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Precise quantitative determination of phytosterols, stanols, and cholesterol metabolites in human serum by capillary gas–liquid chromatography

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Abstract

Total lipid extraction, solid-phase extraction, saponification, derivatization to trimethylsilyl ether derivatives, then capillary gas–liquid chromatography were used for quantitative analysis of sitosterol, campesterol, stigmasterol, sitostanol, campestanol, lathosterol, desmosterol, and lanosterol in human serum. Details of quality control integral to the accuracy and precision of analyses are included. The method limits of detection and quantitation, respectively, ranged from 0.05 μg/ml and 0.2 μg/ml for sitostanol to 0.4 μg/ml and 1.2 μg/ml for campesterol and campestanol. Analytes were measured at concentrations of 120 ng/ml to 6 μg/ml with standard deviations of 0.02 to 0.12 μg/ml for 55 analyses of a control serum sample conducted over a 2-month period.

Keywords: Phytosterols; Stanols; Sitosterol; Campesterol; Stigmasterol; Sitostanol; Campestanol; Lathosterol; Desmosterol; Steroids

1. Introduction

Phytosterol supplements decrease serum cholesterol and may reduce the risk of cardiovascular disease [1]. In a feeding trial involving a stanol-supplemented margarine spread [2], a method was needed for sensitive and precise determination of sitosterol, campesterol, stigmasterol, sitostanol, campestanol, lathosterol, and desmosterol in human serum. Quantitation of serum sterols and stanols (5,6-dihydrosterols) is complicated by the presence of numerous other lipids and low concentrations (0.2–13 μg/ml) of the analytes relative to cholesterol (~1500–3000 μg/ml). A method [3,4] previously reported for measurement of phytosterols, stanols, and cholesterol metabolites [5] lacked sufficient detail about preparation of serum samples, chromatographic conditions, and analytical sensitivity, and was not readily reproduced. Other reports [6,7] do not include validation of the separation and quantification of phytosterols and stanols as well as cholesterol metabolites (e.g. desmosterol, lathosterol). Sample clean-up was not reported in some of

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the published methods, and others used thin-layer chromatography. Carryover and column/injector contamination are problems in the absence of serum sample clean-up prior to chromatography. Very polar compounds (e.g. phospholipids) are an important consideration in sterol analysis when semi-polar or polar stationary phases are employed, especially when analyzing a large number of samples.

A 60-m capillary column with a moderately polar stationary phase (14% cyanopropylphenyl–86% dimethylpolysiloxane) was used, and a one-step solid-phase extraction was added to remove highly polar compounds from the saponified total lipid extract. Epicholesterol was used as an internal standard. Since the primary concern was to detect changes in low levels of serum sterols and stanols as a result of dietary supplementation, we required a very precise assay. Consequently, numerous quality control measures were implemented. In this paper we give a detailed description of the methodology and quality control employed for the precise quantitative analysis of sitosterol, campesterol, stigmasterol, sitostanol, campestanol, lathosterol, and desmosterol in human serum.

2. Experimental

2.1. Materials

2.1.1. Chemicals

Epicholesterol was obtained from Steraloids (Wilton, NH, USA). Beta-sitosterol, campesterol, stigmasterol, sitostanol, fucosterol, lathosterol, lanosterol, and desmosterol, cholestanol, squalene, pyrogallol, cyclohexane, and pyridine were purchased from Sigma Chemical (St. Louis, MO, USA). Campestanol was from Research Plus (Bayonne, NJ, USA), and bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA w/1% TMCS) was obtained from Alltech Associates (Deerfield, IL, USA). Hexane, chloroform, methanol, isopropanol, sodium chloride (ACS grade), and potassium hydroxide (NF/FCC grade) were from Fisher Scientific (Fairlawn, NJ, USA). Absolute ethanol was obtained from AAPER Alcohol and Chemical (Shelbyville, KY, USA). All solvents were HPLC grade and used without further purification.

2.1.2. Serum samples

Serum samples (1–2 ml) frozen in 2-ml cryovials were obtained from a human feeding trial conducted at Pennsylvania State University (University Park, PA, USA) and Pennington Biomedical Research Center (Baton Rouge, LA, USA). Subjects consumed a stanol-supplemented margarine that provided 3 g per day stanols and 16 g per day fat in a controlled feeding trial; details of the study are reported elsewhere [2]. Serum samples were stored at −80°C until analyzed.

2.2. Instrumentation

2.2.1. Gas chromatography

Gas chromatography was performed with a Perkin Elmer GC Autosystem™ (Perkin Elmer, Norwalk, CT, USA), an Rtx®-1701 (14% cyanopropylphenyl–86% dimethylpolysiloxane) capillary column (60-m×0.25-mm I.D., 0.25-μm film thickness; Restek, Bellefonte, PA, USA), and a flame ionization detector with hydrogen (45 ml/min) and air (450 ml/min) as fuel source. Hydrogen at 1.18 ml/min was the carrier gas. Operating conditions were: injection temperature, 280°C; detector temperature, 280°C; oven temperature, 265°C; split ratio, 9:1 (split vent flow 9.44 ml/min); injection volume, 1 μl; head pressure 21 p.s.i. Detector signal output was monitored by computer (Dell Dimension XPS D233; Dell Computer, Round Rock, TX, USA) and all chromatograms and data were generated and processed using Turbochrom™ Workstation version 6.0.2.1 software (Perkin Elmer).

2.3. Quality control measures

2.3.1. Preparation of glassware

All glassware (and other labware, such as stir bars and spatulas) was scrupulously cleaned and rinsed with distilled deionized water and acetone, then immediately before use, rinsed with chloroform, then ethanol, and allowed to air dry. All glass test tubes were silanized with 5% dimethyldicholorosilane in hexane (Sigma).

2.3.2. Other measures to prevent contamination

Since sterols are ubiquitous in the lab (e.g. in skin secretions, rubber septa, vegetable oils and fats), and because the levels of these compounds in serum are

low (ng to μg/ml), it is essential to prevent sample contamination to preclude falsely elevated values of the analytes. Precautions against cross-contamination are especially necessary in laboratories where vegetable oils or other materials containing significant levels of phytosterols are analyzed. Tight control of measuring volumes and weights is essential to ensure the precision and accuracy of the assay in the ng to μg range.

2.3.3. Calibration of pipettes and balances

For all critical volume measurements (e.g. standard solutions, serum aliquots taken for analysis), automatic pipettes were carefully calibrated for accuracy and precision immediately before use, using the solvent of the solution to be dispensed (e.g. chloroform or ethanol for standards, water for serum), and a five-decimal place analytical balance, calibrated with certified standard weights. The pipetter was held in the hand 5 min to warm it to a constant temperature before calibration. To determine accuracy, the weighed volumes were compared to the expected mass, determined from the density of the liquid at the measured temperature of the liquid aliquoted [8]. For preparing standard mixtures and for performing standard additions to serum samples, volumetric pipettes were used.

2.3.4. Preparation of standard solutions

2.3.4.1. Internal standard. Test tubes containing internal standard were prepared in batches. Epicholesterol (3 mg) was weighed to the nearest 0.01 mg into a 1-l volumetric flask, brought to volume with absolute ethanol, and thoroughly mixed. The solution was dispensed in 1.6-ml aliquots into each of approximately one-hundred 25×150 mm glass test tubes using a bottle-top dispenser that had been calibrated for accuracy and precision (Section 2.3.3), yielding 5 μg epicholesterol per tube (the exact amount of internal standard was calculated for the exact weights and volumes measured). The tubes were capped under nitrogen and stored at <10°C for up to 2 months. Immediately prior to use, the solvent was evaporated at 60°C with a stream of nitrogen.

2.3.4.2. Reference and calibration standards. For each component, a 6 μg/ml stock standard solution was made. Three mg of component was weighed to the nearest 0.01 mg and diluted to 500 ml in a volumetric flask, using absolute ethanol for sterols and chloroform for stanols. Calibration standard solutions containing 30 μg/ml of the analyte and 50 μg/ml epicholesterol were prepared in batches. Solvent was evaporated at 60°C with a stream of nitrogen, then the mixture was derivatized with 1 ml pyridine/BSTFA as described for samples (Section 2.4.4). The solution was distributed among ten autosampler vials with volume reduction inserts (~100 μl/vial) and stored at −60°C. These standards were used to determine calibration factors and to check instrument calibration throughout the study.

Two reference standard mixtures were prepared and run with each batch of samples to determine run-specific retention times for the analytes. 'Reference A' contained 5 μg/ml each of sitosterol, lathosterol, campestanol, stigmasterol, and sitostanol. 'Reference B' contained 5 μg/ml each of desmosterol, campesterol, lanosterol, and fucosterol.

2.3.5. Preparation of quality control material

Accutrol™ Human Serum (normal, lyophilized, #A2034; Sigma) was used as a control material. No standard serum with certified or reference values for phytosterols could be found, and homogeneous fortification of the serum with sitostanol was not feasible. Because of the potential heterogeneity of reconstituted aliquots of lyophilized serum (due to variable loss of dried material during reconstitution), and because we required a large number of homogeneous aliquots to adequately monitor assay precision, we prepared a composite of reconstituted Accutrol™ serum and dispensed a batch of 380 1.25-ml aliquots.

One-hundred vials of the lyophilized Accutrol™ serum (from the same lot) were reconstituted and combined. In batches of ten at a time, vials were removed from the refrigerator and each was reconstituted with 5 ml of distilled deionized water, mixed well, and allowed to stand for 10–20 min at room temperature (20–23°C), then placed in the refrigerator (2–4°C).

Within 3 days, a composite of the reconstituted serum was made in a walk-in cold room (7°C). As much as possible of it was transferred from each of the 100 serum vials to a 500-ml glass reagent bottle, which was then covered and stirred at medium–high speed for 1 h. The serum was then dispensed in 1.25-ml aliquots into 380 2-ml microcentrifuge tubes

in batches of ten at a time, using a new pipette tip after every ten samples. The tubes were stored at −80°C.

2.4. Sample preparation

2.4.1. Total lipid extraction

Serum samples were thawed overnight (12–20 h) at 2–4°C then mixed thoroughly. Using a calibrated pipetter, 0.90 ml serum was dispensed into a test tube containing the internal standard (5 μg epicholesterol).

Total lipid was extracted by a modified Folch procedure [9], using chloroform–methanol (2:1, v/v) followed by a 0.9% saline wash, as follows. After mixing the sample, 0.9 ml of methanol was added, then tubes were capped and vortexed at high speed for 15 s. Samples were then shaken for 10 min on an orbital shaker at 300 rpm. If phases separated before orbital shaking, the tubes were first shaken briefly by hand. Chloroform (2×9.0 ml) and 6.75 ml 0.9% (w/v) aqueous sodium chloride were added, tubes were capped tightly and vortex mixed at high speed for 15 s, then shaken at 300 rpm on the orbital shaker for 10 min (if phases began to separate before orbital shaking, tubes were first shaken by hand for a few seconds to remix). Samples were then centrifuged at 200 *g* at 20°C for 10 min to facilitate phase separation.

2.4.2. Saponification

The chloroform (lower) layer from each total lipid extraction was transferred to a 25×150 mm test tube. Care was taken not to transfer any of the emulsion or solid material at the interface between the upper and lower phases, by pushing out any material that entered the pipette tip before drawing in the chloroform solution. A new pipette tip was used for each sample.

The total lipid extract was saponified by a modification of a method previously described by Thompson and Merola [10]. Solvent was evaporated completely from the total lipid extract at 60°C with a stream of nitrogen. Ethanol with 3% (w/v) pyrogallol (8 ml) was added to each sample. Tubes were capped and vortexed at high speed for 10 s, then 0.5 ml of 1.28 g/ml aqueous potassium hydroxide was added. Tubes were capped tightly, vortexed for 10 s at high speed, sonicated for 30 s, then heated in a test tube rack in a 85–89°C water bath for 30 min, ensuring that the bottom half of the tubes were immersed. Tubes (in rack) were shaken and inverted vigorously after 1, 2, 5, 10, 15, 20, 25, and 30 min of heating (a second test tube rack was held firmly over the top during shaking to hold the tubes). The capped tubes were sonicated again for 30 s, cooled under running tap water, then slowly opened.

Cyclohexane (20 ml), then 12 ml distilled deionized water were added to each sample. Tubes were recapped, shaken vigorously for a few seconds, then rocked 15 min on a platform mixer at maximum speed (Thermolyne Vari-Mix platform mixer; Barnstead/Thermolyne No. 48725). No residual material was evident in the bottom or on the sides of tubes after shaking. Samples were centrifuged at 200 *g* at 20°C for 10 min to accelerate phase separation. For each sample, 17.0 ml (2×8.5 ml) of the cyclohexane (upper) layer was transferred to a 25×150 mm test tube, with care taken not to include any of the black, aqueous phase. Cyclohexane was evaporated at 60°C with a stream of nitrogen. Chloroform (0.5 ml) was added to each sample, then samples were vortexed for 10 s at high speed.

2.4.3. Solid-phase extraction

A modification of the method of Kaluzny et al. [11] was used to separate the neutral lipid fraction (containing sterols and stanols) from more polar lipids. Bond Elute LRC® aminopropyl solid-phase extraction (SPE) cartridges 10 ml/500 mg (Varian Associates, Harbor City, CA, USA) were used. The SPE cartridges were attached to a vacuum manifold (Visaprep DL™; Supelco, Bellefonte, PA, USA) set at slightly less than 1″ Hg with valves closed. Cartridges were conditioned with 4 ml hexane, which was discarded. A 16×125 mm glass test tube was placed under each outlet. With vacuum valves closed and using a new pipette tip for each sample, sample was transferred into the SPE cartridge, directly onto the adsorbent, repeating until as much as possible had been transferred. With vacuum set at slightly less than 1″ Hg, valves were opened one by one until the sample solution had just completely entered the adsorbent, then turned off until all cartridges had been set. Elution solvent (chloroform–isopropanol, 2:1, v/v, 0.5 ml) was added to each

cartridge. The solvent was then drawn under vacuum through the cartridges one at a time until it reached just above the column bed, then valve was closed until all samples had been taken to this stage. An additional 3.5 ml of the elution solvent was added to each tube, then all valves were opened and the solvent was allowed to pass completely (dropwise) through the cartridges. The vacuum was then increased to draw the columns dry (~8″ Hg for at least 2 min).

2.4.4. Derivatization

Solvent was completely evaporated from samples at 60°C with a stream of nitrogen. Freshly prepared derivatization reagent (pyridine–BSTFA with 1% TMCS, 1:1, v/v), 100 μl, was pipetted into each tube and vortex mixed for 10 s. The reagent was then carefully swirled around and ~2″ up the sides of each tube. Tubes were allowed to stand at least 1 min, vortex mixed for 3 s, then allowed to stand again for at least 1 min. Each sample was transferred using a 9″ glass pasteur pipette into a 100-μl limited volume insert in an autosampler vial, sealed with a PTFE/silicone septum cap, and stored at <10°C until assayed by GC (within 1 week).

2.4.5. GC analysis and quantitation

Analyte retention times were determined using commercially available standards. Analysis of selected serum samples without added epicholesterol internal standard confirmed lack of sample constituents that co-eluted with the internal standard. In routine assays, component peaks were identified by the adjusted retention time, which compensated for any shifts in expected retention times based on that of the internal standard [12]. A relative search window (based on a percent of the absolute retention time) was then applied to the adjusted retention time as the criteria for identification. The size of the relative search window was determined based on the degree of absolute retention time shift that was expected during a run and the proximity of other peaks expected in the samples.

After determining the method was linear over the expected concentration ranges (below), analytes were quantified based on a single point response factor (RF):

$$\mathrm{RF} = \frac{R_C/R_{IS}}{M_C/M_{IS}}$$

where R_C is the peak area for the component, R_{IS} is the peak area for the internal standard, M_C is the amount of component and M_{IS} is the amount of internal standard added to the sample. For each analyte, the RF was determined at 3 μg as the average of triplicate analysis of standards. In samples, each component (M_C) was determined using the response factor for that component and R_C and R_{IS} determined from the GC analysis.

Baseline events (timed events in the instrument method which effect integration of peaks) were set up in the standard (routine) Turbochrom™ method file based on optimal consistency of peak integration for the control sample. In sample runs, individual attention was paid to the chromatograms to avoid and correct any obvious integration problems (usually as a result of small peak size and interfering peaks), by modifying baseline events as necessary.

2.5. Linearity, limit of detection, and limit of quantitation studies

The expected concentration ranges for desmosterol, lathosterol, campesterol, sitosterol, stigmasterol, campestanol, sitostanol, lanosterol, and fucosterol were tested for linearity. For each compound, standard solutions were made at four or five different concentrations between 0.05 μg/ml and 25 μg/ml. Each standard solution was derivatized and assayed in duplicate at all concentrations, except in quintuplicate at 0.75 μg/ml for desmosterol, lanosterol, fucosterol, campestanol, and sitostanol and at 5 μg/ml for campesterol, sitosterol, and stigmasterol. For each compound, a linear regression was performed on the data (component/internal standard area ratio vs. component/internal standard mass ratio). All curves were linear, above the limit of detection, in the concentration range tested ($r^2 = 0.99$ or greater in all cases).

The method limit of detection (MLOD) and limit of quantitation (MLOQ) for sitosterol, campesterol, lathosterol, and desmosterol were determined from results of the repeated analyses ($n = 55$) of 0.9-ml aliquots of the Accutrol™ control serum composite. For each component, the MLOD was calculated as

three times the standard deviation of the mean and the MLOQ was estimated as ten times the standard deviation of the mean of the 55 measurements.

Since sitostanol and campestanol were not present in the control serum and lanosterol and stigmasterol were barely detectable, the MLODs and MLOQs for these components were determined by analysis of Accutrol™ serum spiked with known amounts of standards, as follows.

A chloroform solution containing 0.5 μg/ml of stigmasterol, sitostanol, and lanosterol and 1 μg/ml of campestanol was prepared. One ml of the solution was added to each of seven 0.9-ml Accutrol™ composite aliquots, which were then assayed as described above, except for the volume of chloroform added during the total lipid extraction which was reduced by the volume of standard added. MLOD was calculated as three times the standard deviation of the mean, and MLOQ was calculated as ten times the standard deviation of the mean of the seven measurements.

We also checked the method performance in assaying smaller serum aliquots, which may be of interest when the sample volume is limited. Seven 0.5-ml and seven 0.25-ml aliquots of the control serum composite unspiked and spiked with sitostanol, campestanol, stigmasterol, and lanosterol in amounts near the MLOQ determined for analysis of 0.9 ml serum were assayed. A chloroform solution of sitostanol, campestanol, stigmasterol, and lanosterol was added to three 0.25 ml and three 0.5-ml aliquots of the Accutrol™ control composite such that final concentrations were 1 μg/ml campestanol and 0.5 μg/ml sitostanol, stigmasterol, and lanosterol. Additionally, seven serum samples from a clinical feeding trial [2], in which sitostanol was detected, were re-assayed in 0.25-ml aliquots and compared to the results from analysis of the 0.9-ml aliquots.

2.6. Recovery studies

Recovery analysis was based on fortification of samples of the control serum composite. For the analytes not present in the control material, fortification was performed at four different concentrations and reported for all concentrations at or above the MLOQ determined for the individual compounds. For the compounds present in the control serum, samples were fortified at one concentration, which was approximately the same as that determined from analysis of the control serum. For these samples, the values calculated for the unfortified control sample analyzed and chromatographed in the same assay were subtracted from the fortified control sample values to determine recovery:

$$\%R = \frac{M_{fc} - M_c}{M_f} \times 100$$

where $\%R$ is percent recovery, M_{fc} is the raw amount in μg of component determined in the fortified sample, M_c is the raw amount in μg of component in the unfortified material, and M_f is the fortification amount in μg.

2.6.1. Precision studies

For sitosterol, campesterol, lathosterol, desmosterol, and lanosterol, precision was evaluated from the results of repeated analyses of the control sample (Accutrol™). For sitostanol, campestanol, and stigmasterol, precision was estimated from repeated analyses of the control composite spiked with a standard mixture of these components.

2.7. Routine quality control

Prior to analysis of unknown samples, three assay batches of 5–13 aliquots of the serum control material (Section 2.3.5) were analyzed over a period of 2 weeks. A control chart showing the mean and tolerance limits (±3 times the standard deviation) was established for each analyte and used to monitor subsequent assays ([13], pp. 130–132). An aliquot of the control serum composite was assayed with each batch of samples.

3. Results and discussion

3.1. Chromatograms

Representative chromatograms for standards, the control serum, and a serum sample from the diet intervention (which contained sitostanol and campestanol) are shown in Fig. 1.

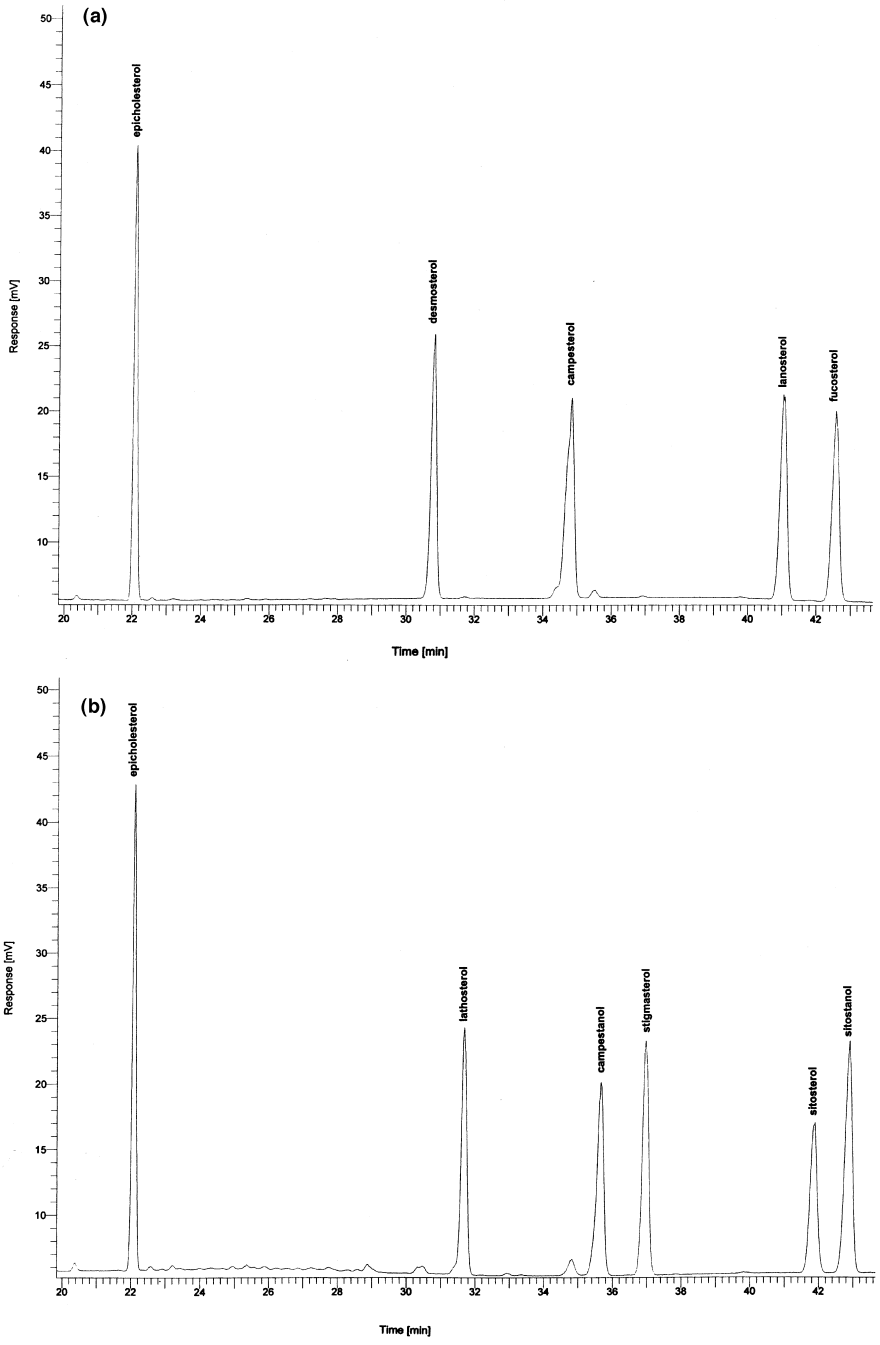

Fig. 1. Representative chromatograms (including epicholesterol internal standard) for reference standards (a and b), Accutrol™ control serum composite (c), and a sitostanol-containing serum sample from the feeding trial (d). Chromatography conditions were as follows: Column: Rtx®-1701 (14% cyanopropylphenyl–86%dimethylpolysiloxane) (Restek, Bellefonte, PA, USA), 60 m×0.25 mm I.D., 0.25-μm film thickness; carrier gas: hydrogen, 1.18 ml/min (linear velocity, 40 cm/s) was the carrier gas; split ratio, 9:1 (split vent flow 9.44 ml/min); column head pressure 21 p.s.i.; injection temperature, 280°C; detector temperature, 280°C; oven temperature, 265°C (45 min), then 10°C/min to 280°C hold 3.5 min.

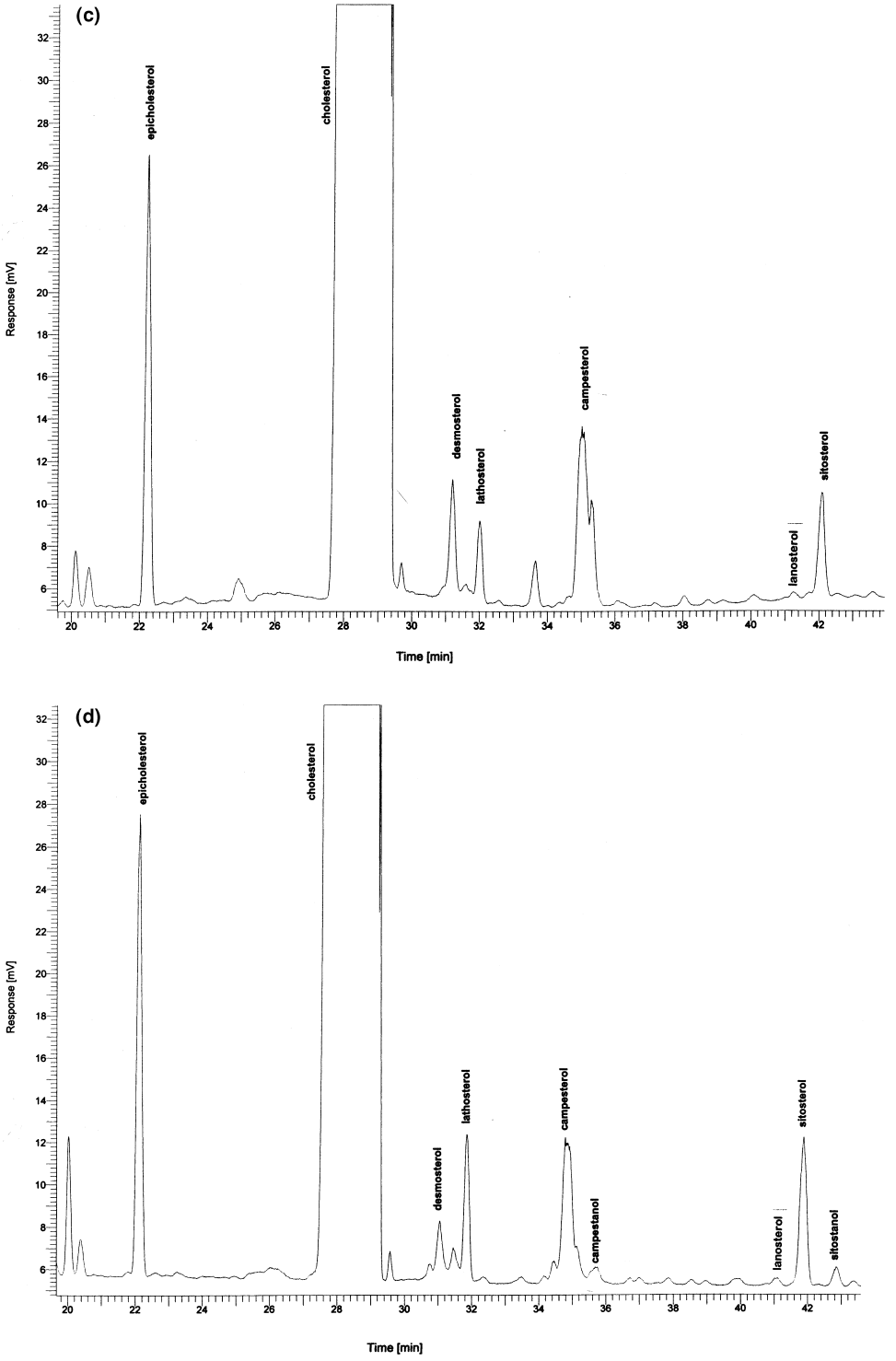

Fig. 1. (*continued*)

Table 1
Retention times (RT), relative retention, and response factors for analytes, internal standard, and other components[a]

Component	RT (min)	Relative retention[a]	Response factor[b]
Epicholesterol (internal standard)	22.16	1.0	
Sitosterol	41.98	1.89	0.8576 (0.0025)
Campesterol	34.92	1.58	0.8816 (0.0025)
Stigmasterol	37.10	1.67	0.8946 (0.0053)
Sitostanol	43.01	1.94	0.8489 (0.0048)
Campestanol	35.79	1.61	0.7596 (0.0036)
Lathosterol	31.79	1.44	0.8504 (0.0019)
Desmosterol	30.92	1.40	0.8794 (0.0025)
Lanosterol	41.17	1.86	0.8704 (0.0053)
Fucosterol	42.72	1.93	0.7479 (0.0014)
Squalene	12.81	0.58	(Not determined)

[a] (Retention time of component)/(Retention time of epicholesterol).
[b] Standard deviation of triplicate determinations is shown in parentheses.

3.2. Retention times and response factors

Table 1 summarizes the retention times, relative retention times, and response factors for sitosterol, campesterol, stigmasterol, sitostanol, campestanol, desmosterol, lathosterol, and also lanosterol, fucosterol, and squalene using the present method. Whereas absolute retention times sometimes varied up to 0.2 min. (e.g. from slight changes in column activity and/or head pressure), relative retention times were consistent within 0.02 min across runs.

3.3. Linearity, limits of detection, limits of quantitation, and analytical ranges

Table 2 summarizes the method linear range, limit of detection (MLOD), and limit of quantitation (MLOQ) for each component (sitosterol, campesterol, stigmasterol, sitostanol, campestanol, desmosterol, lathosterol, lanosterol) in the present study. The method enabled detection of all components at concentrations of <0.4 μg/ml (<1 μmol/l). The greatest sensitivity was for sitostanol (0.05 μg/ml; 0.1 μmol/l) and the least for campesterol and campestanol (0.4 μg/ml; 1 μmol/l). The decreased sensitivity for campesterol and campestanol was largely due to interfering peaks in the region which limited the reliability of peak area estimates at low analyte levels. The MLOQ ranged from 0.2 μg/ml (0.5 μmol/l) for sitostanol to 1.2 μg/ml (3 μmol/l) for campesterol and campestanol. Response was linear in the range analyzed for each component (MLOQ to 10 or 25 μg/ml).

The statistically determined MLOD and MLOQ (defined as three and ten times the standard deviation of replicates, respectively; 13, p. 80) included effects of inconsistent automated peak integration due to background interferences. The statistically determined MLOQ for lanosterol was 0.1 μg/ml, and lanosterol was present in the unfortified control serum at approximately this level. However, uncertainty in quantitation of lanosterol occurred due to occasional incomplete resolution from a nearby peak, and the results for 55 analyses of the control material

Table 2
Analytical parameters for gas chromatography of phytosterols and cholesterol metabolites in serum sample assayed by the present method

Component	Method limit of detection[a]		Method of quantitation[a]		Linear range confirmed
	(μg/ml)	μ mol/l	(μg/ml)	μ mol/l	(μg/ml)
Sitosterol	0.1	0.2	0.4	1	0.4–25
Campesterol	0.4	1	1.2	3	1.2–25
Stigmasterol	0.1	0.2	0.2	0.5	0.2–25
Sitostanol	0.05	0.1	0.2	0.5	0.2–10
Campestanol	0.4	1	1.2	3	1.2–10
Lathosterol	0.2	0.5	0.6	2	0.6–25
Desmosterol	0.1	0.3	0.4	1	0.4–10
Lanosterol	0.2	0.5	0.5	1	0.5–10

[a] For analysis of a 0.9-ml serum aliquot according to the method as described.

Table 3
Recovery of sterols and stanols from serum

Compound	Amount spiked (μg/ml)	Number of replicates	Average recovery[a] (%)
Campestanol	1.1	10	106 (10.8)
	1.7	3	121 (1.1)
Campesterol	6.4	3	105 (2.1)
Desmosterol	2.6	3	74 (0.0)
Lanosterol	1.1	3	97 (0.6)
	1.6	3	106 (1.5)
Lathosterol	1.2	3	100 (2.1)
Sitostanol	0.3	3	117 (3.0)
	0.6	10	104 (4.1)
	1.1	3	107 (7.2)
	1.7	3	106 (1.5)
Sitosterol	2.0	3	102 (0.6)
Stigmasterol	0.3	3	118 (5.9)
	0.6	10	106 (3.0)
	1.1	3	100 (0.6)
	1.7	3	104 (1.7)

[a] Standard deviation of replicates is shown in parentheses.

and control material spiked with various levels of lanosterol (Table 4) suggested that the practical MLOQ was somewhat higher than 0.1 μg/ml. Therefore, the MLOQ and MLOD for lanosterol reported in Table 2 are estimated based on these practical findings.

3.4. Recovery

Recovery of spiked standards is shown in Table 3 and appeared quantitative for lathosterol, campesterol, sitosterol, sitostanol, campestanol, lanosterol, and stigmasterol. Recovery of fortified desmosterol was 74%. The reason for this result is unknown; we did not attempt to repeat the experiment.

3.5. Precision

Table 4 summarizes the mean, standard deviation, and relative standard deviation for sitosterol, campesterol, desmosterol, lathosterol, and lanosterol concentrations assayed in the control material over the course of 23 assay batches by two analysts over a 2-month period. Because sitostanol, campestanol, and stigmasterol are not components of the Accutrol™ serum and could not be readily and homogeneously fortified, no control data were generated for these compounds. For sitosterol, campesterol, lathosterol, and desmosterol, the standard deviation over all analyses ranged from 0.02 μg/ml (lanosterol) to 0.12 μg/ml (campesterol). The within-assay standard deviations were similar to the overall RSD for all components. The relative standard deviation for lanosterol was much higher than for other components (14%), due to the low concentration in the control sample (0.1 μg/ml).

Quality control charts for lathosterol and campesterol in the control serum are shown in Fig. 2.

3.6. Method performance in assay of smaller volumes of serum

Table 5 shows data from the analyses of fortified (sitostanol, campestanol, lanosterol, stigmasterol) and unfortified 0.25-, 0.5-, and 0.9-ml aliquots of the control serum composite. All compounds were for-

Table 4
Summary of assay precision for control serum composite (55 aliquots) assayed in a total of 23 assay batches by two analysts during a 2-month period

Component	Overall mean μg/ml (SD)	Overall RSD[a] (μg/ml)	Average within-assay SD[a] (μg/ml)	Average within-assay RSD (μg/ml)
Sitosterol	1.99 (0.04)	2.2	0.04	1.8
Campesterol	6.18 (0.12)	1.9	0.10	1.6
Lathosterol	1.19 (0.06)	5.0	0.04	3.2
Desmosterol	2.13 (0.04)	1.8	0.06	3.1
Lanosterol	0.12 (0.02)	14.3	0.02	14.9

[a] RSD = Relative standard deviation, as percent of the mean; SD = standard deviation.

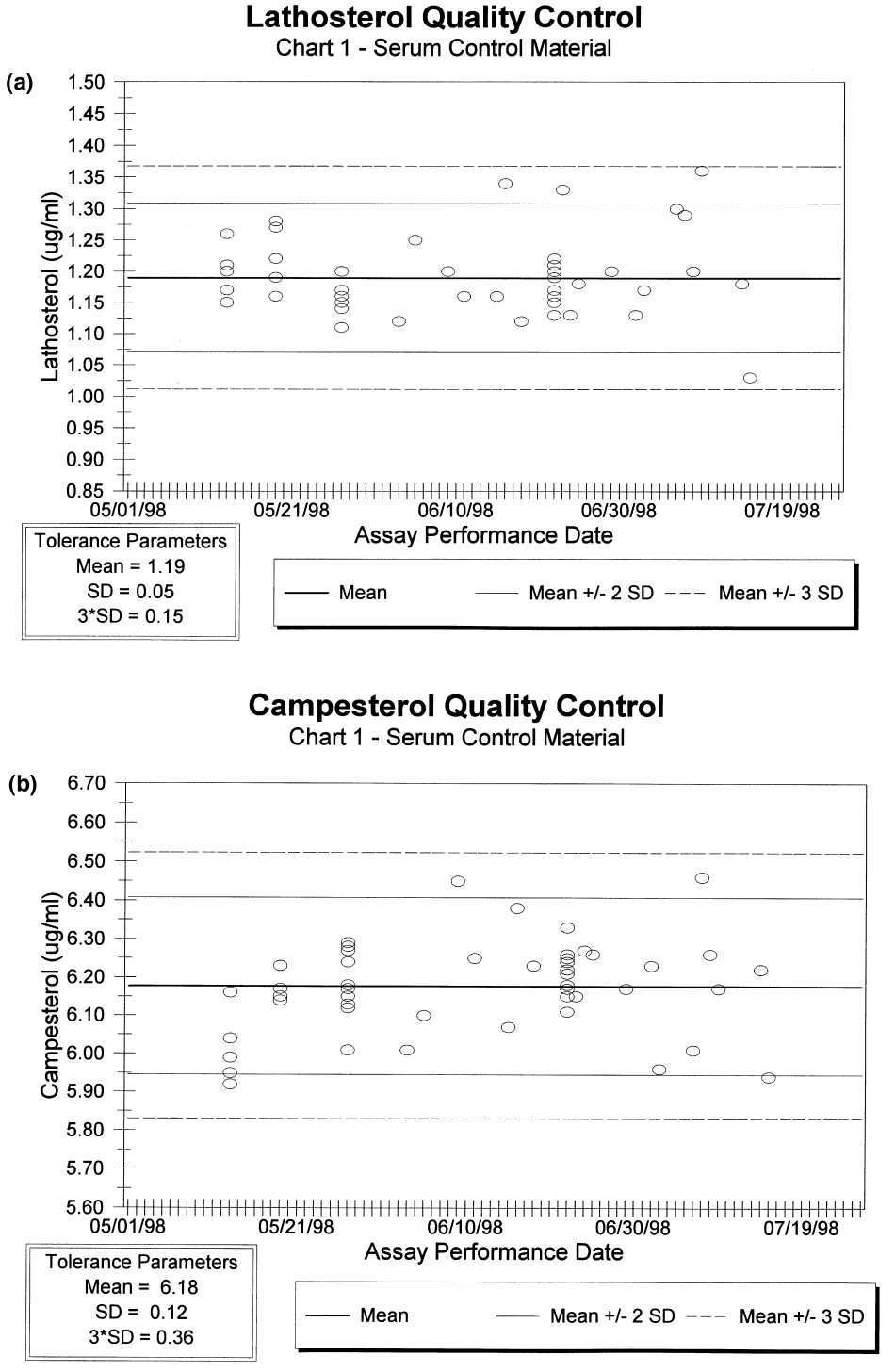

Fig. 2. Examples of quality control charts for Accutrol™ serum control material for a 2-month period. (a) Lathosterol; (b) campesterol. SD = standard deviation.

Table 5
Mean and standard deviation (in parentheses) for analyses of 0.25- and 0.5-ml aliquots and for sitostanol-, campestanol-, and lanosterol-spiked Accutrol™ control serum samples

Accutrol™ sample[a]	Sitosterol (μg/ml)	Campesterol (μg/ml)	Lathosterol (μg/ml)	Desmosterol (μg/ml)	Lanosterol (μg/ml)	Stigmasterol (μg/ml)	Sitostanol (μg/ml)	Campestanol (μg/ml)
0.25 ml	2.00 (0.026)	6.33 (0.140)	1.17 (0.088)	1.94 (0.230)	ND	ND	0.04 (0.105)	ND
0.5 ml	1.92 (0.024)	6.24 (0.164)	1.10 (0.055)	2.14 (0.146)	ND	ND	ND	ND
0.25 ml, fortified[b]	2.01 (0.016)	6.18 (0.034)	1.15 (0.044)	1.92 (0.059)	ND	ND	0.67 (0.045)	0.94 (0.037)
0.5 ml, fortified[c]	1.92 (0.026)	6.09 (0.158)	1.1 (0.07)	1.97 (0.123)	0.08 (0.141)	0.54 (0.003)	0.61 (0.006)	0.97 (0.24)
0.9 ml (QC chart, $n=55$)	1.99 (0.04)	6.18 (0.12)	1.19 (0.06)	2.12 (0.04)	0.12 (0.02)	ND	ND	ND
0.9 ml, fortified[d]	1.88 (0.020)	6.17 (0.159)	1.09 (0.064)	1.94 (0.127)	0.22 (0.010)	0.57 (0.016)	0.63 (0.017)	1.18 (0.117)

[a] Three to ten replicates per sample, as described in text.
[b] Addition of 0.12, 0.11, 0.12, and 0.24 μg of stigmasterol, lanosterol, sitostanol, and campestanol, respectively.
[c] Addition of 0.24, 0.23, 0.24, and 0.48 μg of each of stigmasterol, lanosterol, sitostanol, and campestanol, respectively.
[d] Addition of 0.49, 0.46, 0.49, and 0.97 μg of stigmasterol, lanosterol, sitostanol, and campestanol, respectively.

tified at concentrations near the MLOQ determined for analysis of 0.9 ml (Table 2). Results for sitosterol, campesterol, lathosterol, desmosterol, sitostanol, and campestanol were similar for all volumes analyzed, suggesting that for these components, sensitivity and precision are not compromised by assaying as low as 0.25 ml. In the fortified serum, however, lanosterol and stigmasterol that were measured in the 0.9-ml aliquots were undetectable in the smaller aliquots.

Table 6 summarizes results from analyses of 0.25- and 0.9-ml aliquots of seven sitostanol-containing serum samples (from the feeding trial), which were analyzed to compare the sensitivity of the method in

Table 6
Comparison of sterol concentrations assayed in 0.25-ml vs. 0.9-ml aliquots of serum samples from seven participants in clinical feeding trial [2]

Sample ID	Ml assayed	Sitosterol (μg/ml)	Campesterol (μg/ml)	Lathosterol (μg/ml)	Desmosterol (μg/ml)	Lanosterol (μg/ml)	Stigmasterol (μg/ml)	Sitostanol (μg/ml)	Campestanol (μg/ml)
VT608EI	0.25	1.24	1.80	2.54	1.03	–	–	–	–
	0.90	1.24	2.50	2.92	1.61	0.20	–	0.18	–
VT608F5	0.25	2.24	4.41	2.13	0.90	–	–	–	–
	0.90	2.26	4.22	1.79	0.98	–	–	0.21	–
VT608FY	0.25	1.87	3.09	2.57	1.19	–	–	–	–
	0.90	1.86	3.57	2.67	1.69	0.15	–	0.19	–
VT608FL	0.25	1.55	2.61	4.76	1.77	–	–	0.49	–
	0.90	1.49	3.06	4.82	1.72	0.21	–	0.20	–
VT6087W	0.25	3.26	3.99	3.43	1.18	–	–	0.27	–
	0.90	3.15	5.13	3.69	1.88	0.25	–	0.23	–
VT6088D	0.25	3.37	6.53	3.77	1.34	–	–	0.38	–
	0.90	3.20	6.15	3.87	1.81	0.13	–	0.31	–
VT6088T	0.25	2.55	4.33	2.40	1.01	–	–	–	–
	0.90	2.52	5.12	2.57	1.69	0.19	–	0.23	–

measuring naturally occurring (i.e. unfortified) sitostanol in smaller and larger sample volumes. The 0.25-ml assay failed to detect sitostanol in four of the seven samples and lanosterol in all seven samples. For sitosterol (mean 2.24 μg/ml), stigmasterol (not detected) and campestanol (not detected) results were similar in both analyses. For campesterol, lathosterol, and desmosterol, results were somewhat lower (3–25% on average) for the 0.25-ml aliquots. These results suggest that in a range of actual serum samples the sensitivity of the analysis may be reduced when 0.25-ml vs. 0.9-ml is assayed, especially for lanosterol and sitostanol.

4. Conclusions

A sensitive and precise method was developed for quantitation of phytosterols, stanols, and selected cholesterol metabolites in human serum. Analytes were detected at levels of 120 ng/ml to 6 μg/ml with standard deviations of 0.02 to 0.12 μg/ml. We have communicated the method in detail to facilitate its reproduction in other laboratories.

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