoxime Derivatives of Ia and IIa Analogs

Presumed Fragmentation	*m/e* (Percent Relative Intensity) Pertrimethylsilyl III*b* Methoxime	Pertrimethylsilyl IV Methoxime	Pertrimethylsilyl VI Methoxime	Pertrimethylsilyl V Methoxime
M	773 (10)	861 (0)	627 (0)	715 (0)
M − 15	758 (45)	846 (8)	612 (7)	700 (10)
M − 30	743 (21)	831 (3)	597 (8)	685 (30)
M − 15 − 90	668 (75)	756 (6)	522 (10)	610 (20)
M − 30 − 90	653 (70)	741 (14)	507 (20)	595 (60)
M − 30 − side chain	—	—	524 (10)	524 (75)

[a] Only common major fragments are presented.

spectively. The abundance of the mass fragment at *m/e* 553 is again consistent with the fragmentation trend for I*b* and trimethylsilyl II*b*. As expected from its structure, no derivative was formed with methoxyamine.

A single change of the structure of I*b* and II*b* capable of being derived from metabolic origin reflected definitive and interpretable mass spectral changes as discussed when subjected to the GLC–mass spectral conditions. A combination of these single structural changes should still be reflected by the combined changes in the corresponding mass spectral fragmentation patterns. To test this hypothesis, GLC–mass spectra[15] of the pertrimethylsilyl and pertrimethylsilyl methoxime derivatives of VII, VIII, XI–XIII, and XV were examined (Tables II and IV).

The mass spectrum of VII gave major fragments at *m/e* 729 (M − 15), 714 (M − 30), 598 (M − 15 − side chain), and 582 (M − 30 − neutral side chain), consistent with its structure (Table IV). Its pertrimethylsilyl methoxime derivative (Table VI) also yielded a fragmentation pattern similar to the pertrimethylsilyl methoxime derivative of V. In this case, the mass fragments are interpretable as a combination of methyl and silanol cleavages.

The mass spectral data of the pertrimethylsilyl derivative of XII (Table II) followed closely the combined fragmentation patterns of trimethylsilyl IV and trimethylsilyl X*b*. As expected, a prominent peak at *m/e* 611, representing side-chain and silanol cleavages, was present. Similarly, trimethylsilyl XI gave fragments consistent with its structure (Table II). Side-chain and methyl cleavages giving rise to a fragment at *m/e* 540 appeared to be favorable processes, although the M − 30 peak was also prominent.

Plausible combined metabolic changes on the A and D rings and the side chain resulted in XIII. The pertrimethylsilyl derivative of this synthetic compound exhibited major mass fragments (Table II) at *m/e* 768, 698, 613, 598, and 468, corresponding to M − 30, M − 30 − 90, M − 205 (side chain), M − 15 − 205, and M − 30 − 320, respectively. The last fragment is accountable by a retro-Diels–Alder fragmentation reaction involving the D ring. These fragmentation patterns are essentially the appropriate combinations of those of pertrimethylsilyl derivatives of XI and XII. In addition, no mass spectral changes were observed for this compound after double treatment with methoxime and pertrimethylsilyl formation, indicating a reduction of the carbonyl group.

Combination of side-chain oxidation and demethylation on ring A generated XV (Table II). Major fragments of its pertrimethylsilyl derivatives included the usual methyl and combination of methyl and silanol cleavages. Similar to the acid, the fragment at *m/e* 788 (M − 30) represented one prominent pathway. Nevertheless, a substantial fragment at *m/e* 611 (M − 90 − side chain) marked the demethylation characteristic of the A ring. Consistent with its structure, no methoxime formation was observed.

Table VI—Mass Spectral Analysis [a] of Pertrimethylsilyl Methoxime Derivatives of Ia and IIa Analogs

Presumed Fragmentation	*m/e* (Percent Relative Intensity) Pertrimethylsilyl VIII Methoxime	Pertrimethylsilyl[b] VII Methoxime
M	685 (0)	773 (0)
M − 15	670 (24)	758 (6)
M − 30	655 (28)	743 (60)
M − 73	—	700 (3)
M − 15 − 90	580 (52)	668 (8)
M − 30 − 90	565 (100)	653 (30)
M − 30 − side chain	582 (52)	582 (60)

[a] Only common major fragments are presented. [b] All of the intensities here have been multiplied by 10.

METABOLISM

Both I*a* and II*a* undergo rather extensive hepatic metabolism, giving rise to several fluorescent metabolites (7, 8, 11). Metabolic changes involving *O*-demethylation on the A ring, reduction of the carbonyl group on the side chain, and reductive glycosidic cleavage were proposed on the basis of solid probe mass spectral analysis of the aglycone and other spectroscopic data. With the described GLC–mass spectral techniques, VII, X*b*, XI, and XIII were identified in the hydrolysate of a partially purified rabbit bile 1-butanol extract. The GLC properties of these pertrimethylsilyl and pertrimethylsilyl methoxime derivatives gave excellent chromatographic peaks, and the GLC–mass spectra of these metabolites were identical in all respects with those of the synthetic samples. Details of the isolation and identification of these metabolites, including the evidence of a lack of a metabolic change of the amino sugar moiety on the I*a* metabolites, will be published separately.

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ACKNOWLEDGMENTS

Supported by Contracts N01-CM-23241 and N01-CM-67065 of the Division of Cancer Treatment, National Cancer Institute.

The assistance of Dr. Sue Rotschaeffer, Analytical Facilities, California Institute of Technology, Pasadena, Calif., for the high-resolution mass spectra is appreciated.

NOTES

Limit Test for Determination of Free Ferricyanide and Ferrocyanide in Sodium Nitroferricyanide

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Received January 12, 1978, from the *Research Products and Diagnostics Section, Quality Control Department, Hoffmann-La Roche Inc., Nutley, NJ 07110.* Accepted for publication April 25, 1978.

Abstract □ A simple and rapid procedure for the isolation and determination of free ferricyanide and ferrocyanide in sodium nitroferricyanide (nitroprusside) was developed. The method, employing aluminum oxide column chromatography, was sensitive enough to detect ferricyanide and ferrocyanide at the 0.05% level.

Keyphrases □ Sodium nitroferricyanide (nitroprusside)—prepared solutions, chromatographic separation and spectrophotometric analyses of free ferricyanide and ferrocyanide □ Ferricyanide and ferrocyanide, free—chromatographic separation and spectrophotometric analyses in prepared solutions of sodium nitroferricyanide (nitroprusside) □ Antihypertensives—sodium nitroferricyanide (nitroprusside), prepared solutions, chromatographic separation and spectrophotometric analyses of free ferricyanide and ferrocyanide

Sodium nitroferricyanide (nitroprusside) (I) has been used extensively as an analytical reagent, primarily for the detection of organic compounds such as ketones and aldehydes, and recently it has gained stature as a potent antihypertensive in acute hypertensive crises (1–6). Since the mode of administration is intravenous infusion, a high quality substance is required. As a result, analytical methodology for the determination of purity and potency of I in pharmaceutical preparations is needed. Methodology and specifications are available for reagent grade material (7–9), but these methods are not suitable for a parenteral grade pharmaceutical product.

Two potential contaminants in I are free ferricyanide and ferrocyanide. Existing methods for the analysis of these two impurities are limit tests in which a faint color or turbidity is detected in the solution if the contaminants are present (7, 9). However, at levels near and below 0.05% ferricyanide and ferrocyanide, it is extremely difficult to detect these subtle changes in the sample solution. This paper reports a simple and rapid procedure for the isolation and determination of ferricyanide and ferrocyanide in I at the 0.05% level. The method involves the chromatographic separation of ferricyanide and ferrocyanide from I on an alumina column by a series of elutions of increasing acid strengths. Ferricyanide and ferrocyanide are determined spectrophotometrically at 415 nm.

The limit of 0.05% is realistic because of the relatively lower toxicity of ferricyanide and ferrocyanide *versus* I. The oral LD_{50} in rats for ferricyanide and ferrocyanide is 1600 mg/kg; for I, it is 20 mg/kg (10, 11).

EXPERIMENTAL

Reagents—Neutral aluminum oxide[1], activity grade I, was used as received. All other chemicals were ACS reagent grade or equivalent and were used without further purification.

Column Preparation—Amber glass columns, 1.45 cm i.d., fitted with polytef stopcocks, were each dry filled with 7 g of aluminum oxide.

Standard Solution—A solution containing 15.5 mg of potassium ferricyanide and 19.9 mg of potassium ferrocyanide trihydrate in 100 ml of 1 *N* HNO_3 was prepared.

Sample Solutions—Solutions of sodium nitroferricyanide at a concentration of 200 mg/ml were prepared in 1 *N* HNO_3.

Procedure—Aliquots of 2.0 ml of sample solutions and 2.0 ml of standard solution were placed on individual columns. The columns were eluted with 35 ml of 0.5 *N* HNO_3, which was discarded. Ferricyanide was then eluted with 25 ml of 2 *N* HNO_3, and ferrocyanide was eluted with 25 ml of 6 *N* HNO_3. The absorbances of the solutions were measured in 1-cm cells at 415 nm. If the sample absorbances were lower than the standard absorbances, the sample contained less than 0.05% each of ferricyanide and ferrocyanide.

RESULTS AND DISCUSSION

Complete separation of ferricyanide $[Fe(CN)_6^{3-}]$ and ferrocyanide $[Fe(CN)_6^{4-}]$ from I $[Na_2Fe(CN)_5NO]$ was achieved by column chromatography with aluminum oxide as the adsorbent. Determination at the 0.05% level was achieved by comparing the sample absorbances at 415 nm to those of the standards.

The dependence of ferricyanide and ferrocyanide recovery on the acid strength of the final elution was investigated. To each of several columns, 0.2 mg of ferricyanide was applied and the columns were eluted as pre-

[1] Woelm, ICN Pharmaceuticals.

0022-3549/78/1200-1752$01.00/0