methods

# Measurement of serum total glycerides and free glycerol by high-performance liquid chromatography[1]

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**Abstract** **Serum levels of total glycerides and free glycerol are important indices of lipid metabolism and cardiovascular disease risk. Convenient enzymatic methods of measurement have been available, but they are susceptible to interference. Situations exist in both research and clinical laboratories in which more specific and precise methods are needed. We developed HPLC methods for the measurement of serum total glycerides and free glycerol. For total glycerides, serum was mixed with an internal standard (1,2,4-butanetriol) and treated with alcoholic sodium hydroxide to hydrolyze glycerides to glycerol. After deproteinization with tungstic acid, the glycerol was benzoylated with an optimized Schotten-Baumann reaction and analyzed by HPLC. For free glycerol, serum was equilibrated with the internal standard and deproteinized with tungstic acid to remove the glycerides. The glycerol was benzoylated and analyzed as for total glycerol. Various factors were investigated, and no significant sources of interference were detected. The total coefficients of variation ranged from 0.7% to 2.0% for total glycerides and from 1.7% to 3.2% for free glycerol. The analytical recoveries ranged from 98.5% to 101.6%. In conclusion, simple and reliable HPLC methods for serum total glycerides and free glycerol have been developed. The methods may also be used for the analyses of glycerol or glycerides in other biological samples.** —Li, H., J. Dong, W. Chen, S. Wang, H. Guo, Y. Man, P. Mo, and J. Li. **Measurement of serum total glycerides and free glycerol by high-performance liquid chromatography. *J. Lipid Res.* 2006.** 47: **2089–2096.**

**Supplementary key words** triglycerides • Schotten-Baumann reaction • benzoylation • butanetriol

Blood serum levels of total glycerides (defined as the sum of triglycerides and free glycerol), free glycerol, and triglycerides (the difference between total glycerides and free glycerol) are important indices of lipid metabolism and cardiovascular disease risk. They are now measured in most situations by enzymatic methods. These methods are convenient but susceptible to interference (1). There are some instrumental analytical methods, mostly isotope dilution mass spectrometric methods, for total glycerides (2–4), triglycerides (2, 5), and free glycerol (6, 7). These methods are intended primarily for use as reference or definitive methods. They are reliable but time-consuming and expensive. Situations exist in both research and clinical laboratories in which reliable and simple instrumental analyses of blood total glycerides and free glycerol are needed.

HPLC with ultraviolet light detection is a simple and precise analytical technique, but it has hardly been used for the measurement of glycerol in biological samples. Judd et al. (8) measured plasma glycerol specific activity, and Kiyoshima et al. (9) determined human tissue glycerol by HPLC. The major difficulties in HPLC analysis of glycerol in biological samples are the recovery and derivatization of glycerol. HPLC analysis of glycerol requires precolumn derivatization for separation and detection reasons. The derivatizations often used are esterifications under anhydrous conditions with organic bases as catalysts (8, 9). Glycerol is a highly polar small molecule polyol. For glycerol in biological samples, both the evaporation of the extraction solvent and the purification of the sample or the formed derivatives are tedious.

It has long been known that alcohols can be acylated with acyl chlorides in aqueous alkaline solutions (the Schotten-Baumann reaction). This reaction is especially suitable for the esterification of alcohols in aqueous samples and has been used for the benzoylation of polyols for chromatographic analysis (10–12). But this reaction as usually carried out is less quantitative and can hardly provide an accurate measurement of glycerol.

Hoping that simple and reliable HPLC analysis of blood glyceride and glycerol could be realized by making use of the Schotten-Baumann reaction, we selected butanetriols as candidate internal standards and investigated various

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factors influencing the benzoylation behaviors of the polyols in the reaction. Our investigation led to the establishment of novel HPLC methods for serum total glycerides and free glycerol that showed analytical recoveries of 98.5–101.6% and total coefficients of variation (CVs) of 1.5–2.6% for total glycerides and 2.0–3.3% for free glycerol.

## MATERIALS AND METHODS

### Materials

The glycerol (99.5+%) used for the preparation of calibrators was obtained from Sigma (St. Louis, MO), and the internal standard 1,2,4-butanetriol was from Fluka (Buchs, Switzerland). Benzoyl chloride (99%) was a product of Sigma, and HPLC-grade *n*-hexane, isopropanol, and acetonitrile were products of Labscan (Bangkok, Thailand). Serum pools were obtained from the Laboratory Medicine Department, Beijing Hospital, and stored in ampoules at −70°C until analysis.

### Preparation of calibrators and internal standards

Calibrators of 0.565, 1.129, 2.259, 3.388, and 4.157 mmol/l (50–400 mg/dl triolein) for the measurement of serum total glycerides and 0.028, 0.056, 0.113, 0.226, and 0.339 mmol/l (2.5–30 mg/dl triolein) for free glycerol were prepared by dissolving weighed glycerol in and diluting with water. The calibrators were stored in ampoules at 4°C. Internal standard solutions of 3.4 mmol/l (for total glycerol) and 0.34 mmol/l (for free glycerol) were prepared by dissolving 1,2,4-butanetriol in water.

### Sample preparation

For serum total glycerides, 0.1 ml of serum or calibrator was mixed with 0.1 ml of the internal standard solution, 0.1 ml of 2 mol/l sodium hydroxide, and 0.6 ml of isopropanol. The mixture was incubated at 40°C for 30 min and then mixed with 0.6 ml of 0.33 mol/l sulfuric acid and 0.3 ml of 0.3 mol/l sodium tungstate. The suspension was centrifuged at 1,500 *g* for 10 min. An aliquot of 0.2 ml of the supernatant was mixed with 0.2 ml of 8 mol/l sodium hydroxide and heated in a 100°C water bath for 15 min. After the addition of 0.2 ml of 0.1% sodium dodecyl sulfate, the mixture was shaken with 85 μl of benzoyl chloride dissolved in 0.5 ml of *n*-hexane for 30 min. The hexane phase was used for chromatographic analysis.

For free glycerol, 0.2 ml of serum or calibrator was mixed with 0.2 ml of the internal standard solution. The mixture was allowed to stand for 30 min and then mixed with 0.1 ml of 0.33 mol/l sulfuric acid and 0.1 ml of 0.3 mol/l sodium tungstate. The supernatant prepared by centrifugation was subjected to the same treatment as for total glycerol.

### Chromatographic analysis and calculation

The chromatographic analysis was performed on an HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) consisting of an isocratic pump, an autosampler, and an ultraviolet light detector controlled by the ChemStation. An aliquot of 5 μl of the hexane phase from the sample preparation was injected onto a Spherisorb phenyl column (5 μm, 4.6 × 150 mm) and eluted with *n*-hexane containing 1% isopropanol and 0.5% acetonitrile at a flow rate of 1.5 ml/min. The absorbance at 230 nm of the eluent was monitored. Peak height ratios of glycerol to 1,2,4-butanetriol for the standards were linearly regressed on the corresponding glycerol concentrations, and the resulting equation was used to calculate serum glycerol concentrations.

## RESULTS AND DISCUSSION

### Method principles

The principles of the methods are as follows. Serum was equilibrated with an internal standard (1,2,4-butanetriol). For total glyceride analysis, serum glycerides were hydrolyzed with alcoholic sodium hydroxide and, after deproteinization with tungstic acid, the glycerol was benzoylated with the Schotten-Baumann reaction and analyzed by HPLC; for free glycerol, glycerides, which reside in lipoproteins, were removed by tungstic acid deproteinization and the glycerol was benzoylated and analyzed as for total glycerol. Typical chromatograms for total and free glycerol analysis are shown in **Fig. 1**. The 100°C heating in the sample preparations was to remove the isopropanol (for total glycerides) and to decompose glucose (total glycerides and free glycerol) in the serum sample. The effect of the heating in free glycerol analysis is shown in **Fig. 2**.

### Derivatization and internal standard selection

A major challenge of this study was the precolumn derivatization. Schotten-Baumann benzoylation was considered the choice of reactions because of its applicability to aqueous samples. The reaction has normally been carried out by shaking alkaline (sodium hydroxide) polyol solution with benzoyl chloride and then extracting the formed benzoates with organic solvents (10–12). We first tried this procedure but found the reaction to be far from quantitative, and the recovery of glycerol was influenced by almost all factors involved, such as the amount of benzoyl chloride used, the concentration of the alkaline solution, the speed and duration of the shaking, the type of extraction solvent, and the properties of glycerol samples (calibrator vs. serum).

The performance of a chromatographic analysis would largely depend on the closeness of physical and chemical behaviors of an internal standard to the analyte, and the latter would depend on the properties of the internal standard and the efficacy of the derivatization procedure. We then selected two butanetriols (1,2,4-and 1,2,3-butanetriol), which would be structurally most similar to glycerol and unlikely to be present in serum (13), as candidate internal standards and investigated various factors influencing the absolute and relative recoveries of glycerol (expressed as the chromatography peak area of glycerol and the peak area ratio of glycerol to internal standard) for purposes of optimizing benzoylation conditions and examining internal standard properties. Our major observations are described below.

*Amount of benzoyl chloride.* Benzoylation was performed with variable amounts of benzoyl chloride, and the amount of benzoyl chloride influenced not only the glycerol peak areas but also the glycerol-to-butantriol peak area ratios. The relationships between benzoyl chloride amount and glycerol areas and area ratios under the specified conditions are shown in **Fig. 3**. Glycerol areas increased with the increase of benzoyl chloride until the amount of benzoyl chloride was close to half that of sodium hydroxide, when the glycerol areas reached their highest level and the area ratios remained consistent. Further increase of benzoyl

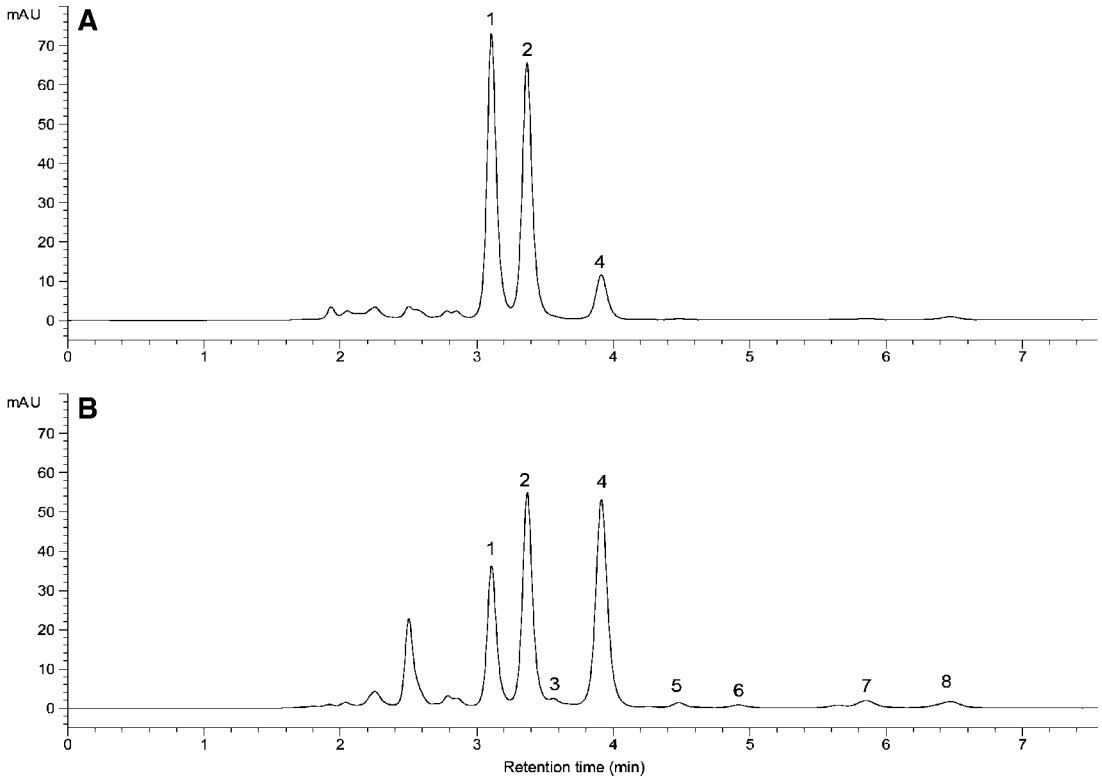


**Fig. 1.** HPLC chromatograms of serum total glycerides and free glycerol measurements. A and B show chromatograms of total glycerides and free glycerol, respectively, from pooled serum. Chromatographic conditions were as follows: column, Spherisorb phenyl (5 mm, 4.6 × 150 mm); mobile phase, *n*-hexane-isopropanol-acetonitrile (98.5:1:0.5); flow rate, 1.5 ml/min; detection, ultraviolet light at 230 nm. Peaks are as follows: 1, glycerol; 2, 1,2,4-butanetriol; 3–8, unidentified serum constituents. mAU, milliabsorbance units.

chloride resulted in no glycerol area increase but caused chromatographic interference (too large a peak in front of glycerol that influenced the quantitation of glycerol). The area ratio of glycerol to 1,2,4-butanetriol was less influenced than that of glycerol to 1,2,3-butanetriol.

Presumably, the major reaction during benzoylation is the conversion of benzoyl chloride to sodium benzoate by sodium hydroxide (only a small part reacts with the polyols). One molecule of benzoyl chloride consumes two molecules of sodium hydroxide. Thus, the greatest theoretical use of benzoyl chloride should be half the moles of sodium hydroxide. Excess benzoyl chloride will not be decomposed and will cause chromatographic interference.

*Sodium hydroxide concentration.* Similarly, the sodium hydroxide concentration influenced both the glycerol areas and the area ratios. With a benzoyl chloride-to-sodium hydroxide mole ratio of 0.9:2, the effect of sodium hydroxide concentration is shown in **Fig. 4**. The highest glycerol peak areas were obtained when the concentration of sodium hydroxide was 3–4 mol/l. Again, 1,2,4-butanetriol showed behaviors more similar to glycerol than did 1,2,3-butanetriol. The formed sodium benzoate could not be solubilized when sodium hydroxide concentration was 4.5 mol/l or greater.

*Timing of the addition of extraction solvent and type of extraction solvent.* As described above, the Schotten-Baumann reaction is normally carried out by shaking alkaline polyol solution with benzoyl chloride and then extracting the formed benzoates with organic solvents. It was initially found that the shaking was critical and that different speeds or durations of shaking resulted in variable glycerol areas and area ratios. It was also observed that after a nonpolar extraction solvent (*n*-hexane or cyclohexane) was added, both the glycerol areas and the area ratios were resistant to shaking. We then tried to shake the polyol solution with benzoyl chloride in the presence of hexane. Glycerol areas and area ratios of glycerol to 1,2,4-butanetriol obtained by benzoylation with and without hexane as a function of shaking time are shown in **Fig. 5**. The presence of hexane made the benzoylation more consistent and practicable.

*Use of detergent.* When preliminary procedures were tentatively applied to measuring serum glycerol, unacceptable analytical recoveries were observed. It was observed that the interface between the aqueous solution and the hexane in serum samples was different from that in calibrators, suggesting a difference in surface properties between the two solutions. Sodium dodecyl sulfate was then used to harmonize the surface properties, and the analytical recoveries were corrected, as shown in **Fig. 6**.

The absolute recovery of glycerol also increased by 20–30% using the detergent. The reaction yield were comparable to that obtained with an anhydrous benzoylation based on the procedure of Judd et al. (8).

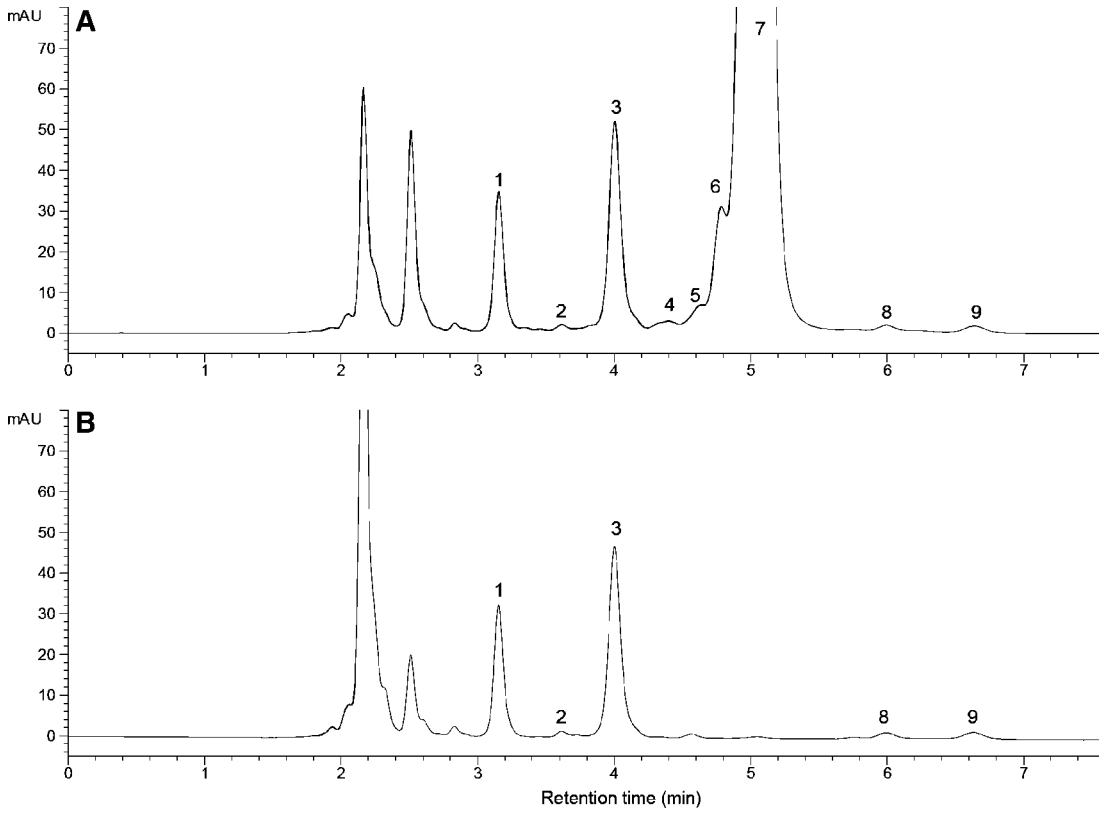


**Fig. 2.** HPLC chromatograms of serum free glycerol. A: Chromatogram of a pooled serum sample that did not undergo the 100°C heating treatment during sample preparation (see Materials and Methods). B: Chromatogram of the same sample that was treated. Chromatographic conditions were as described for Fig. 1. Peaks are as follows: 1, glycerol; 6, 7, glucose; 2–5, 8, 9, unidentified serum constituents. mAU, milliabsorbance units.

*Presence of protein.* If the benzoylation could be performed in the presence of proteins, the sample preparation for the intended HPLC analysis could be further simplified. Albumin was added to the reaction system, and the benzoylation of the polyols was tested. In part of the experiment, the aqueous solution was first heated at 100°C for 10 min before the benzoylation, to mimic a possible condition for serum triglyceride hydrolysis. Although untreated protein did not seem to affect the benzoylation, the heat- and alkali-treated protein did influence the benzoylation.

Based on these observations, 1,2,4-butanetriol was chosen as the internal standard and a procedure for the benzoylation of serum glycerol was established. A sodium hydroxide concentration of 3 mol/l and a benzoyl chloride-to-sodium hydroxide molar ratio of 0.9:2 were used, and the reaction was carried out in the presence of a nonpolar solvent and a detergent. The procedure showed the following features: *a*) a glycerol absolute recovery (reaction yield) close to that of anhydrous benzoylation; *b*) glycerol relative recoveries resistant to the manipulations involved; and *c*) benzoylation and extraction of the analytes performed in a single shaking step and no sample or derivative purifications needed. This procedure enabled simple HPLC analysis of serum total glycerides and free glycerol.

The optimized benzoylation procedure could also efficiently benzoylate other polyols or carbohydrates but showed poor capability of benzoylating monools (ethanol, cholesterol, etc.).

### Chromatography

The chromatographic separation of glycerol and 1,2,4–butanetriol benzoates was performed with a normal-phase elution mode on a phenyl column. The separation finished in 5 min, as shown in Fig. 1. Reverse-phase chromatography required much longer run times for the resolution of the benzoates, and normal-phase chromatography on silica columns needed longer equilibration times and showed poorer stability.

### Linearity and precision

The linear correlation between glycerol concentration ($x$) (the five calibrators, each in duplicate) and peak height ratio ($y$) of glycerol to the internal standard in ~20 analytical runs for total glycerides and free glycerol was assessed by linear regression analysis. The slopes, intercepts, and standard errors of the $y$ estimate, slopes, and intercepts are shown in **Table 1**.

Four serum pools were analyzed in triplicate in three independent runs for the estimation of the precision of the HPLC methods. The results are shown in **Table 2**. The within-run and total CVs for total glycerides ranged from 0.5% to 1.3% and from 0.7% to 2.0%, respectively, and those for free glycerol ranged from 1.3% to 2.6% and from 1.7% to 3.2%. Analyses on four control sera in a period of ~2 years showed long-term CVs of 1.5–2.6% for total glycerides and 2.0–3.3% for free glycerol (**Table 3**).

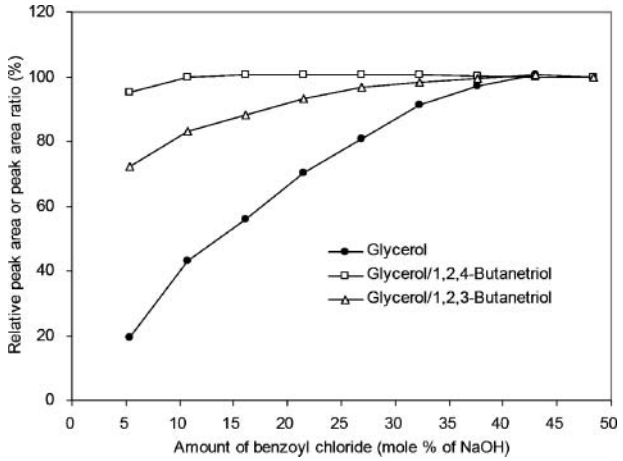


**Fig. 3.** Effect of benzoyl chloride amount on the benzoylation of glycerol and butanetriols. Glycerol and 1,2,4-butanetriol or 1,2,3-butanetriol (all ~0.05 mmol/l) in 3 mol/l sodium hydroxide solution containing 0.05% sodium dodecyl sulfate were shaken with different amounts of benzoyl chloride dissolved in hexane for 30 min. The formed benzoates were chromatographed. Peak areas and peak area ratios are expressed as percentages of those when benzoyl chloride amount was 48% of sodium hydroxide. The data presented are means of duplicate determinations.

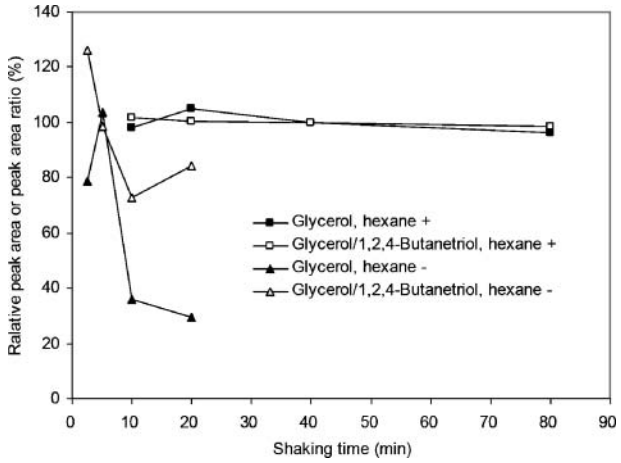


**Fig. 5.** Effect of shaking time on the benzoylation of glycerol and 1,2,4-butanetriol with and without the presence of hexane. Glycerol and 1,2,4-butanetriol (both ~0.05 mmol/l) in 3 mol/l sodium hydroxide solution containing 0.05% sodium dodecyl sulfate were shaken with benzoyl chloride (at 45% of sodium hydroxide) dissolved in hexane (hexane +) for 10–80 min, or the polyols shaken with benzoyl chloride for 5–20 min and then extracted with hexane (hexane −). The formed benzoates were chromatographed. Peak areas and peak area ratios are expressed as percentages of those at 20 min shaking time with hexane. The data presented are means of duplicate determinations.

### Accuracy

Known amounts of glycerol were added to serum pools, and the glycerol concentrations of the pools with and without added glycerol were analyzed in triplicate. The original glycerol levels, the amounts of added glycerol, and the analytical recoveries are shown in **Table 4**. The average recoveries were 100.0% and 99.7% for total glycerides and free glycerol, respectively.

There are other factors that may influence the accuracy of the analyses but cannot be revealed by the analytical recovery. These factors may include incomplete hydrolysis of glycerides, the release of phospholipid glycerol during the sample preparation, the presence of glycerides or lipoproteins in the supernatant for free glycerol analysis, and coelution of serum constituents with glycerol or the internal standard.

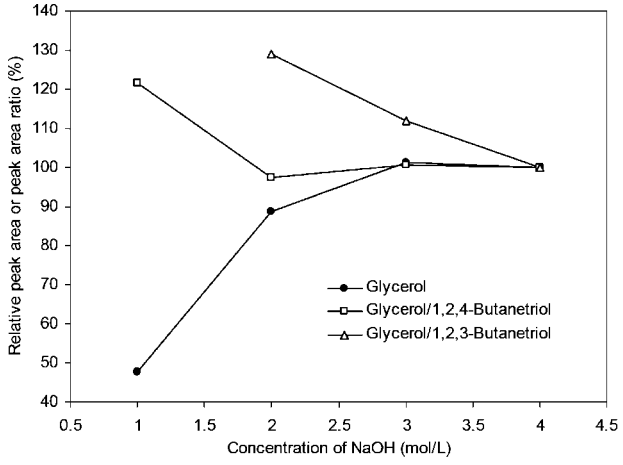


**Fig. 4.** Effect of sodium hydroxide concentration on the benzoylation of glycerol and butanetriols. Glycerol and 1,2,4-butanetriol or 1,2,3-butanetriol (all ~0.05 mmol/l) in 1–4 mol/l sodium hydroxide solution containing 0.05% sodium dodecyl sulfate were shaken with benzoyl chloride (at 45% of the respective sodium hydroxide) dissolved in hexane for 30 min. The formed benzoates were chromatographed. Peak areas and peak area ratios are expressed as percentages of those when sodium hydroxide concentration was 4 mol/l. The data presented are means of duplicate determinations.

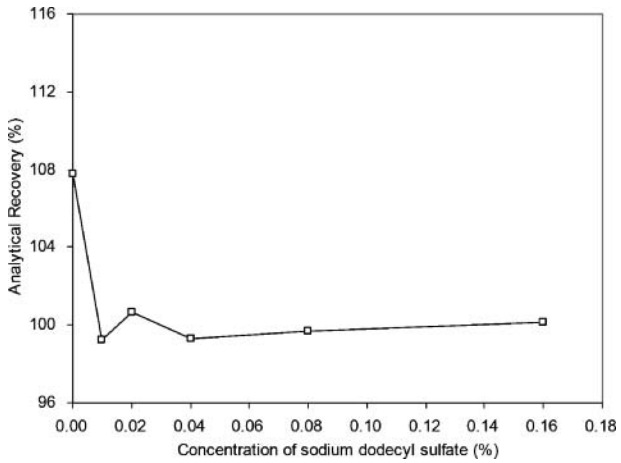


**Fig. 6.** Effect of sodium dodecyl sulfate on the analytical recovery of total glycerides. Pooled serum was analyzed for total glycerides according to the procedure described in Materials and Methods, except that variable amounts of sodium dodecyl sulfate (final concentration of 0–0.16% in the aqueous solution) were added. The serum was analyzed with and without the addition of glycerol, and the analytical recovery was calculated. The data presented are means of duplicate determinations.

TABLE 1. Linearity between glycerol concentration and peak height ratio of glycerol to the internal standard

| Variable | Total Glycerol | Free Glycerol |
|---|---|---|
| Slope | 0.2926–0.3092 | 3.0662–3.1868 |
| Intercept | −0.0060–0.0200 | −0.0003–0.0112 |
| SEM of estimate | 0.0038–0.0074 | 0.0033–0.0150 |
| SEM of slope | 0.0008–0.0016 | 0.0091–0.0689 |
| SEM of intercept | 0.0023–0.0045 | 0.0017–0.0147 |
| $r^2$ | 0.9999–0.9998 | 0.9999–0.9990 |

Aliquots of a pooled serum mixed with 1,2,4-butanetriol were hydrolyzed for 10, 20, 40, and 80 min, and the peak area ratios were measured. No significant differences were observed among the peak area ratios, suggesting that hydrolysis of serum glycerides could actually be finished within 10 min under our conditions.

Phospholipids can easily be hydrolyzed to glycerolphosphate by alcoholic hydroxide alkali. Further hydrolysis of glycerolphosphate by alkali to glycerol is difficult. Although unlikely, possible glycerol release from phospholipids during the 100°C heating in the total glycerol sample preparation was tested. Serum samples were prepared with a longer heating time (60 min), and no significant glycerol increase was detected. Possible phospholipid hydrolysis to glycerol was also tested at a high phospholipid level. Phosphatidylcholine was added to a serum sample at a concentration of 1,000 mg/dl, and the sample was analyzed for total glycerides. Again, no significant glycerol increase was detected. Glycerolphosphate may be hydrolyzed by mineral acids. The supernatants generated by tungstic acid precipitation in the total glycerol analysis were stored overnight at room temperature. Glycerol levels remained the same.

In serum free glycerol analysis, if serum glycerides were not completely removed by deproteinization, free glycerol would be falsely increased. The supernatants after the deproteinization were washed with tetrachloromethane and analyzed for free glycerol. No significant changes were detected.

Serum constituents that are coeluted with glycerol or the internal standard and have an absorption at 230 nm will interfere with the chromatographic analysis. These possible constituents may include substances that have a chromophore themselves and substances that can be benzoylated and extracted into hexane. A pooled serum sample was processed according to the sample preparation procedure except that no benzoyl chloride was incorporated. The hexane layer was chromatographed, and no detectable chromophore-containing substances were observed. The only possible substances thus would be nonionic polyhydroxyl compounds. A series of polyols and sugars that could be endogenously or exogenously present in serum were benzoylated and chromatographed under our conditions. Their retention times and resolutions are listed in **Table 5**. They all were well resolved with glycerol and 1,2,4-butanetriol.

TABLE 2. Precision of HPLC analysis of serum total and free glycerol

| Glycerol | Serum Pools | Mean | CV | |
|---|---|---|---|---|
| | | | Within-Run | Total |
| | | *mmol/l* | % | |
| Total glycerol | Pool 1 | 0.938 | 1.2 | 2.0 |
| | Pool 2 | 1.776 | 1.3 | 1.9 |
| | Pool 3 | 2.788 | 0.5 | 0.7 |
| | Pool 4 | 3.974 | 1.0 | 1.1 |
| Free glycerol | Pool 1 | 0.083 | 2.6 | 2.8 |
| | Pool 2 | 0.111 | 1.6 | 1.7 |
| | Pool 3 | 0.144 | 2.1 | 3.2 |
| | Pool 4 | 0.266 | 1.3 | 2.4 |

CV, coefficient of variation.

TABLE 3. Long-term precision of HPLC analysis of serum total and free glycerol

| Glycerol | Controls | Runs | Mean | Total CV |
|---|---|---|---|---|
| | | | *mmol/l* | % |
| Total glycerol | Q01 | 27 | 2.214 | 1.89 |
| | Q02 | 27 | 1.693 | 2.20 |
| | Q07 | 8 | 0.658 | 2.71 |
| | Q08 | 13 | 1.378 | 1.45 |
| Free glycerol | Q02 | 10 | 0.149 | 3.31 |
| | Q03 | 10 | 0.146 | 2.04 |
| | Q08 | 8 | 0.350 | 2.77 |

### Detection limit

For a total glyceride sample of ~1 mmol/l or a free glycerol sample of ~0.1 mmol/l analyzed under our conditions, the signal-to-noise (defined as six times the standard deviation of the baseline) ratio given by the ChemStation ranged from 400 to 600, suggesting that our methods can detect total glycerides in samples at concentrations as low as ~2 μmol/l and free glycerol at ~0.2 μmol/l. This detection limit would enable the methods to be adapted to measure tissue or cell samples.

### Comparison with enzymatic methods

A control serum was analyzed for total glycerides in ~90 runs with an enzymatic method and 13 runs with the HPLC method over a period of 2–3 years. The enzymatic method was performed with an enzymatic total glycerides reagent (Biosino Biotech Co., Ltd., Beijing, China) on Photometer 5010 (Boehringer Mannheim). This method has been standardized by the U. S. Centers for Disease Control and Prevention and National Heart, Lung, and Blood Institute Lipid Standardization Program since 1997. The enzymatic and HPLC averages were 1.379 (CV, 3.1%) and 1.378 (CV, 1.5%), respectively. In a mini survey, four fresh frozen serum pools were shipped to 11 clinical laborato-

TABLE 4. Analytical recovery of HPLC analysis of serum total and free glycerol

| Glycerol | Serum Glycerol | Added Glycerol | Mean Recovery |
|---|---|---|---|
| | *mmol/l* | | % |
| Total glycerol | 1.418 | 1.129 | 99.9 |
| | 1.418 | 2.259 | 100.0 |
| Free glycerol | 0.090 | 0.113 | 100.1 |
| | 0.090 | 0.226 | 99.3 |

TABLE 5. Retention times of polyols and carbohydrates

| Polyols and Carbohydrates | Retention Time | Resolution[a] |
|---|---|---|
| | *min* | |
| 1,2-Propanediol | 2.51 | |
| Ethylene glycol | 2.74 | |
| 1,3-Propanediol | 2.77 | 2.8 |
| Glycerol | 3.11 | 2.1 |
| 1,2,4-Butanetriol | 3.37 | 1.5 |
| Erythritol | 3.56 | |
| Arabitol | 4.49 | |
| Ribitol | 4.75 | |
| Glucose[b] | 5.11, 4.78 | |
| Sorbitol | 6.23 | |
| Mannitol | 6.52 | |
| Inositol | 7.35 | |

[a] With immediate next-eluting polyol.
[b] Glucose eluted as two peaks.

ries in the Beijing area that measure triglycerides by an enzymatic method (Kyowa Medex Co., Ltd.) that eliminates free glycerol by preincubation. The pools were measured in triplicate with patient samples in each laboratory. The total glyceride and free glycerol levels of the pools were also measured in duplicate in four runs with the HPLC method, and triglycerides were calculated by subtraction. The enzymatic and HPLC triglyceride averages and CVs are listed in **Table 6**. The two averages agreed with each other within 3%.

### Measurement of serum total glycerides and free glycerol in Beijing residents

Serum levels of total glycerides and free glycerol in 480 Beijing residents (unselected outpatients of the hospital, 247 men and 233 women, 21–83 years of age) were measured with the HPLC methods, and triglycerides were calculated. The means and percentiles are listed in **Table 7**. The triglycerides and free glycerol in different gender and age groups are shown in **Fig. 7**. The distributions were all skewed and could be log-transformed to approximate normal distributions. Analysis of the log-transformed data showed the following: in both men and women, free glycerol levels were positively correlated with triglycerides (men, $r = 0.19$, $P < 0.05$; women, $r = 0.15$, $P < 0.05$), and both triglycerides (men, $r = 0.16$, $P < 0.05$; women, $r = 0.50$, $P < 0.001$) and free glycerol (men, $r = 0.33$, $P < 0.001$; women, $r = 0.26$, $P < 0.001$) were positively correlated with age; triglycerides were significantly higher in men (Student's $t$-test = 5.37; $P < 0.001$), but free glycerol levels were significantly higher in women (Student's $t$-test = 5.37; $P < 0.001$).

TABLE 6. Serum triglycerides analyzed with HPLC and enzymatic methods

| | Enzymatic | | HPLC | |
|---|---|---|---|---|
| Pools | Average | CV | Average | CV[a] |
| | *mmol/l* | % | *mmol/l* | % |
| Pool A | 2.018 | 4.0 | 2.071 | 1.4 |
| Pool B | 1.509 | 2.7 | 1.531 | 1.6 |
| Pool C | 1.145 | 6.7 | 1.130 | 2.4 |
| Pool D | 0.922 | 5.8 | 0.946 | 2.7 |

[a] Calculated from the combined SD of total and free glycerol analysis.

TABLE 7. Total glycerides, free glycerol, and triglycerides in Beijing residents

| | | | Percentiles | | | | |
|---|---|---|---|---|---|---|---|
| Sample | Variable | Average | 10 | 25 | 50 | 75 | 90 |
| | | | *mmol/l* | | | | |
| All | Total glycerides | 1.381 | 0.671 | 0.846 | 1.228 | 1.697 | 2.303 |
| | Free glycerol | 0.082 | 0.047 | 0.058 | 0.075 | 0.100 | 0.125 |
| | Triglycerides | 1.299 | 0.596 | 0.765 | 1.152 | 1.614 | 2.224 |
| Men | Total glycerides | 1.514 | 0.762 | 0.990 | 1.359 | 1.836 | 2.473 |
| | Free glycerol | 0.074 | 0.044 | 0.052 | 0.069 | 0.084 | 0.107 |
| | Triglycerides | 1.440 | 0.688 | 0.919 | 1.271 | 1.751 | 2.385 |
| Women | Total glycerides | 1.240 | 0.608 | 0.745 | 1.052 | 1.502 | 2.047 |
| | Free glycerol | 0.090 | 0.050 | 0.063 | 0.081 | 0.111 | 0.133 |
| | Triglycerides | 1.150 | 0.538 | 0.669 | 0.975 | 1.397 | 1.917 |

### Limitations and other potential applications

The HPLC methods described here are primarily for serum total glycerides and free glycerol analysis. Because only nonionic polyols could interfere with the analysis, it is assumed that these methods could be equally used on plasma samples. One limitation of these methods is that they cannot be used for the analysis of some control serum materials that contain large amounts of polyhydroxyl substances (e.g., polyethylene glycol, sucrose) as preservatives. The content of polyhydroxyl substances in the materials

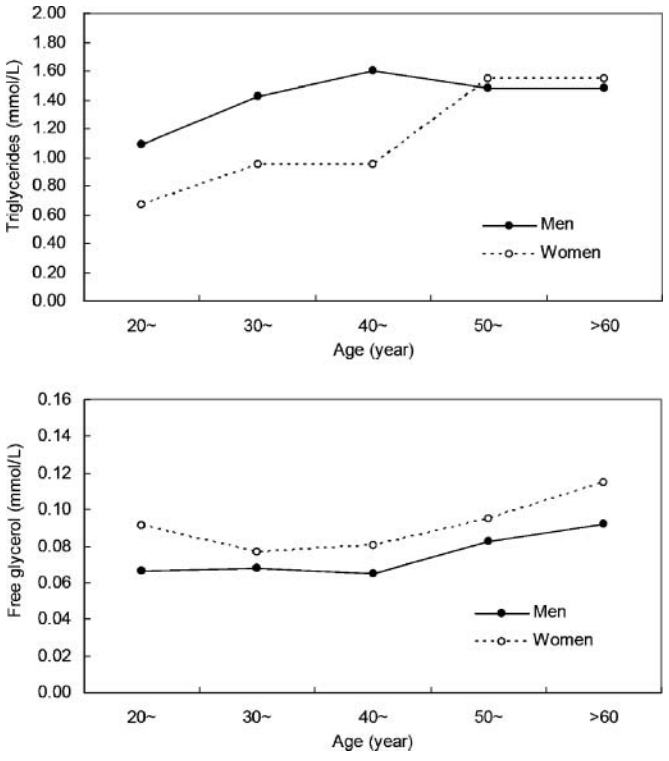


**Fig. 7.** Triglycerides and free glycerol in gender and age groups of Beijing residents.

may be as great as 20%. Chromatographic interference was observed when the methods were applied to these materials.

As discussed above, the methods are specific, precise, and sensitive. It is assumed that they might be adapted to measure glycerol in other biological samples. Additionally, the optimized benzoylation procedure might be of use for the analysis or recovery of other polyols and sugars in biological samples.

## Conclusions

HPLC methods for measuring serum total glycerides and free glycerol have been developed. They are simple, precise, and specific and can be used in research or clinical laboratories in which precise and specific serum total glycerides and glycerol measurements are needed. The optimized Schotten-Baumann benzoylation procedure may also be used for the analysis or recovery of glycerol in other biological samples. The HPLC methods are incapable of analyzing some control materials that contain large amounts of polyhydroxyl substances as preservatives.

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