Drawbacks in the use of 23-nor-deoxycholic acid standards

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Summary 23-Nor-deoxycholic acid is widely used as internal standard in gas–liquid chromatographic studies of bile acids. Two batches of this compound, submitted to conventional alkaline hydrolysis of bile acid conjugates, were found to be transformed into a product with chromatographic properties different from those of "authentic" 23-nor-deoxycholic acid. To identify this "new" product a comparison was made between chromatographic properties, mass spectra, and NMR spectra of 23-nor-deoxycholic acid before and after alkaline hydrolysis. The results indicate that the "new" compound is the true 23-nor-deoxycholic acid while the product present in the two batches examined is a monoacetate derivative.—**Attili, A. F., M. Angelico, L. Capocaccia, U. Pièche, and A. Cantafora.** Drawbacks in the use of 23-nor-deoxycholic acid standards. *J. Lipid Res.* 1982. **23:** 211–215.

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23-Nor-deoxycholic acid (NDC) is widely used as internal standard in gas–liquid chromatography (GLC) for the determination of bile acids in biological fluids (1, 2). Fortuitously, we observed that the trifluoroacetate (TFA) derivative of NDC, submitted to conventional conditions of the alkaline hydrolysis step in GLC analysis of bile acids, had a different relative retention time from that of the TFA derivative of "authentic" NDC.

Lepage, Fontaine, and Roy (3) recently reported, on the other hand, that alkaline hydrolysis of NDC does not lead to the formation of any artifactual product. Stimulated by these puzzling observations, a systematic study was carried out in order to identify the structure of NDC and of the "hydrolyzed" NDC.

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; MS, mass spectrometry; TFA, trifluoroacetate; TMS, trimethylsilyl; NDC, 23-nor-deoxycholic acid standard; NMR, nuclear magnetic resonance.

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MATERIALS AND METHODS

Chemicals

23-Nor-5β-cholanic acid,3α,12α diol (23-nor-deoxycholic acid) was obtained from Steraloids Inc., Wilton, NH (Batch N. 7101, mp 218–220°C) and from Research Plus Steroid Laboratories Inc., Denville, NJ (no batch number, mp 213–218°C). Deoxycholic acid was obtained from Supelco Inc., Bellefonte, PA.

Alkaline hydrolysis

Method 1 (mild alkaline hydrolysis): 4 ml of 2 N NaOH and 4 ml of aqueous methanol (1:1 by vol) were autoclaved for 4 hr at 115°C (1).

Method 2 (strong alkaline hydrolysis): 4 ml of 5 N NaOH and 4 ml of methanol were autoclaved at 120°C for 4 hr (4).

Method 3: 5 ml of 1.25 N NaOH were autoclaved at 115°C for 4 hr. After cooling the hydrolysis mixtures were acidified to pH 1 with 5 N HCl and then extracted three times with three volumes each of ethyl ether. The ethereal extract was evaporated to dryness.

Methylation

Bile acid methyl esters were obtained by adding 2 ml of 2,2-dimethoxypropane, 3 ml of methanol, and 0.05 ml of concentrated HCl (5) to the dry extract. This mixture was kept at 37°C for 1 hr and then dried under a gentle stream of nitrogen.

Derivatization

TFA derivatives were obtained by adding 0.1 ml of trifluoroacetic anhydride to the bile acid methyl esters. This solution was kept at 37°C for 15 min and then dried under nitrogen. Partial trimethylsilyl ether (TMS) derivatives were prepared as described by Eneroth et al. (6). Acetate derivatives were prepared as described by Roovers, Evrard, and Vanderhaege (7).

Gas–liquid chromatography (GLC)

Glass columns were packed with either 3% QF$_1$ on Chromosorb WHP-DMCS, 80/100 mesh, for TFA or acetate derivatives or with 1.25% HI-EFF-8BP on Chromosorb WHP-DMCS, 100/120 mesh, for TMS derivatives. Column length: 1.8 m × 2 mm ID. Operating conditions: oven, 230°C; injector, 240°C; flame ionization detector, 250°C; carrier gas (nitrogen) flow rate, 30 ml/min. A Hewlett-Packard model 5840A gas chromatograph was used.

Thin-layer chromatography (TLC)

TLC was carried out using the solvent system S11 of Eneroth (8) (trimethylpentane–ethylacetate–acetic acid

10:10:2, by vol). Precoated silica gel plates (SIF$_{254}$, Carlo Erba, Milan, Italy) 0.25 mm thick were employed.

Mass spectrometry (MS)

The mass spectra of bile acid derivatives were obtained with an LKB model 2091 mass spectrometer interfaced to a PDP 11 computer (Digital Equipment Corp., Maynard, MA). Ion source was kept at 250°C. The accelerating voltage was 3500 V. The energy of bombarding electrons was 70 eV. Samples were analyzed as methyl esters by direct inlet of 5–10-μg aliquots. Probe temperature was raised from 25 to 180°C in 7 min. Spectra were recorded from m/e 0 to 600 in 2 sec.

Nuclear magnetic resonance (NMR)

The ^{1}H and ^{13}C NMR spectra of underivatized NDC and "hydrolyzed" NDC were obtained with a Varian XL-100/15 instrument (Varian Associates, Palo Alto, CA) using dimethyl sulfoxide-d$_6$ as solvent and tetramethyl silane as internal standard.

RESULTS AND DISCUSSION

NDC, when submitted to mild (method 1) or strong (method 2) alkaline hydrolysis is completely transformed (yield 99.5%) into a product displaying different chromatographic characteristics from those obtained with NDC. This also occurs if hydrolysis is carried out, as in routine studies in our laboratory, in the absence of methanol (method 3). We improperly called this product "hydrolyzed" NDC.

The TFA derivative of "hydrolyzed" NDC showed a retention time relative to deoxycholic acid different from that of NDC (0.74 and 1.08, respectively). The chromatogram in **Fig. 1** was obtained by submitting 200 μg of NDC to alkaline hydrolysis and then adding a further 200 μg of NDC to the cooled hydrolysis mixture.

"Hydrolyzed" NDC is formed in an alkaline pH and fails to form at the same temperature and pressure in neutral aqueous milieu.

The TLC characteristics of underivatized NDC and "hydrolyzed" NDC are also different. In fact, with the TLC solvent system S11 of Eneroth (8), NDC and "hydrolyzed" NDC, as extracted from hydrolysis milieu, showed R_f values of 0.36 and 0.30, respectively, thus confirming that the formation of "hydrolyzed" NDC is exclusively due to alkaline hydrolysis.

Attempts were then made to identify the structure of "hydrolyzed" NDC.

As a first step, the methyl esters of NDC and "hydrolyzed" NDC were submitted to MS. Comparison of mass spectra (**Fig. 2**) showed that the two compounds displayed similar fragmentographic patterns. The main
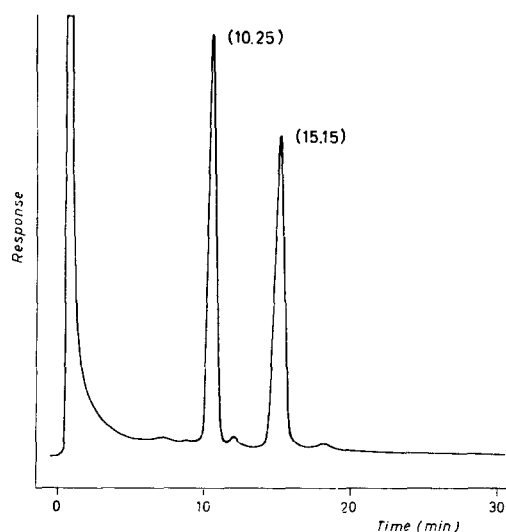

Fig. 1. GLC separation of methyl ester TFA derivatives of "hydrolyzed" NDC (RT 10.25 min) and NDC (RT 15.15) on 3% QF$_1$ column.

difference observed was the intensity of the m/e 356 fragment, corresponding to the loss of two water molecules from the molecular ion (m/e 392). This fragment was hardly evident in either spectra. At higher values of m/e only NDC presents a very weak signal (I = 0.45%) at m/e 416.

The mass spectra exclude that the marked chromatographic differences between the compounds are due to severe modification of the molecule during hydrolysis. The similarity of mass spectra may be compatible with two possibilities: conformational changes or the presence in one of the two compounds of a group easily ionizable by electron impact.

To discriminate between these hypotheses, NMR spectra of the two compounds were recorded. Surprisingly, the ^{13}C NMR spectrum of NDC (the "authentic" standard) had 24 separately resolved signals (**Fig. 3**), two of which corresponded to carboxylic carbons and thus did not correspond to 23-nor-deoxycholic acid. One (δ_e = 174.0 ppm) can be assigned to the carboxylic end of the molecule, the other (δ_e = 169.6 ppm) to a carbon linked by an esteric linkage to one of the hydroxyl groups of the molecule. Another signal (δ_e = 20.897) could be assigned to a methyl group of an acetate unit (9). The presence, in the molecule of NDC, of a methyl group linked to a carboxylic carbon was further confirmed by determining the ^{1}H NMR spectrum which showed a characteristic signal at δ_e = 2.02 ppm. The ^{13}C NMR spectrum of "hydrolyzed" NDC corresponded to that of 23-nor-deoxycholic acid, having 23 separately resolved signals, one of which, with a δ_e of 174.3 ppm, corresponded to the carboxylic carbon.

It can thus be reasonably concluded that NDC is a monoacetate derivative of 23-nor-deoxycholic acid, while
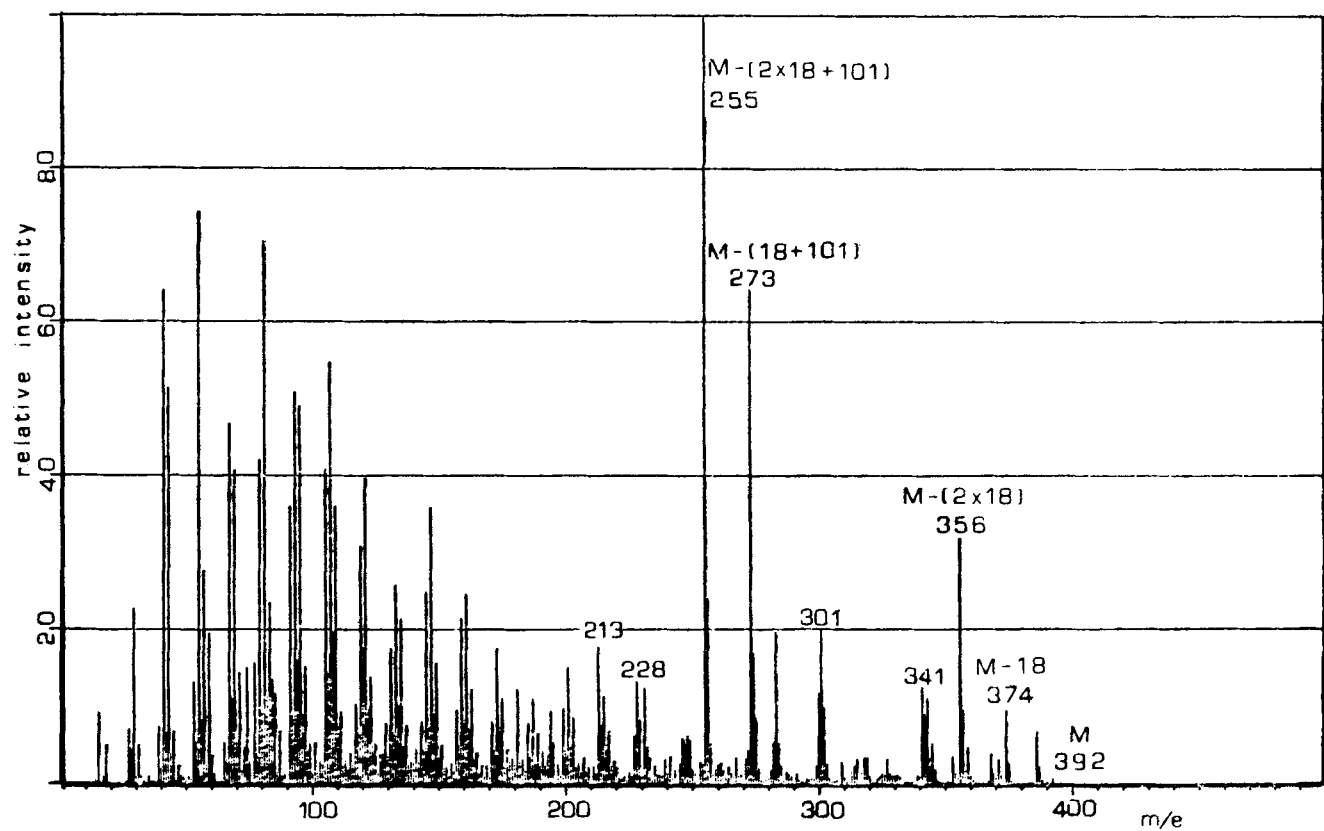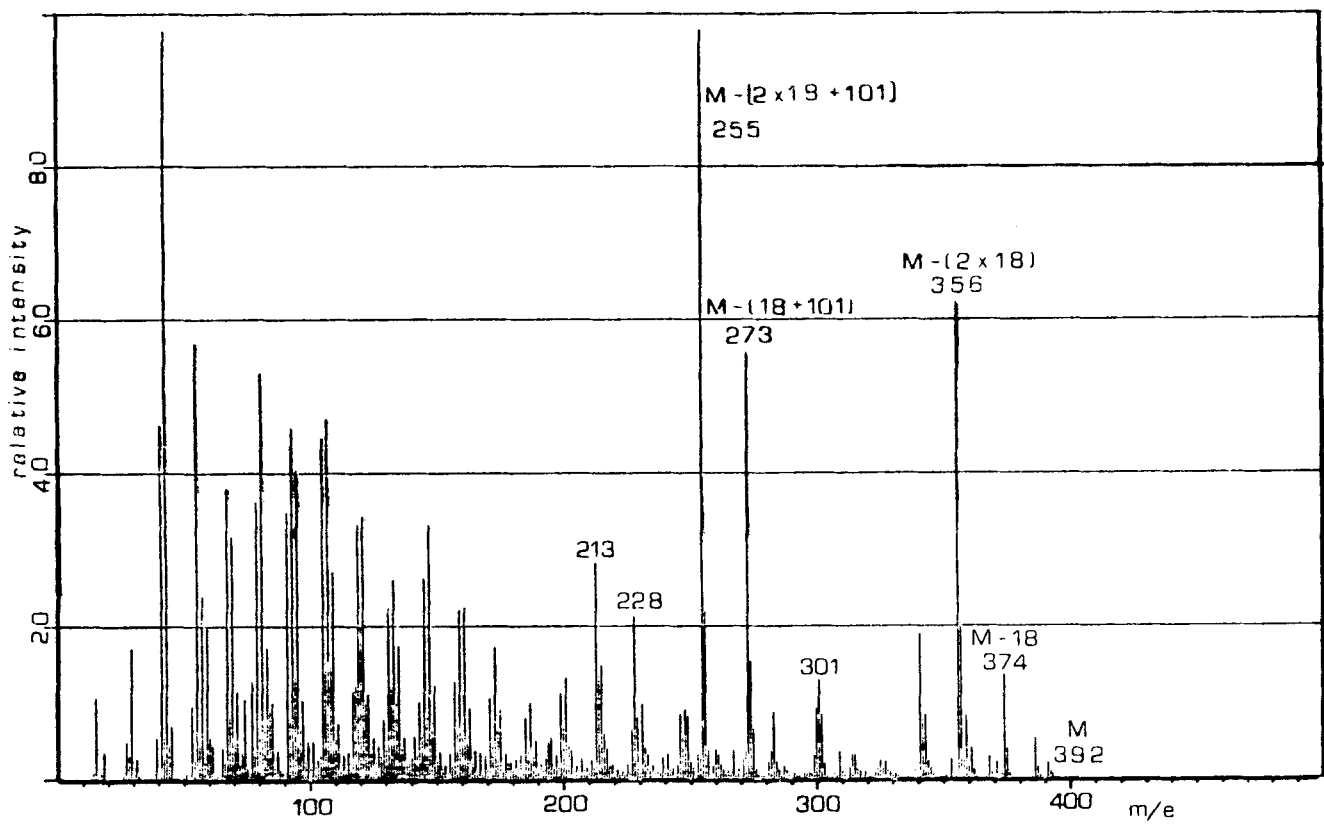
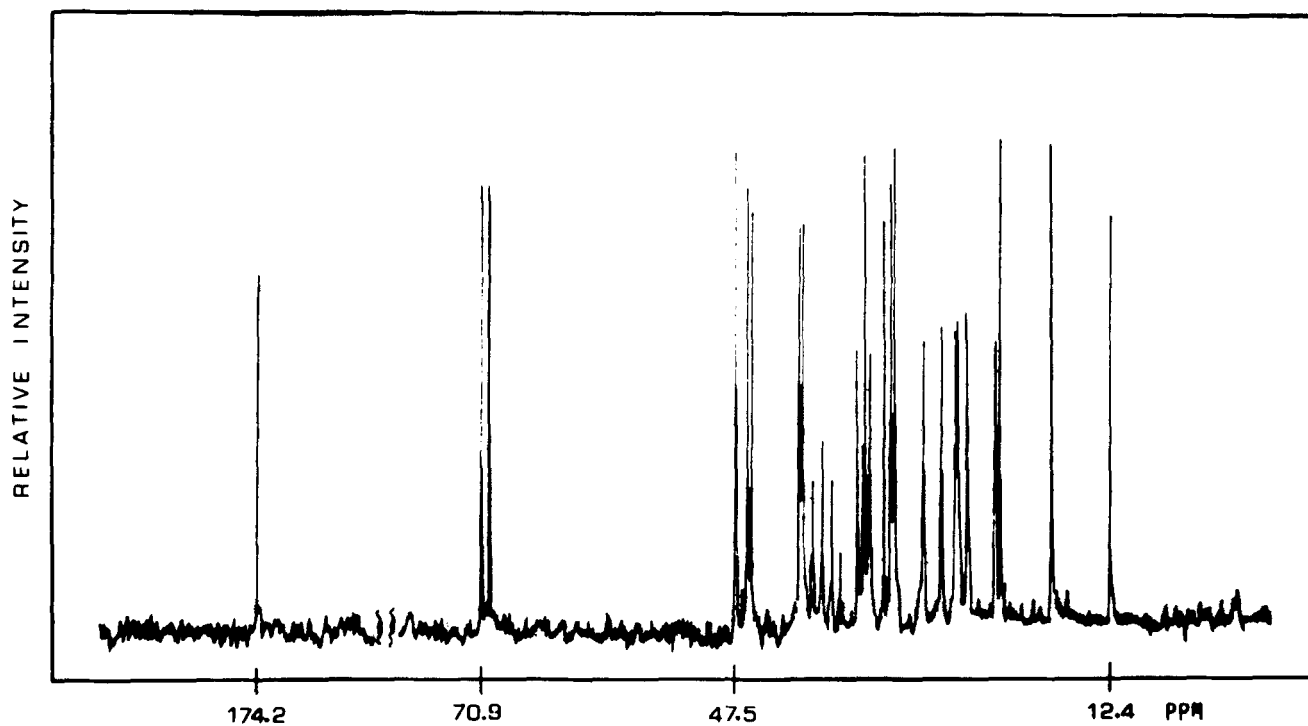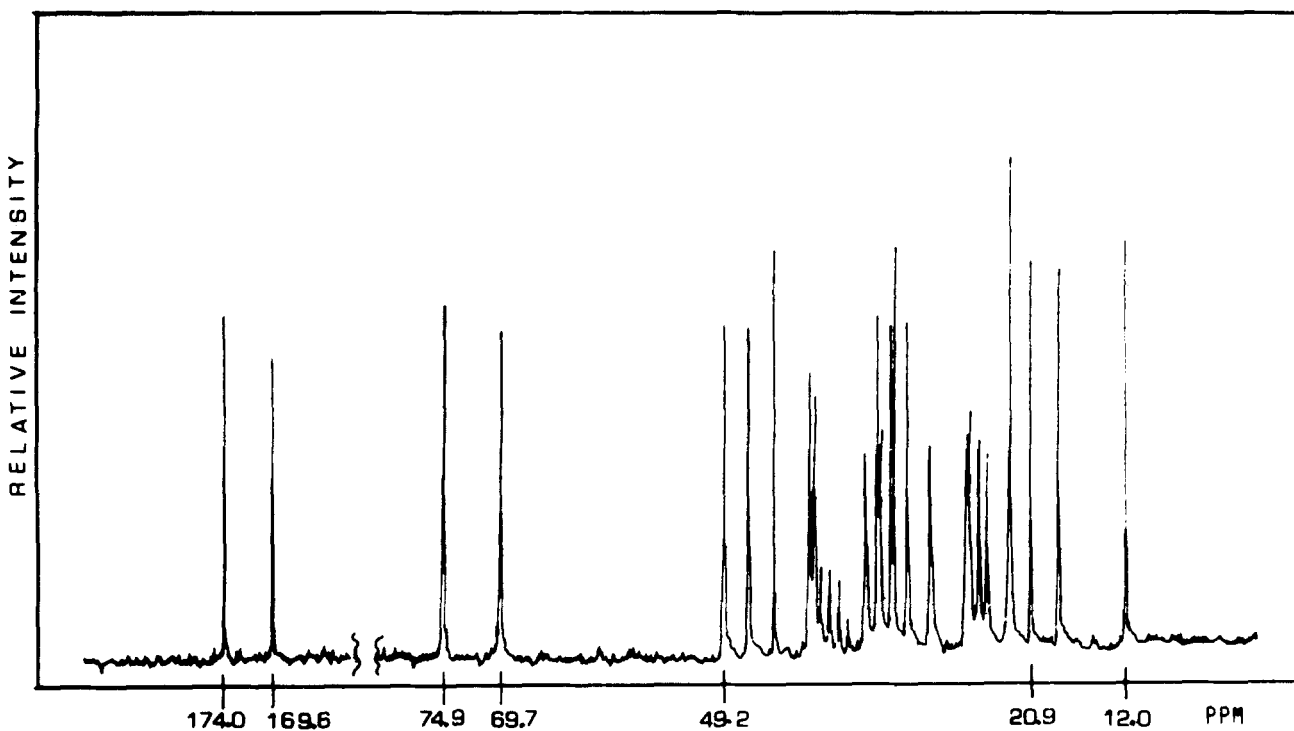**Fig. 2.** Mass spectra of methyl esters of NDC (upper) and "hydrolyzed" NDC (lower).

Fig. 3. ^{13}C NMR spectra of NDC (upper) and "hydrolyzed" NDC (lower).

the "hydrolyzed" NDC almost certainly corresponds to the structure of the authentic substance. The fact that the ^{13}C spectrum of NDC shows only 24 peaks instead of the 25 expected, can be explained by supposing an accidental overlapping of two resonances of the backbone carbons.

In the light of our conclusions, differences between MS spectra can be explained as follows. As far as concerns NDC, the fragment at m/e 356 is derived from the loss of one molecule of water and one molecule of acetic acid, and in "hydrolyzed" NDC from the loss of two water molecules. The corresponding peak is higher in NDC than in "hydrolyzed" NDC since the loss of hydroxyl groups is less likely than carboxylic acid loss from the ester group (10). Furthermore, according to Szczepanik, Hachey, and Klein (11), the presence of a fragment at m/e 416 may be attributed to a nor structure with 1 AcO + 1 C = C.

Derivatization experiments can also confirm this hypothesis. In fact, the retention times of TFA derivatives correspond to a monoacetate mono-TFA derivative in NDC and to a di-TFA derivative in "hydrolyzed" NDC. Since acetate groups tend to slow elution and TFA groups to accelerate elution, with respect to simple methyl esters on QF columns, it is clear why "hydrolyzed" NDC was eluted first and NDC later. GLC analysis of acetate derivatives displayed the same retention time for NDC and "hydrolyzed" NDC because both compounds gave rise to the same derivative. This is in agreement with the results of Lepage et al. (3), who reported that rigorous alkaline hydrolysis of NDC did not lead to the formation of any artifactual product and that recoveries were excellent. TMS derivatives also displayed a marked difference in retention time on HI-EFF-8BP column (relative retention times with respect to deoxycholic acid of NDC and "hydrolyzed" NDC were 1.52 and 0.71, respectively) as silylation cannot displace the AcO group in the NDC.

In conclusion, the product present in the two batches was a monoacetate derivative of 23-nor-deoxycholic acid. Conversely, the product obtained during alkaline hydrolysis, which we considered as an artifact, was indeed the true 23-nor-deoxycholic acid.

This finding is of no consequence for those who use acetate derivatives for GLC analysis but may be of considerable importance when TFA or TMS derivatives are used.■

REFERENCES

1. van Berge-Henegouwen, G. P., R. N. Allan, A. F. Hofmann, and P. Y. S. Yu. 1977. A facile hydrolysis-solvolysis procedure for conjugated bile acid sulfates. *J. Lipid Res.* 18: 118–122.
2. Roda, E., R. Aldini, G. Mazzella, A. Roda, C. Sama, D. Festi, and L. Barbara. 1978. Enterohepatic circulation of bile acids after cholecystectomy. *Gut.* 19: 640–649.
3. Lepage G., A. Fontaine, and C. C. Roy. 1978. Vulnerability of bile acids to alkaline hydrolysis. *J. Lipid Res.* 19: 505–509.
4. Palmer, R. H., and M. G. Bolt. 1971. Bile acid sulfates. I. Synthesis of lithocholic acid sulfates and their identification in human bile. *J. Lipid Res.* 12: 671–679.
5. Ali, S. S., and N. B. Javitt. 1970. Quantitative estimation of bile salts in serum. *Can. J. Biochem.* 48: 1054–1057.
6. Eneroth, P., B. Gordon, R. Ryhage, and J. Sjövall. 1966. Identification of mono- and dihydroxy bile acids in human feces by gas–liquid chromatography and mass spectrometry. *J. Lipid Res.* 7: 511–523.
7. Roovers, J., E. Evrard, and H. Vanderhaege. 1968. An improved method for measuring human blood bile acids. *Clin. Chim. Acta.* 19: 449–457.
8. Eneroth, P. 1963. Thin-layer chromatography of bile acids. *J. Lipid Res.* 4: 11–16.
9. Stothers, J. B. 1972. Carbon-13 NMR spectroscopy. Academic Press, New York. 439–441.
10. Dias, J. R., and B. Nassim. 1980. Mass spectral fragmentation of bile acid ester derivatives. *J. Org. Chem.* 4: 337–345.
11. Szczepanik, P. A., D. L. Hachey, and P. D. Klein. 1976. Characterization of bile acid methyl ester acetate derivatives using gas–liquid chromatography, electron impact, and chemical ionization mass spectrometry. *J. Lipid Res.* 17: 314–334.