Sequential microenzymatic assay of cholesterol, triglycerides, and phospholipids in a single aliquot

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The assay of multiple analytes in a single aliquot can be advantageous from both measurement and economic standpoints. The objective of this study was to develop a simple and sensitive microenzymatic method for the determination of three biologically important lipids. Triglycerides (as glycerol), phospholipids (as choline), and total cholesterol (as unesterified cholesterol) were assayed, in that order, by sequential addition of sample, reagents, and microbial enzymes directly into a single microtiter plate well, accompanied by continuous monitoring of a common reporter reaction in which hydrogen peroxide is quantified either by colorimetry with 4-aminoantipyrene and 3-hydroxy-2,4,6-triiodobenzoic acid or by ultraviolet fluorometry with p-hydroxyphenylacetic acid. The detection limit of the method is in the subnanomole mass range for all three lipids. Results obtained with either fluorescence or colored endpoints were in excellent agreement with alternative individual chemical and enzymatic procedures.

INDEXING TERMS: multi-analyte analysis • fluorescence • colorimetry • glycerol • choline • lipoproteins • microtiter plate assay • 3-hydroxy-2,4,6-triiodobenzoic acid • p-hydroxyphenylacetic acid

Measurements of cholesterol, triglycerides (TG), and phospholipids (PL) in combination are commonly required in experimental work, and are included in the repertoire of most clinical chemistry laboratories.1 Enzymatic assays based on kinetic or endpoint colorimetry or on ultraviolet (UV) absorbance are often used for quantifying lipids in biologic fluids [1–4]. These techniques are reliable, provided that such fluids are available in sufficient volume and have adequate masses of the lipids to yield significant changes in light extinction. However, difficulties with detection limits often arise when these methods are applied to matrices with low analyte abundance. Although organic solvent extraction may be used to concentrate lipids, this is usually time consuming, labor intensive, and impractical in a nonspecialist laboratory or when large numbers of samples are to be analyzed. Consequently, there is a need for simple and sensitive techniques for the direct quantification of cholesterol, TG, and PL in combination in small samples and dilute media.

The common practice for deriving a lipid profile in a sample of interest is to make individual measurements of TG, PL, and cholesterol by using separate aliquots for each assay. An alternative approach that would be advantageous in terms of both analytic speed and economic cost would be to assay the lipids sequentially in the same aliquot. Provided that prior analysis of one lipid does not affect the concentration or measurability of the other lipids further down the chain, and as long as each reaction goes to irreversible completion with a stable equilibrium, it should be possible to link the different reactions in a temporal sequence, and thereby to quantify the mass of each lipid in a single aliquot by summation. Specific enzymatic assays are already available for the separate determinations of total cholesterol (TC), TG, and PL based on peroxidase, H2O2, and chromogenic reporters [5–7], but use of all three assays sequentially in a single aliquot has not been achieved. In this paper we describe a simple peroxidase-based microassay in which TG, PL, and TC mass are determined, in that order, by sequential addition of the sample, indicator reagents, and microbial enzymes into a single microtiter plate well, coupled with intermittent monitoring of a common equilibrium reaction product by either visible light colorimetry or ultraviolet fluorometry. Three incremental changes in signal intensity are recorded to the point where stable plateaus are reached, and the TG, PL, and TC masses are calculated by subtracting one plateau from another. By using a triply halogenated chromophoric substrate we have substantially improved the sensitivity of the colorimetric procedure compared with existing methods, such that the detection limits for TG, TC, and PL extend well into the subnanomole...
mass range. Both the colorimetric and fluorometric procedures have excellent reproducibility for all three lipids and, because they are microtiter plate-based, they can readily attain high throughputs and be automated. The fluorometric method is applicable to both aqueous-based media and isopropanol extracts, making it suitable for use with cell extracts.

**PRINCIPLE OF THE PROCEDURE**

The reaction sequences and key enzymes are outlined in Fig. 1. Glycerol is the first reaction product to be assayed, followed by choline and then unesterified cholesterol (UC). The colorimetric/fluorometric signal generated during the first step is directly proportional to the amount of glycerol liberated from TG by the action of lipoprotein lipase (LPL) (plus the contribution of any free glycerol already present). The signals generated in the second and third steps are directly proportional to the masses of choline released from choline-containing PL by the action of phospholipase D (PLD), and of UC released from cholesteryl esters (CE) by the action of cholesteryl esterase (CEST) (plus the contributions of any free choline and UC already present). In the fluorometric procedure a strongly fluorescent adduct, 6,6',dihydroxy-3,3'-biphenyldiacetic acid, is produced by the reaction of H$_2$O$_2$ with 3-hydroxyphenylacetic acid (HPA) (excitation max 315 nm; emission max 425 nm). In the colorimetric detection mode a intensely pink dye (absorbance peak ~492 nm) is generated by the oxidative condensation of 3-hydroxy-2,4,6-triiodobenzoic acid (HTIBA) with 4-aminoantipyrine (AAP). Both reporter reactions are catalyzed by horseradish peroxidase (HRPO).

**Materials and Methods**

**KEY ENZYMES, CHEMICALS, AND CALIBRATOR**

The following were purchased from Sigma Chemical Co., St. Louis, MO, and Dorset, UK: cholesterol oxidase (COX; from *Pseudomonas fluorescens*, cat. no. C7149; EC 1.1.3.6), CEST (from *P. fluorescens*, cat. no. C9406; EC 3.1.1.13), glycerol kinase (GK; from *Cellulomonas* spp., cat. no. G6142; EC 2.7.1.30), glycerol-3-phosphate oxidase (GPO; from *Streptococcus thermophilus*, cat. no. G4388; EC 1.1.3.21), LPL (from *Pseudomonas* spp., type XIII, cat. no. L9518; EC 3.1.1.3), choline oxidase (CHOX; from *Alcaligenes* spp., cat. no. C5896; EC 1.1.3.17), PLD (from *Streptomyces chromofuscus*, type VI, cat. no. P8023; EC 3.1.4.4), HPRO (type VI-A, cat. no. P6782; EC 1.1.1.7), ATP (Tris salt, from equine muscle, cat. no. A0520), bovine serum albumin (BSA; Fraction V, essentially fatty acid-free, cat. no. A6003), p-hydroxyphenylacetic acid (cat. no. H4377), AAP (cat. no. A2814), Triton X-100 (peroxide and carbonyl free, cat. no. X-100-PC), magnesium chloride hexahydrate (cat. no. M2393), and calcium chloride dihydrate (cat. no. C7902), HTIBA (cat. no. 6065H-3) and N-(2-hydroxyethyl)piperazine-N'-2-(ethanesulfonic acid) (HEPES; cat. no. 6001H) were obtained from Research Organics, Cleveland, OH. Precinorm L® special lipid control serum (cat. no. 781827) was from Boehringer Mannheim, E. Sussex, UK. Compounds used in the interference studies were from Sigma.

**EQUIPMENT**

A Bio-Tek Ceres UV900 HDI computerized microtiter plate reader with an integral forced-air heating system was purchased from Bio-Tek Instruments (Winooski, VT). Absorbance was measured at 492 nm with the spectrophotometer set at 37 ºC by using flat-bottomed virgin polystyrene microtiter plates (96F, cat. no. 2-69620; Nunc, Roskilde, Denmark). A Fluoroskan II fluorescence microtiter plate reader, equipped with Version 4.0 software, was purchased from Labsystems (Helsinki, Finland). Fluorescence intensity was measured in black 96-well polystyrene microtiter plates (Labsystems, cat. no. 9502257) by using an excitation wavelength of 315 nm (half bandwidth 35 ± 4 nm) and an emission wavelength of 425 nm (half bandwidth 25 ± 3 nm). The photon integration time was set at 1 s/well. Instead of utilizing the integral plate heater in the instrument for maintaining a constant 37 ºC environment (which was unsuitable for this type of assay, principally on account of its unevenness across the microplate surface), we performed all the analyses with the fluorometer enclosed in a 37 ºC forced-air incubator.

**WORKING SOLUTIONS**

The following reagents were made up in a sample diluent consisting of 100 mmol/L HEPES, pH 7.4, in 150 mmol/L NaCl containing 10 g/L fatty acid-free BSA, and stored at 0 ºC (ice water) for no more than 3 weeks: (a) colorimetric background reagent: per liter, AAP 0.02 mol, HTIBA 0.02 mol, and HRPO 4 kU; (b) fluorometric background reagent: per liter,
HPA 20 mmol and HRPO 4 kU; (c) TG reagent: per liter, LPL 20 kU, GPO 2 kU, GK 1 kU, ATP 800 μmol, MgCl_2 · 6H_2O 40 mmol; (d) PL reagent: per liter, PLD 100 kU, CHOX 10 kU, CaCl_2 · 2H_2O 40 mmol, Triton X-100 20 g; (e) cholesterol reagent: per liter, CEST 0.5 kU, COX 1.0 kU, Triton X-100 20 g.

**ASSAY PROTOCOL**

The assay protocol is as follows:

1) Aliquot aqueous specimens and calibrators (dilutions of Precinorm L) into replicate wells of a microtiter plate with sufficient sample diluent to bring the total volume to 200 μL. Routinely we quantify TC, TG, and PL in 1 μL of plasma in duplicate. [Since 1-μL volumes of specimen and calibrator are difficult to aliquot precisely even with a mechanical diluter/dispenser, we usually perform a sample predilution step into a separate microtiter well, in which 10 μL of plasma is diluted with 90 μL of sample diluent; subsequently, 10 μL of the diluted specimen plus 190 μL of sample diluent are assayed for lipid content. We perform all dilutions and dispensing with a Hamilton (Reno, NV) Microlab 1000 instrument fitted with 100-μL (sample) and 2.5-mL (reagent) syringes.] The calibration curve is constructed by serial double dilutions of Precinorm L in sample diluent, plus a zero calibrator consisting of sample diluent alone. For a typical assay of plasma specimens the calibration curve is made up of seven points (excluding zero), with the highest point corresponding to 5 μL of Precinorm L per well. In instances in which lipids dissolved in 1000 mL/L isopropanol are analyzed, the maximum sample volume is ≤50 μL/well, and diluted calibrators are reconstituted in the same solvent as sample unknowns.

2) Add 25 μL of colorimetric or fluorometric "background" reagent, shake the plate gently, discharge any air bubbles by passing a gentle stream of air over the plate, and, after equilibrating at 37 °C for 15 min, determine the baseline (endogenous) fluorescence or absorbance (reading 1). [For reagent additions in partial plate assays we use an Eppendorf repeater pipette (Model 4780; Brinkman Instruments, Westbury, NY) fitted with a 1.25-mL syringe tip; for full plate and multiple plate assays we use a Model 868.250 8-channel dispenser (Socorex, Lausanne, Switzerland).]

3) Initiate the first reaction sequence by pipetting 25 μL of TG reagent into all wells. Shake the plate and intermittently (~5-min intervals) monitor development of fluorescence or color, until a plateau in the reader output signal is achieved (reading 2), i.e., until the signal intensity changes ≤5% of the previous value. Between readings the plate is maintained at 37 °C in the dark.

4) Add 25 μL of PL reagent to initiate the second reaction sequence. Shake the plate and follow development of signal intensity, until a second plateau is reached (reading 3).

5) Add 25 μL of TC reagent to start the final reaction sequence. Shake the plate and record signal intensity until a third plateau is attained (reading 4).

6) Calculations: For each well, reading 4 minus reading 3 gives the response for TC content; reading 3 minus reading 2 gives the response for total choline content; and reading 2 minus reading 1 gives the response for total glycerol content. With this calculation all signals are automatically corrected for endogenous or background fluorescence/absorbance. Lipid concentrations in sample unknowns are calculated by interpolating on a calibration curve fitted to the calibrator points, by using either a linear least-squares regression model (if the dose–response function is linear) or a second-order polynomial regression model (if dose–response function is curved).

**REACTION TIME COURSE**

To assess the time course of the reactions and stability of the equilibria, we followed development of fluorescence intensity or absorbance at short intervals. Serial dilutions of Precinorm L were used to provide varying amounts of TG, PL, and TC in each well.

**OPTIMIZATION OF ASSAY CONDITIONS**

Because our method involves several commercially available reagents that had not been evaluated for lipid assays, we did experiments to optimize the enzyme activities and chemical concentrations in each of the three steps by using an intermediate mass of Precinorm L (2 μL per well) as the source of TG, PL, and TC. Each step was separately optimized by serial double-diluting every reagent in the assay diluent, and then comparing the signal intensities obtained with these dilutions with that obtained with buffer alone. When the optimum concentration of a particular reagent had been determined, we kept that concentration constant while optimizing the concentration of the next reagent. Our criterion for optimization was defined as the minimum amount of reagent required to achieve a relatively fast reaction rate (plateau within 1 h).

**DOSE-RESPONSE CURVES**

To determine the dose–response characteristics of each phase of our sequential procedure, we diluted Precinorm L twofold over a 64-fold range in sample diluent (highest amount, 5 μL/well) and analyzed for TG, PL, and TC, in that order, in quadruplicate. Curves of absolute signal intensity vs lipid concentration in each well were fitted by using the Statview 512+ program from Brainpower (Calabasas, CA) on an Apple Macintosh II personal computer.

**SENSITIVITY, DETECTION LIMIT, AND IMPRECISION**

The maximum sensitivity of each assay was defined as the greatest slope of the linear portion of the calibration curve. The theoretical limit of detection, defined as the smallest quantity of each lipid that could be distinguished from the diluent blank with 95% confidence, was estimated by interpolating the mean + 2 SD signal intensity value of the zero calibrator on an averaged calibration curve, determined in quadruplicate in a single run. We evaluated the imprecision of our procedure by using one plasma and two artificially reconstituted quality-control pools containing low, medium, and high concentrations of all three lipids. The two nonplasma pools were prepared by diluting a total lipoprotein fraction (d < 1.21 kg/L supernate) with 50 g/L BSA. The pools were stored, together with serially diluted Precinorm L calibrators, at −80 °C in multiple aliquots.
Each pool/calibrator was thawed once, analyzed, and then discarded. Within-run imprecision was determined by assaying each pool by using the triple-step fluorometric and colorimetric assays 10 times in a single microtiter plate. Between-run imprecision was determined in 6–9 separate assays (duplicate measurements) performed over 6 weeks with freshly prepared reagents. Enzymes were from a single lot and were stored at −80 °C in multiple small aliquots.

VALIDATION
As there is no recognized reference procedure for measurement of TG, it was difficult to establish the validity of the TG assay in our procedure by a parallel independent methods comparison approach. However, we compared total glycerol values determined by the first step of our sequential fluorometric method in 16 plasma samples (TG range 0.42–6.37 mmol/L) with values obtained in the same samples by a Centers for Disease Control and Prevention-standardized Clinical Lipid Laboratory that uses a nonglycerol blanked, Trinder-based kit on a centrifugal analyzer.

The validity of the PL assay in our method was established by comparing split-sample PL concentrations determined in the second step of the colorimetric procedure with inorganic phosphate values obtained by the perchloric acid/ammonium molybdate method [8]. We assayed in duplicate 30 samples of lipoproteins (10 each of VLDL, LDL, and HDL) prepared from nonhuman primate plasma by ultracentrifugation and Superoxide-6 (Pharmacia, Uppsala, Sweden) gel permeation chromatography.

We established the validity of the TC assay in our procedure by comparing values in eight serum samples obtained in the final step of the fluorometric method with values obtained by a gas–liquid chromatographic (GLC) technique. The GLC measurements were performed in duplicate on untreated and saponified samples by using a minor modification of the method of Ishikawa et al. [9] and a Hewlett-Packard (Palo Alto, CA) Model 5890 GLC fitted with an autosampler and flame ionization detector. Separations were carried out at 240 °C with an inlet and detector temperature of 270 °C by using a J&W Scientific (Folsom, CA) 15-m megabore column coated with a 1-μm film of 500 mL/L phenylmethylpolysiloxane. 5α-Cholestane was used as the internal standard. Saponification was with tetramethy lammonium hydroxide–isopropanol, and extraction was with tetrachloromethane:methylbutyrate (1:3 by vol). Cholesterol analysis by our enzymatic procedure was conducted on 1-μL aliquots in duplicate, and all samples and calibrators underwent TG and PL reactions before addition of the TC assay reagent. In a separate assay, UC in the samples was quantified with a single-step enzymatic colorimetric reaction in the presence of COX alone; CE was calculated as the difference between TC and UC. The assays were calibrated with Precinorm L by using the manufacturer’s assigned target values for UC and TC.

INTERFERENCE STUDIES
A variety of compounds commonly used in lipid laboratories (e.g., sodium azide, EDTA-Na2, KBr, NaBr, Mn2+, Mg2+, polyethylene glycol 6000, dextran sulfate M, 15 000, n-ethylmaleimide, and iodoacetate), as well as bilirubin, ascorbate, and hemoglobin, were evaluated for interference in both the enzymatic fluorometric and colorimetric assays. Each compound was dissolved in sample diluent, adjusted to pH 7.4, and then serially diluted twofold over a 64-fold range. Aliquots of these solutions were then added to microtiter wells containing 2 μL of Precinorm L, either before initiation of the sequential enzymatic measurement phases or at the end of the last determination step. We used this approach to distinguish interference with the enzymatic/chemical reactions from interference with the detection of the fluorescence or absorbance signals.

APPLICABILITY
To investigate the suitability of our procedure for measuring tissue lipids, we applied it to extracts of cultured cells. Human skin fibroblasts, human hepatoma HepG2 cells, and murine J774 macrophages were grown in monolayer for 4 days. After washing with ice-cold phosphate-buffered saline, the monolayers were treated with 500 μL of isopropanol in situ for 30 min at ambient temperature in a covered box placed on a slowly rotating orbital shaker (15 rpm). The isopropanol extracts were then transferred to glass tubes, capped, and centrifuged (1500g for 30 min) to pellet any debris, and 50-μL aliquots of the supernates were analyzed for TC, TG, and PL by our method. In previous experiments with radiolabeled cells we had found that isopropanol extraction gives recoveries >90% for all three lipid classes (data not shown). Total cell protein was measured according to Lowry et al. [10]. To standardize our assay, we extracted the lipids from undiluted and serially diluted Precinorm L by using isopropanol (10 vol for every vol of aqueous calibrator), and assayed these extracts in the same manner as the cell extracts.

Results

REACTION TIME COURSE
Figure 2 illustrates the increases in UV fluorescence (panels A and B) and visible light extinction (panels C and D) during the three phases of our sequential procedure. In the first and second phases (analysis of total glycerol followed by choline), the detector signals reached a maximum within 50 min and 80 min, respectively. The final analysis step (for cholesterol) was the fastest, the signal reaching a maximum within 30 min. The time to reach plateau was inversely related to lipid mass in each well. In the absence of lipid (i.e., solvent blank), the output signals in the colorimetric, but not the fluorometric, assay exhibited a shallow upward creep during each reaction phase. This can be explained by the gradual condensation of HTTBA and AAP to give the chromophoric quinone diimide dye, which is known to occur in the absence of HRPO and H2O2; HPA is much more stable in this regard. When colorimetric signals for wells containing varying amounts of lipids were subtracted from solvent blanks, the plateaus in each phase were highly consistent over time, and the effect of the shallow creep was eliminated. In wells with very high lipid concentrations, the signal plateaus were more stable with fluorescence detection than with visible light extinction. This was not due to instability in the equilib-
rrium of the colorimetric reaction, but reflected the limited
ability of our UV-Vis microtiter plate reader to precisely
quantify very low levels of light transmission. There was
an abrupt rise in visible range absorbance upon addition of the PL
enzymic reagent in the second phase because of the absorption
at 492 nm of the yellow pigments present in CHOX.

OPTIMIZATION OF ASSAY CONDITIONS
Curves of UV fluorescence intensity and absorbance as a
function of incubation time are shown in Figs. 3 (TG assay) and
4 (TC assay). Depicted are the results for optimization of
enzyme, detergent, cation, and fluorophore concentrations by
fluorometry, and for optimization of chromophore substrate
concentration by colorimetry. Optimization of the reagent
concentrations in our PL assay has been described elsewhere
[11]. To ensure absence of any substrate/product interference
and reagent incompatibilities between different measurement
steps, we subsequently retasted the optimum concentrations in
the setting of the complete triple assay, using both fluorometric
and colorimetric detection modes. As illustrated in Fig. 3, for a
target incubation time of 30 min, maximal signal intensities for
the TG assay were achieved by using 500 mU/well LPL, 25
mU/well GK, 50 mU/well GPO, and 20 nmol/well ATP.
Detector signals were highly dependent on the concentration
of all four components, and were abolished in the absence of any
one. Also, when HRPO and HPA or HTIBA and AAP were
omitted, the fluorescence or visible light signals did not exceed
background. Varying the amount of HRPO between 40 and
2500 mU/well, and of AAP between 0.078 and 5.0 mmol/well,
had minimal effect on signal intensity (<40 mU/well HRPO
and <0.078 mmol/well AAP were not tested). Because HRPO
and AAP are relatively inexpensive and available at high specific
activity/purity, we chose to use 100 mU/well and 0.625 mmol/well,
respectively, in the final formulation. Optimum concentra-
tions of HPA and HTIBA were the same at 0.625 mmol/well.
Increasing the amount of HTIBA to fivefold molar excess over
AAP slowed the reaction and gave lower final plateaus. Devel-
opment of signal intensity was delayed, but not totally abolished,
in the absence of added Mg\(^{2+}\). Nevertheless, because Mg\(^{2+}\)
 together with ATP are obligatory cofactors for GK activity [12],
we included 1 mmol/well Mg\(^{2+}\) to avoid the possibility that GK
activity might be suppressed during analysis of specimens con-
taining divalent metal-cation chelators.

As shown in Fig. 4, for an incubation time of 30 min during
TC determination, maximal signal intensities were achieved by
using 25 mU/well COX and 12.5 mU/well CEST. Without any
CEST, some increase in the signal was observed, in direct
proportion to the amount of UC present. The optimum masses
of HPA, HRPO, HTIBA, and AAP were similar to those for the
tG reaction step. However, unlike in the TG assay, where
Triton X-100 appeared unnecessary (and indeed exerted an
inhibitory effect at concentrations >0.1 mg/well), the presence
of the detergent in the TC assay was essential for CE hydrolysis;
the optimum concentration chosen was 1 mg/well. Although
several commercial TC test kits include sodium cholate to
enhance CE hydrolysis, we found no effects on reaction velocity,
equilibrium stability, or background noise that could not be
mimicked by using Triton X-100.
Fig. 3. Optimization of enzymes and chemicals for assay of TG.
Each panel shows the dependence of (y-axis) fluorescence intensity (panels A-H, FU) or absorbance (panels I and J, mAU) vs (x-axis) component concentration at various incubation times (min): panels A-D and I and J: 4 (C), 6 (D), 8 ( ), 10 ( ), 20 ( ), 30 ( ); panels E-H: 5 (C), 10 ( ), 20 ( ), 25 ( ); Precinorm L, 2 µL/well, was used as the source of lipid. Each point is the mean of duplicate determinations.

DOSE-RESPONSE CURVES
Dose-response curves obtained by analyzing varying amounts of Precinorm L (from 5 to 0.078 µL/well) are shown in Fig. 5. In all three phases of the assay, excellent linearity was obtained over 0.1–2.0 nmol of lipid per well. However, at >2 nmol/well the calibration curves were curvilinear, particularly when using fluorometry. Nevertheless, all points on the calibration curve could be fitted accurately (r >0.999) by using a second-order polynomial regression model, and we routinely used the entire curve for interpolation of unknowns. Traditionally, nonlinear calibration curves in enzymatic endpoint assays are censured because they are thought to reflect incomplete, nonequilibrium reactions. However, we do not feel that failure to achieve linear calibration curves is a shortcoming of our assay, as in experiments with serial dilutions of hyperlipidemic serum or lipoproteins, or with aqueous solutions of glycerol, choline, and UC; we

Fig. 4. Optimization of enzymes and chemicals for assay of cholesterol.
Each panel shows the dependence of (y-axis) fluorescence intensity (panels A-F, FU) or absorbance (panels G and H, mAU) vs (x-axis) component concentration at various different incubation times (min): 4 ( ), 6 ( ), 8 ( ), 10 ( ), 20 ( ), 30 ( ); panels F: 5 ( ); Precinorm L, 2 µL/well, was used as the source of lipid. Each point is the mean of duplicate determinations.
observed complete parallelism of concentration–response throughout the entire (curvilinear) calibration curve, irrespective of how long each reaction was continued after reaching a plateau. Moreover, addition of further aliquots of concentrated sample to previously analyzed, plateaued wells (with lipid masses in the curved region of the curve) resulted in further rapid increments of detector signal, indicating that the reactions were not exhausted and that the preceding reactions had gone to equilibrium (data not shown). Thus, although at high analyte concentrations the calibration curves for each of the three steps of our procedure are nonlinear, the method gives linear results when samples are interpolated on calibration curves obtained under identical conditions. Note that the slope of the straight portion of the PL calibration curve is about twice those of the TG and TC curves, reflecting the fact that 2 mol of H$_2$O$_2$ are released for every mole of choline oxidized, whereas glycerol and cholesterol each produce only 1 mol of H$_2$O$_2$ per mole.

**Sensitivity, Detection Limit, and Imprecision**

The maximum sensitivities of the fluorometric assay were: TG, 3.8 fluorescence units (FU)/nmol; PL, 6.1 FU/nmol; and TC, 3.6 FU/nmol (see insets, Fig. 5). Analogous values for the assays by colorimetry were: TG, 0.063 arbitrary units (AU)/nmol; PL, 0.118 AU/nmol; and TC, 0.071 AU/nmol. The minimum detectable masses of TG, PL, and TC using fluorescence were 250, 125, and 250 pmol, respectively. With colorimetry the detection limits were 500, 250, and 500 pmol, respectively.

Results for within- and between-assay imprecision are presented in Table 1.

**Validation**

Agreement between total glycerol values in 16 plasma samples by our three-step assay and a commercial Trinder-based assay run on a centrifugal analyzer was excellent ($r = 0.996$, Fig. 6A). Slight deviations from unity in the slope and from zero in the intercept were probably due to the use of different calibrators (and manufacturer-assigned TG values) in the two methods, and to the fact that our procedure, by including background readings, corrects for the presence of endogenous peroxides and nonspecific chromophores. Adjustment of the values from our assay by a factor of 1/0.850 yielded deviations between the two techniques of 0.03–13.31% (absolute difference divided by mean of both results times 100).

Results for the validation of the PL phase of our procedure are shown in Fig. 6B, and indicate excellent agreement between the enzymatic and chemical methods.

Results for the validation of TC step are displayed in Fig. 6C. The mean and 1 SD of UC concentration were very similar to the enzymatic and chemical techniques (enzymatic fluorometry, 1.55 ± 0.40 vs GLC, 1.30 ± 0.37 mmol/L), and the same was true for TC concentration (5.44 ± 1.23 vs 5.52 ± 1.24). After pooling all the values the intermethod correlation coefficient was 0.995; regression analysis gave a slope of 0.930 and intercept of 0.317.
INTERFERENCE STUDIES
In interference studies, sodium azide, EDTA-Na₂, Mn⁺⁺, Mg²⁺, n-ethylnalmemide, and iodoacetate had no significant effects at concentrations up to 1 μmol/well (data not shown). NaBr and KBr, at concentrations up to 500 nmol/well, and polyethylene glycol and dextran sulfate, at concentrations up to 10 mg/well, also exhibited negligible interference. This is true irrespective of whether the chemicals are present at the outset (a test of their effects as inhibitors/potentiators of the enzymatic reactions) or are added at the end (a test of their effects on the final signal intensities). Ellman’s reagent, 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), is a potent quencher of fluorescence at the wavelengths used here and cannot be used with this signal detection mode, though low concentrations (<1 μmol/well) are tolerable in the colorimetric mode (data not shown). Isopropanol is the only organic solvent we have tested for interference; maximum permissible levels are 50 μL/well. Bilirubin up to 1 nmol/well, ascorbate up to 5 nmol/well, and hemoglobin up to 5 μg/well had no effect on either the fluorometric or colorimetric assays.

APPLICABILITY
We applied our procedure to measurements of lipids in isopropanol extracts of cultured cells and obtained the following values (nmol/mg protein, representative individual dishes): fibroblasts: TC 80, TG 88, PL 46; HepG2 cells: TC 86, TG 79, PL 77; J774 macrophages: TC 131, TG 48, PL 111. These results demonstrate that our method can be applied to unconcentrated isopropanol extracts of cultured cells. The values for TC in the three different cell types are in close agreement with published values obtained by nonenzymatic techniques [13, 14].

Although available techniques for assaying TC, TG, and PL are highly specific and reliable, they are time consuming, expensive and (or) insensitive, and, in the case of nonenzymatic methods, require considerable expertise, hazardous reagents, or expensive instruments [8, 9, 15, 16]. They can also be difficult to automate. What we propose is a simple microtiter plate-based enzymatic procedure for the sequential measurement of TC, TG, and PL in the same aliquot, which has several useful features: (a) sensitivity to the subnanomole mass range, (b) good reproducibility and precision, (c) excellent agreement with traditional enzymatic and nonenzymatic assays, (d) high throughput relative to manual procedures, and (e) compatibility with both aqueous-based matrices and isopropanol extracts. The method is based on the fluorometric or colorimetric detection of H₂O₂ via a single equilibrium reaction product, generated through microbial enzymatic oxidation of glycerol, choline, and UC, both preexisting and liberated in that order, from TG, choline-containing PL, and CE by the actions of LPL, PLD, and CEST, respectively. Sequential measurements of all three lipids in a single well offer several advantages: (a) conservation of samples, (b) reduced costs, (c) saving of technician time, and (d) adaptability for automation on microtiter plate-based robotic systems. Moreover, technicians need not learn several dissimilar protocols for obtaining lipid profiles.

None of the enzymatic reaction sequences in our assay is novel [5–7], and kits based on LPL/GPO/GK (for TG), PLD/CHOX (for PL), and CEST/COX (for TC) with the Trinder–Emerson colorimetric reporter reaction [17, 18] are commercially available. The coupling of HPA and H₂O₂ to give a highly fluorescent adduct was first described by Guilbault et al. in 1968.

### Table 1. Within-assay and between-assay imprecision for determination of TG, PL, and cholesterol by the sequential microenzymatic method.

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<td><strong>Within-assay imprecision (n = 10)</strong></td>
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<td>PL</td>
<td>1.18 ± 0.026</td>
<td>2.2</td>
<td>2.30 ± 0.032</td>
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<td>3.0</td>
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<tr>
<td>TG</td>
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<td>3.5</td>
<td>1.03 ± 0.029</td>
<td>2.8</td>
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<td>2.7</td>
<td>2.35 ± 0.033</td>
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<tr>
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<td>2.59 ± 0.060</td>
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<td><strong>Between-assay imprecision</strong></td>
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<td>Fluorescence</td>
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<td>0.97 ± 0.019</td>
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<td>2.66 ± 0.065</td>
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<tr>
<td>Colorimetry</td>
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<td>TG</td>
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<td>1.02 ± 0.040</td>
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<td>2.6</td>
<td>2.57 ± 0.067</td>
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QC pool 1 is plasma, and QC pools 2 and 3 are dilutions of a total lipoprotein fraction (d <1.21 kg/L supernate) in BSA.
Since then, several groups [11, 20–25] have described enzymatic assays for TC, TG, or PL, using this compound and UV fluorometry. Huang et al. [20] first described an enzymatic fluorometric assay for serum TC using homovanillic acid as the fluorophore. However, their method required the sequential addition of seven reagents and determination of H₂O₂ by kinetic fluorometry. Later improvements [21, 23] included the use of HPA as an autooxidation-resistant fluorophore, single-reagent addition, and measurement of endpoint or steady-state fluorescence. These workers validated their assays for TC and UC in organic solvent extracts of cells, but did not do so for aqueous-based media. Furthermore, because their methods involved a single-cuvette spectrophotofluorometer and reaction volumes in the milliliter range, they are expensive in time and reagents. We were the first to develop a single-reagent rapid enzymatic microassay for PL involving an automated microtiter plate fluorometer [11], which is applicable to both aqueous-based matrices and isopropanol extracts. Mendez et al. [25] described an enzymatic fluorometric assay for glycerol with a linear range of 0.05–35 nmol based on the HRPO/HPA reporter system. In the final step of their procedure the reaction mixture is alkalized to pH 10 to boost fluorescence intensity. Although alkalization may be advantageous under some circumstances (e.g., to clarify turbid assay mixtures), gross alkalization would be unsuitable for our procedure, since the pH optima of the lipid-modifying enzymes that we use are close to neutral [26–29]. In our experience, alkalization also increases the imprecision of the assay because of the additional pipetting step. Furthermore, the signal enhancement achieved is often nonuniform; i.e., the amplification of fluorescence is not a constant function of the preexisting fluorescence. This can be a problem when calibrators and samples are in different solvents, or when different samples have varying amounts of fluorescence-quenching compounds.

Sequential measurement of more than one lipid in a single aliquot is not completely novel. Procedures for sequentially measuring two lipids in a single cuvette have been described [30–33]. However, some of these assay UC and CE only, and one was restricted to measurements in organic solvents. All were designed for use with either single-cuvette scanning-type spectrophotometers or expensive clinical chemistry analyzers. Zoppi [32] described a procedure for assaying TG by using oxidation of NADH for its indicator reaction, followed by cholesterol determination by a Trinder-type reaction. This approach may have some avoidable drawbacks [30]: low reliable measurement range intrinsic to NADH-coupled reactions relative to peroxidase chemistries; requirement for high glycerol or pyruvate concentrations in the cholesterol reagent (to prevent the peroxide formed in the second step from reacting with the relatively high concentration of NADH that may remain from the first step); and a potential for differential matrix interference effects through use of UV absorbance for one analyte and visible range absorbance for another. Sharma et al. [30] described a sequential procedure for quantifying TG and TC, using a common Trinder-type reporter reaction and endpoint, single-wavelength, visible-light spectrophotometry. Their use of α-cyclodextrin in the TG reagent to scavenge free fatty acids appears to be a promising way to reduce sample turbidity, but there are no data on whether α-cyclodextrin affects PL determinations. Furthermore, these workers did not automate their procedure, scale it down, or apply it to present-generation computer-controlled equipment. Our present report is the first to describe measurement of TC, TG, and PL in a single aliquot based on a microtiter plate format.

A critical feature of our method is the stability of the chromophore/fluorophore after oxidation with H₂O₂ in the presence of peroxidase. Of the numerous water-soluble Trinder-type reporters we tested [including phenol, dichloro-
phenol, 3,5-dichloro-2-hydroxybenzene sulfonate, N-ethyl-n-(2-hydroxy-3-sulfopropyl)-m-aniline, and N-ethyl-n-(2-hydroxy-3-sulfopropyl)-m-toluidine, only the hydrogen donor HTIBA was found to be substantially free from bleaching once it had condensed with AAP to form the quinone diimide dye. Any measurable bleaching in the presence of HTIBA usually occurred in the final stages of the sequence (during the TC phase), and only when the absorbances were >2.5 AU (in which case the samples were reanalyzed after dilution). The reason for color fading is not clear, but it may involve reduction of the quinone diimides to the quinone diamides [34] and (or) oxygen depletion [35]. Of the fluorophores we tested, HPA and p-hydroxyphenyl propionic acid were indistinguishable in terms of reaction kinetics and stability after oxidation with H₂O₂, but the reaction kinetics with homovanillic acid were too slow to give an efficient assay.

We use the sequence TG→PL→TC for two principal reasons. First, the PL and TC steps require surfactant for the efficient hydrolysis of PL and CE, but Triton X-100 at its optimum of 1 mg/well significantly inhibits the TG reaction kinetics (Fig. 3, panel F). Second, most plasma samples in a routine laboratory contain low molar concentrations of TG relative to PL and TC, and therefore yield low glycerol signal intensities. Because our assay involves subtracting superimposed signals from sequential reactions, greater precision is achieved when low-yield reactions precede high-yield ones. The TC step is placed last because it is more robust than the others, being unaffected by high concentrations of detergent, divalent cations, hydrolyzed lipids, shifts in pH, or reduced oxygen tension. It is worth mentioning that for samples that do not require TG assay, we get equivalent results if PL is measured before or after TC (data not shown).

Although our method is designated for quantification of TC, TG, and PL, the same sequential principle can be adopted for determination of free and TG-glycerol, UC and CE, and free and PL-choline in a single aliquot, by separating the lipase and the oxidase reactions into paired reagents (see Fig. 1). Thus far it has not been possible to link the TG and PL reactions with a sequential assay for both UC and CE, because the Pseudomonas-derived LPL hydrolyzes some CE in addition to TG. We have not investigated other types of TG lipase. However, as lipases from Rhizopus arrhizus and porcine pancreas appear not to hydrolyze CE [36], these might be suitable. It should be noted that the tandem use of PLD and CHOX quantifies choline-containing PL only: phosphatidyl choline, sphingomyelin, and lysophosphatidyl choline (comprising >95% of the PL in human plasma [7]). We advise caution against using our procedure for profiling lipids in matrices that are rich in phosphatidyl glycerol in addition to choline PLs, as there would be simultaneous generation of signals from both choline and glycerol; substitution of phospholipase D with phospholipase C plus alkaline phosphatase may then be more appropriate [37]. Pseudomonas-derived COX is equally reactive towards Δ⁴- and Δ³-3-hydroxy cholestanes [38] and hence in our assay will produce signals not only with cholesterol but also compounds such as β-sitosterol, 7-dehydrocholesterol, 5α-cholestan-3β-ol, and desmosterol. Although absolute specificity for cholesterol is therefore not achieved, our method is certainly more specific than the common nonenzymatic assays [39, 40].

The excellent sensitivity and detection limit of our procedure, using either colorimetry or fluorometry, mean that samples with low analyte abundance can be assayed without concentration. For example, it should be possible to obtain an accurate lipid profile by colorimetry using only 2 μL of interstitial fluid or 60 μL of cerebrospinal fluid [41, 42]. Our method should also facilitate studies in small animals, when only micro-liters of blood may be available [43], and should be useful for the estimation of serum total lipids, a parameter often used as a denominator for expressing concentrations of lipid-soluble organochemicals, toxicants, and vitamins [44, 45]. Because of its low detection limit, our method is also relatively insensitive to the effects of interfering agents and lipid solvents.

We calibrate our procedure with a lyophilized pooled human serum preparation, Precinorm L, which is widely used as a quality-control matrix for lipid assays. It has assigned target values for TC, UC, TG, and choline-containing PL, determined by analogous enzymatic colorimetric techniques. A moderately hyperlipidemic serum sample or an ultracentrifugally isolated lipoprotein fraction (d <1.21 kg/L) might also be used. Precinorm L contains unidentified fluorescent components and yellowish colored pigments, producing higher background signals than most plasma or lipoprotein samples. However, because our method includes a basal reading step before each lipolysis step, and because all subsequent readings are corrected for background, the final measured lipid concentrations in wells containing Precinorm L are unaffected. We recommend against using calibrators of pure glycerol, choline, and UC, because their reaction kinetics are much faster than those of TG, PL, and CE, which need to be hydrolyzed before the oxidase/peroxidase sequences. For the same reason, use of the "pure" calibrators provided in most commercial test kits is precluded. Since glycerol, choline, and proprietary surfactants are commonly used in kits to aid component solubilization and stabilization, we recommend validating each commercial calibrator for lipid composition, using kinetic absorbometry/fluorometry, before calibration with them. Theoretically, it should be possible to develop a triple lipid assay, involving pretreatment of samples with a combination of LPL, PLD, and CEST, followed by analysis of the mixture for total glycerol, choline, and UC by our sequential summation procedure. In this case a solution of glycerol, choline, plus UC would be an appropriate calibrator. However, because the hydrolysis of CE and PL in our procedure requires a nonionic detergent, the LPL reaction would have to precede these other reactions. Pure triolein, 1,α-phosphatidylcholine, and cholesteryl oleate are unsuitable as calibrators, since they are expensive, difficult to maintain in solution without additives, and readily oxidize in aqueous solution. Furthermore, their behavior during the assay procedure may not accurately mirror those of the naturally occurring mixtures of lipids in biological samples [27, 46].

All the enzymes and chemicals required for our procedure are commercially available in high specific activity and homogeneity. The enzymatic reagents are quickly prepared from frozen stock solutions, and in the presence of 10 g/L fatty
acid-free BSA are stable at 0 °C (ice water) for ~3 weeks. HTIBA and AAP slowly condense in the presence of HRPO, even at 0 °C in the absence of exogenous H₂O₂. Therefore, we prepare fresh background reagent for each assay and keep it in ice water in the dark when not in use. Both the colorimetric and fluorometric methods incorporate a background reading step, in which any endogenous H₂O₂ and chromophores/fluorophores in samples produce a signal. Because this signal is variable, it will result in errors if not corrected for. For this reason, we recommend against adding the reporter components simultaneously with the enzymes of the first step. It should also be noted that Triton X-100 is subject to oxidation by free radicals to yield peroxides, and can therefore produce high background fluorescence and color signals [24], for which reason we recommend using peroxide-free and carbonyl-free Triton X-100. Although the fluorometer that we used is not normally marketed with filters optimized for detection of the HPA/H₂O₂ adduct, one of the four standard filter pairs (emission 355 nm, excitation 460 nm) can be used. Custom-synthesized narrow-band-pass filters set at peak emission 315 nm and excitation 425 nm will yield three- to fourfold higher signal-to-noise ratios and sensitivities.

The detection limit of our method is limited by the chromogenic/fluorescence yield of the chromophore/fluorophore-peroxide adducts, and by the sensitivity of the optical detection components in the microtiter plate readers. In colorimetry, the detection limit could certainly be enhanced by improving the transmissivity of the microtiter plates and by making the wells narrower and deeper. We have not determined the molar absorptivity (ε) of HTIBA, and could find no published data, but its chromogenic yield with H₂O₂ is expected to be similar to that of 3-hydroxy-2,4,6-trihromobenzoic acid (29 400 L mol⁻¹ cm⁻¹), based on their structures and atomic substituents [47]. This can be compared with ε for phenol, which is 13 000 L mol⁻¹ cm⁻¹ [34]. There are other Trinder-type hydrogen donors with ε greater than HTIBA, but the one we have tried (2-hydroxy-3,5-dichlorobenzene sulfonate, 52 000 L mol⁻¹ cm⁻¹) exhibits pronounced bleaching at high degrees of coupling.

What are the relative merits of using UV fluorescence over visible range absorbometric detection in our procedure? First, the fluorometric mode has a twofold lower detection limit than the colorimetric one. Second, spectrophotofluorometers usually have broader measurement ranges than visible light readers. Third, carotenoid pigments, hemolysis products, and turbid solutions tend to affect epifluorescence at the wavelengths used here much less than transmitted absorbance, although bichromatic visible light readings can be used to overcome this deficiency. On the other hand, microtiter plate fluorometers tend to be much more expensive than visible range microplate readers. Additionally, as they typically come fitted with single-measurement light paths and require relatively long photon counting times to achieve good signal-to-noise ratios, fluorometers tend to be slower than multichannel optical readers, precluding rapid kinetic analysis across multiple wells. Our results indicate that, in terms of measurement precision, fluorometry and colorimetry are comparable.

For the combined determination of TG, PL, and TC mass by our procedure, the direct reagent cost per specimen is currently ~$5. Including solvent blanks, seven serially diluted calibrators, and three quality-control pools, one technician can pipette and analyze 34 samples in duplicate in about 3 h. With the aid of an independent 37 °C incubator, a robotic dispensing/transferring device, and a dedicated computer for data capture/reduction, there is no reason why multiple plates cannot be run together on a single microplate reader with staggered readings.

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References


