Quantitation of Osteolytic Phytosteryl Acetates in Human Serum[1] (37456)

J. O. WHITNEY, H. HORN,[2] AND G. S. GORDAN

Department of Medicine, University of California, San Francisco, Ca. 94143

In 1967, we reported that nanogram amounts of short-chain esters of campesterol, stigmasterol, β-sitosterol and 7-dehydrositosterol, identified by gas–liquid chromatography (GLC)/mass spectroscopy (MS) in human breast cancer extracts, could also be detected in serum (1). Either or both stigmasteryl acetate and 7-dehydrositosteryl acetate were found in the serum of 25 breast cancer patients, but in only 10 out of 43 normal women. Because these compounds possess potent calcium mobilizing activity, we postulated that they may play a role in human breast cancer, a condition frequently associated with hypercalcemia. We were unable to achieve quantitation by GLC, however, due to inconsistent losses during extraction and purification.

Since that time, the presence of phytosterols in breast cancer tissue and serum has been confirmed (2, 3) but Haddad *et al.* (3) found no significant difference in the concentration of free phytosterols in the plasma of breast cancer patients, lactating mothers and normal women. They were unable to detect stigmasteryl acetate. The campesterol and β-sitosterol content of rat hepatoma, moreover, was shown to be similar to that of normal rat liver, but phytosteryl acetates were not measured (4).

We have found that addition of suitable internal standard to serum before extraction makes possible quantitation of phytosteryl acetates at the nanogram level with acceptable precision by GLC. Application of this method provided evidence that stigmasteryl acetate and 7-dehydrositosteryl acetate are not peculiar to breast cancer.

Materials and Methods. In order to determine the precision of the method, authentic stigmasteryl acetate was added to pooled plasma. Four concentrations (1.0, 10.0, 100.0, and 1000.0 ng/ml of plasma) were measured.

All glassware was silanized and solvents were redistilled before use. Standards were obtained from commercial sources or were prepared from free sterols by acetylation in pyridine. Exact composition of standards was determined by GLC.

Cholesteryl acetate was used as an internal standard. Relative retention time and relative detector response of the phytosteryl acetates to cholesteryl acetate are shown in Table I.

Internal standard (2.5 μg/ml) was added to 10.0 ml of serum before extraction by the procedure of Folch *et al.* (5). Each extract, applied as a band, was subjected to thin-layer chromatography (TLC) on 0.5 mm Silica Gel G in petroleum ether:ethyl ether:acetic acid (90:10:1). Bands were sprayed with 0.001% aqueous Rhodamine 6G and viewed under ultraviolet light. The steryl acetates were separated from both free sterols and other short- and long-chain steryl esters, scraped from the plate, and eluted with chloroform. The eluate was dried under nitrogen and redissolved in 5 μl of chloroform, all of which was used for GLC.

GLC was performed with a Hewlett Packard Model 402 Biomedical Gas-Chromatograph equipped with a hydrogen flame ionization detector. Six foot U-shaped columns, 2.0 mm i.d., were packed with 3.8% SE-30 on 80–100 mesh Gas-Chrom Q. Column temperature was 240° and helium was the carrier gas. Peaks were identified by comparing their retention times (relative to cholesteryl acetate) with those of known standards. Peak areas were measured by

[1] Aided by grants from the U.S. Public Health Service (CA,10166) and from the A. J. Stone Fund.

[2] On Sabbatical leave from the Department of Endocrinology, Hebrew University, Hadassah Medical School, Jerusalem, Israel.

TABLE I. Relative Retention Time (RRT) and Relative Detector Response (RDR) of the Phytosteryl Acetates to Cholesteryl Acetate.[a]

Compound	RRT	RDR
Campesteryl acetate	1.307 ± 0.006	1.054 ± 0.021
Stigmasteryl acetate	1.410 ± 0.005	1.048 ± 0.033
β-Sitosteryl acetate	1.625 ± 0.003	0.962 ± 0.031

[a] Values are mean of 6 determinations ± 1 SD.

triangulation and concentrations calculated in relation to the area of the internal standard.

All samples were from fasting subjects. Nine normal women and nine normal men were included in the study for comparative purposes. Since phytosterols could originate in the diet, ten vegetarians who had taken strict vegetarian diets for at least one year were tested. Eleven patients with disseminated breast cancer were studied. One of these patients was examined twice; another on three occasions. Four patients with benign breast disease, fibroadenoma or dysplasia, were investigated.

Results and Discussion. Cholesteryl acetate proved to be an adequate internal standard. It could not be detected in five separate samples of pooled plasma. It behaved like the phytosteryl acetates in extraction and TLC but was adequately separated by GLC. A relatively large amount of standard (2.5 μg) was used so that any endogenous impurities contributed only neglibly to the area of the standard. At this concentration the standard acted as a carrier preventing the loss or irreversible absorption of nanogram amounts of phytosteryl acetate during extraction and TLC. Ten milliliters of serum

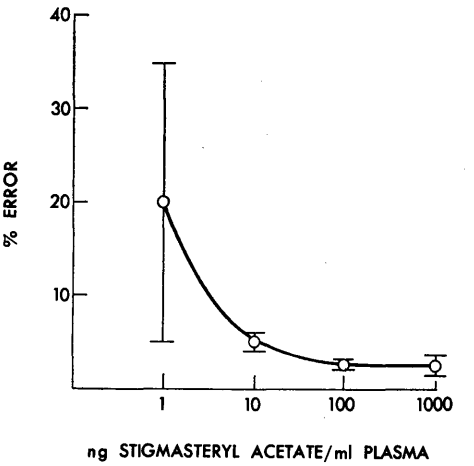

FIG. 1. % error in the GLC measurement of various concentrations of stigmasteryl acetate added to 10 ml of pooled blood plasma before extraction. Each point is the mean of 3 or 4 extractions ± 1 SD.

was the smallest sample size which gave reproducible results. With lesser volumes, losses in extraction and purification left amounts of phytosteryl acetates too small for flame detection.

As shown in Fig. 1, stigmasteryl acetate could be measured with at least 95% precision from 10 to 1000 ng/ml. Below 10 ng, the error of measurement was greater. Table II gives the mean serum concentration of phytosteryl acetates in the categories studied. In no instance was 7-dehydrositosteryl acetate detected. Stigmasteryl acetate and the other phytosteryl acetates investigated were not elevated in breast cancer patients. We conclude that these compounds are not directly

TABLE II. Concentration of Phytosteryl Acetates in the Serum of Normal Men and Women, Vegetarians, Breast Cancer Patients and Patients with Benign Breast Disease.[a]

Subjects	Campesteryl acetate (ng/ml)	Stigmasteryl acetate (ng/ml)	β-Sitosteryl acetate (ng/ml)
Normal men (9)[b]	27.8 ± 18.8	29.6 ± 13.8	16.7 ± 8.8
Normal women (9)	2.2 ± 1.6	23.3 ± 17.0	10.1 ± 3.0
Vegetarians (5 men, 5 women)	7.4 ± 3.9	7.3 ± 3.2	9.6 ± 5.1
Breast cancer patients (14)	2.7 ± 0.6	5.6 ± 1.2	5.9 ± 1.3
Benign breast disease (4)	2.0 ± 0.8	1.0 ± 0.5	3.0 ± 0.6

[a] Values are mean ± SEM.

[b] Numbers in parentheses represent number of individual samples.

related to breast cancer.

It is now well established that free phytosterols are absorbed to a small extent by the human intestine (6, 7). Normal men and women and vegetarians showed great variation in phytosteryl acetate content, perhaps reflecting a dietary origin. There was less variation in the phytosteryl acetate content of breast patients, suggesting that they have different dietary patterns than normal subjects.

It is noteworthy that the concentration of stigmasteryl acetate in all subjects studied is in the same range as that of campesteryl acetate and β-sitosteryl acetate. Investigations of the free phytosterol content of human serum have shown that campesterol and β-sitosterol are present in far greater quantities than stigmasterol (3, 7). If the phytosterols are solely of dietary origin and equally absorbed, one would expect that the relative amount of phytosteryl acetates would be similar to the amount of free compounds. Since stigmasteryl acetate is present in concentrations similar to or higher than the other phytosteryl acetates, acetylation of stigmasterol may exceed that of the other phytosterols. It is equally likely that stigmasteryl acetate is more abundant in food than other phytosteryl acetates and is absorbed directly by the intestine without previous hydrolysis.

Summary. A gas-chromatographic method for the quantitation of phytosteryl acetates in human serum at the nanogram level is described. Cholesteryl acetate is utilized as an internal standard added to the serum before extraction. Using this method, serum phytosteryl acetate levels of breast cancer patients are not significantly different from those of other subjects.

1. Gordan, G. S., Fitzpatrick, M. E., and Lubich, W. P., Trans. Ass. Amer. Phys. **80**, 183 (1967).

2. Day, E. A., Malcom, G. T., and Beeler, M. F., Metabolism **18**, 646 (1969).

3. Haddad, J. G., Couranz, S. J., and Avioli, L. V., J. Clin. Endocrinol. **30**, 174 (1970).

4. Nes, W. R., Thompi, N. S., and Lin, J. T., Cancer Res. **32**, 1264 (1972).

5. Folch, J., Lees, M., and Sloane Stanley, G. H., J. Biol. Chem. **226**, 497 (1957).

6. Gould, R. G., Jones, R. J., LeRoy, G. V., Wissler, R. W., and Taylor, C. B., Metab. Clin. Exp. **18**, 652 (1969).

7. Salen, G., Ahrens, E. H., and Grundy, S. M., J. Clin. Invest. **49**, 952 (1970).

Received April 3, 1973. P.S.E.B.M., 1973, Vol. 143.