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Determination of "free" glycerol in human serum reference materials by isotope-dilution gas chromatography–mass spectrometry

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ABSTRACT

Serum free glycerol analyses are an important part of the preparation and evaluation of human serum reference materials used for the quality assurance of triglyceride assays. However, enzymatic kits for free glycerol analysis obtained from different vendors have, on occasion, provided different results for a given sample. In an effort to establish the "target" glycerol content of selected reference materials, we have established a method for the analysis of serum free glycerol by using isotope-dilution gas chromatography–mass spectrometry, incorporating [1,3-$^{13}C_2$]glycerol as the internal standard. The use of a simplified serum extraction and clean-up procedure resulted in (uncorrected) recoveries of glycerol averaging about 90% before derivatization, and in estimated concentrations for spiked serum pools that corresponded closely to the expected values. A comparison of enzymatic and gas chromatographic–mass spectrometric results for several reference serum pools suggest that the latter method is of value in evaluating and validating routine enzymatic methods for free glycerol analysis.

INTRODUCTION

Serum free glycerol analyses have been used in assessing adipose lipolysis as an index of fat mobilization [1,2] and in monitoring therapeutic plasma glycerol levels [3,4]. In addition, serum free glycerol may be important in confounding enzymatic triglyceride analyses by contributing to "blank" levels. The need to correct serum triglyceride assays for free glycerol content under routine conditions remains somewhat controversial [5–7]; however, such corrections are essential for reference analyses and in certain other circumstances [6,7].

There is no established reference method or methodological principle for serum free glycerol assays. Enzymatic methods, which essentially duplicate triglyceride methods while omitting lipase, are relatively simple and precise and have been used effectively in many studies. We have noted, however, that enzymatic glycerol kits from different suppliers may yield substantially different results with certain serum samples or reference materials. These differences appear to be matrix-specific, since they may be observed with some but not all serum samples or reference pools, and generally not with aqueous glycerol standards. Consequently, it can be difficult to determine the true glycerol level of particular serum samples or pools by using only enzymatic meth-

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ods. Thus, to help us select a suitable method for making routine enzymatic "blank" corrections in triglyceride reference analyses, we undertook to develop a confirmatory procedure based on an alternative method.

Glycerol is water-soluble, hygroscopic, of low molecular mass, and at least somewhat volatile, making it relatively difficult to recover and analyze from an aqueous medium. Although procedures based on both gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been reported for the analysis of serum free glycerol [1,3,4,8], the identification of an appropriate and reliable internal standard can be difficult. Many problems in the analysis of free glycerol, however, can be avoided or minimized by using an isotopically labeled form as the internal standard. In this report we describe a method for analyzing serum free glycerol by isotope-dilution gas chromatography–mass spectrometry (ID-GC–MS) in which [1,3-$^{13}C_2$]glycerol is used as an internal standard, and we compare the results obtained by this method with those obtained by using three enzymatic kits in the analysis of selected serum triglyceride reference pools.

EXPERIMENTAL[a]

Standards and reagents

Glycerol (99.5+%) for the preparation of standards was obtained from Aldrich (Milwaukee, WI, USA), Fisher Scientific (Norcross, GA, USA), or (as aqueous dilutions) from New England Reagent Laboratory (East Providence, RI, USA). [1,3-$^{13}C_2$]Glycerol (99 atom%) was a product of MSD Isotopes (St. Louis, MO, USA), and [1,3-$^{14}C_2$]glycerol was obtained from DuPont–New England Nuclear Research Products (Boston, MA, USA). [1,3-$^{14}C_2$]Glyceryl trioleate was synthesized from labeled glycerol and oleoyl chloride essentially as described by Björkhem *et al.* [9]. The product was purified by solvent extraction, silica column chromatography, and preparative thin-layer chromatography (TLC) with diethyl ether–hexane solvent mixtures. Radiochemical purity of the final product was ≧99% as judged by analytical TLC and autoradiography. Derivatization reagents were obtained from Pierce (Rockford, IL, USA). The human serum pools analyzed in this study were reference materials prepared at the Centers for Disease Control (CDC) (Atlanta, GA, USA).

Glycerol analysis by ID-GC–MS

The internal standard (I.S.) stock solution was prepared by dissolving [1,3-$^{13}C_2$]glycerol in water to a final concentration of about 1.25 mg/ml. Aliquots of serum or aqueous glycerol standards were then assayed by placing 200 μl of well mixed serum or standard solution in a 1.5-ml microcentrifuge tube and adding 10 μl of the I.S. stock solution. The tubes were tilted and mixed as needed to assure dispersal of the I.S. in the sample solutions, then placed on a mechanical mixer at room temperature for 20 min.

To each sample, 800 μl of methanol were added. The samples were capped and vortex-mixed for 30 s, allowed to stand for 10 min, then centrifuged at 10 000 rpm (*ca.* 8165 *g*) for 10 min. The supernatant was recovered in a clean glass tube, 1 ml of hexane was added, and the solution was again vortex-mixed briefly and centrifuged. After discarding the hexane supernatant, the methanol–water infranatant was recovered in a tapered glass sample vial. The samples were carefully dried under a stream of nitrogen at 30–35°C, then allowed to stand overnight at room temperature in a desiccator. The following morning, 50 μl of N,O-bis(trimethylsilyl)acetamide (Tri-Sil BSA) were added to each sample. The samples were capped with septa and aluminum crimp seals, and heated at 55°C for 1 h. After cooling the samples to room temperature, aliquots were taken for analysis by GC–MS.

The samples were analyzed by using split injection on a 12 m × 0.2 mm methyl silicone column (Ultra-1) with a 0.33-μm film installed via the direct capillary interface in a Hewlett-Packard 5790/5970A benchtop GC–MS system. The

[a] Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the US Department of Health and Human Services.

initial oven temperature of 100°C was held for 1 min after injection, and then the oven was programmed to 250°C at 20°C/min. We maintained the injection port at 250°C and the capillary interface at 260°C. Samples were assayed by using selected-ion mode (SIM) analysis with an ionization energy of 70 eV, monitoring ions at m/z 218.1 and 220.1 at 2.8 Hz, and with an electron multiplier voltage of 1200–1400 V.

Enzymatic analyses

Enzyme assays of free glycerol were carried out by using kits from three different manufacturers, according to the vendors' suggested conditions for temperature, sample and reagent volume, incubation time, and wavelength. Serum blanks were incorporated in all analyses. The sample free glycerol values were calculated from the regression parameters obtained from four-point standard curves (10.4, 20.8, 52, and 104 μg/ml glycerol) determined for each set of samples, rather than from the two-point standard mean slope calculation as used by the Abbott VP clinical analyzer in the normal operating mode. In each run, duplicate analyses from three vials from each of the four CDC pools were made by

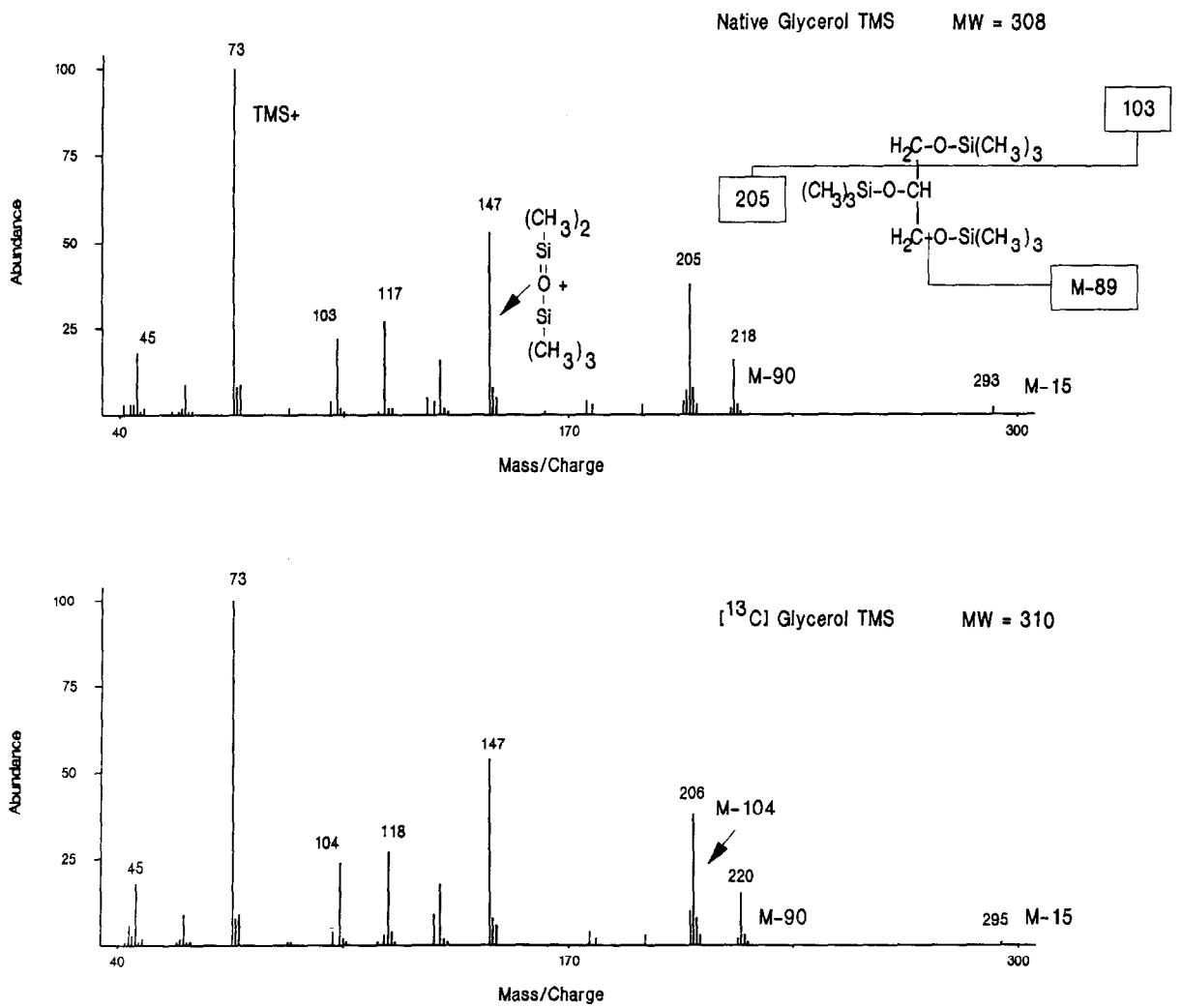

Fig. 1. Mass spectra of native and 1,3-$^{13}C_2$-labeled glycerol as the trimethylsilyl (TMS) ethers.

using each of the three enzyme kits. Enzymatic analysis runs were repeated weekly for six consecutive weeks. The source of the kits was as follows: kit A from Abbott Labs. (North Chicago, IL, USA); kit B from Sclavo (Wayne, NJ, USA); kit C from Technicon (Atlanta, GA, USA).

Lipase contamination

To evaluate contaminating lipase activity, we added [1,3-$^{14}C_2$]glyceryl triolein in 5 μl of ethanol to 25 μl of serum. After the addition of 250 μl of water or the enzyme reagent, the mixture was incubated at 37°C for 15 min. The samples were extracted for 10 min with 5 ml of chloroform–methanol (2:1, v/v). After extraction, 1 ml of 0.2 *M* KCl was added, and the samples were mixed and centrifuged. The aqueous phase was recovered, the volume was measured, and the radioactivity of 100-μl aliquots was determined in triplicate. For control purposes, aliquots of the organic phase were also monitored, both directly and after their concentration and fractionation by TLC with hexane–diethyl ether–acetic acid (80:20:1, v/v) as the developing solvent.

RESULTS

The mass spectra of native and labeled glycerol as the trimethylsilyl (TMS) ethers are given in Fig. 1. No molecular ion could be detected in these analyses, and the M − 15 fragment was very weak. The highest mass ion of reasonable abundance in native glycerol-TMS that also retained both of the carbon atoms that were labeled in the internal standard (carbons 1 and 3) was the M − 90 fragment at *m/z* 218 resulting from the loss of trimethylsilanol. The fragment at *m/z* 205 was more abundant, but it involved the loss of one of the terminal (labeled) carbon atoms, as indicated in the inset to Fig. 1. Therefore, we chose the *m/z* 218 and 220 pair for quantitation by GC–MS using SIM.

Representative extracted-ion chromatograms from the SIM analysis of a serum pool extract are shown in Fig. 2. The glycerol-TMS ether eluted within 3 min under these analytical conditions and was well resolved from later-eluting contaminants. Analysis of sample extracts by TLC confirmed that the methanol was also extracting significant amounts of lipid that required high temperatures and greatly extended GC run times to elute from the column. Therefore, we further extracted the methanol–water supernatant with hexane to remove most of this contaminating lipid. Under our conditions, a total run time of 20 min was sufficient to clear the column of remaining coextracted contaminants.

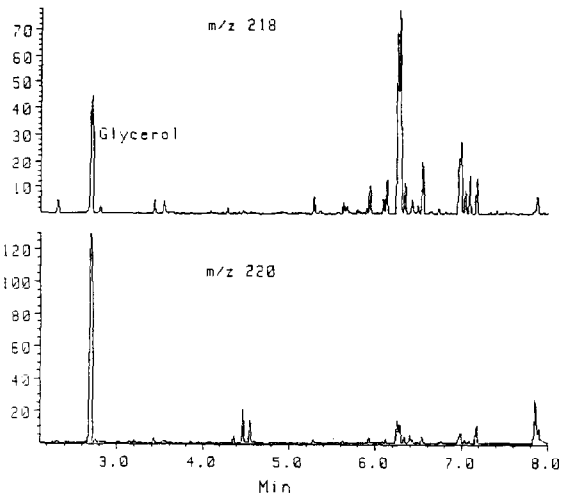

Fig. 2. Representative selected-ion chromatograms for the analysis of a serum sample by gas chromatography–mass spectrometry using selected-ion mode detection. The calculated glycerol content of this sample was 50 μg/ml.

Calibration curves were constructed from aqueous standards processed along with each group of serum samples. Although the mass of the internal standard was only 2 a.m.u. greater than that of native glycerol, the calibration curves were apparently linear through at least 60 μg/ml; thus, these curves accommodated all of the pools examined in this study. The regression equation for these data was $y = 0.0157x + 0.0268$, $r = 0.9993$ ($n = 11$). At least one water blank was included with each set of samples, and a small glycerol contribution in the blank was usually observed. However, this contribution remained minimal and uniform when the glassware was carefully cleaned and the solvents were screened before use.

We monitored the recovery of glycerol from

the extraction and clean-up procedure by spiking a serum sample with [1,3-$^{14}C_2$]glycerol. Overall recoveries up to the derivatization step averaged 91%; about 9% of the labeled material remained in the sample tube residue, and <0.05% could be detected in the hexane extracts. As indicated in Table I, the recovery of glycerol from a spiked serum pool sample analyzed by the ID-GC–MS method averaged 99%.

Table II depicts the results for free glycerol analysis of four serum reference pools analyzed by three different enzymatic kits and by the ID-GC–MS method. The enzymatic results were obtained from a series of runs in which each pool was analyzed in triplicate over a period of six weeks. In general, the results obtained with kit C showed good agreement with the GC–MS value for all four pools, whereas the results obtained with kit A were slightly higher, and those obtained with kit B were substantially higher. Most pools had coefficients of variation (C.V.s) for the ID-GC–MS method of about 2–3%, with the exception of pool 4, which had a notably higher C.V. (5.4%). The GC–MS data included multiple aliquots from individual vials, and an evaluation of these data by an analysis of variance suggested that an unusually large vial-to-vial variability component was associated with pool 4, although, somewhat surprisingly, the enzymatic data for this pool had relatively low C.V.s. These low C.V.s might have resulted because different sets of vials were analyzed in the enzymatic and GC–MS assays. Previous enzymatic analyses of pool 4 had also shown large variability.

TABLE I

GLYCEROL CONCENTRATION OF A SPIKED SERUM POOL ANALYZED BY ID-GC–MS (n = 3)

Glycerol added (μg/ml)	Final concentration (μg/ml)		Observed as % of expected
	Expected	Observed	
0	–	9.6	–
7.8	17.4	17.0[a]	98
15.6	25.2	25.6	102
31.2	40.8	40.7	100
46.8	56.4	54.9	97
Mean			99

[a] n = 2.

TABLE II

ENZYMATIC AND GC–MS FREE GLYCEROL VALUES MEASURED ON FOUR POOLS

Assay[a]	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	n
Pool 1			
Kit A	12.1 ± 0.96	7.9	18
Kit B	30.7 ± 11.41	37.2	18
Kit C	10.4 ± 0.89	8.5	18
GC–MS	10.5 ± 0.21	2.0	12
Pool 2			
Kit A	11.0 ± 1.81	16.5	18
Kit B	27.5 ± 10.82	39.4	18
Kit C	8.9 ± 1.49	16.7	18
GC–MS	9.2 ± 0.22	2.4	11
Pool 3			
Kit A	28.8 ± 2.06	7.1	18
Kit B	60.6 ± 25.99	42.9	18
Kit C	20.4 ± 1.60	7.9	18
GC–MS	21.2 ± 0.31	1.5	9
Pool 4			
Kit A	53.6 ± 3.11	5.8	18
Kit B	77.6 ± 12.88	16.6	18
Kit C	50.7 ± 2.75	5.4	18
GC–MS	52.9 ± 2.86	5.4	18

[a] Kits A, B, and C are enzymatic glycerol analysis methods from three different suppliers.

In all cases, the results from kit B diverged from the other methods, yielding both higher mean values and larger C.V.s. The latter was mainly the result of a substantial between-lot variability in kit B. As indicated in Table III, the within-lot C.V.s for kit B were comparable with those for the other enzymatic methods for all four pools, although the mean values were substantially higher when lot I was used. Lot II provided relatively lower mean values for each pool, but the values remained much higher than the levels obtained with the other kits or by GC–MS.

As indicated in Table IV, the discrepant results from kit B may have resulted, at least in part, from lipase contamination, because the recovery of radioactivity in the aqueous phase was sub-

TABLE III

FREE GLYCEROL ANALYSIS WITH KIT B BY LOT (n = 9)

Pool	Lot	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)
1	I	41.5 ± 3.73	9.0
	II	19.9 ± 0.92	4.6
2	I	37.7 ± 3.60	9.5
	II	17.3 ± 0.80	4.6
3	I	84.8 ± 10.44	12.3
	II	36.3 ± 0.53	1.5
4	I	88.9 ± 6.09	6.8
	II	66.3 ± 5.19	7.8

stantially higher when [1,3-$^{14}C_2$]glyceryl triolein was incubated with the reagents in this kit than with those in the others. By contrast, assays conducted with labeled triolein using kits A or C did not yield radioactivity in the aqueous phase that differed significantly from the blank. Lipase contamination in lot I of kit B may have been even greater, but we did not have enough reagent available to examine that lot. It should be noted, however, that although lipase contamination in kit B would be consistent with the results we obtained with the four reference pools, this explanation alone does not suffice, because the discrepancy between the results from this kit and the GC–MS data that we observed with triglyceride reference materials was not as apparent when fresh serum samples were analyzed. The reason for this anomaly is currently under investigation.

TABLE IV

HYDROLYSIS OF [1,3-^{14}C]GLYCERYL TRIOLEIN (n = 4)

Source	% dpm aqueous	Difference from blank[a]
Blank	0.48 ± 0.31	–
Kit A	0.55 ± 0.35	p = 0.76 (N.S.)[b]
Kit B (lot II)	3.63 ± 1.91	p = 0.04
Kit C	0.78 ± 0.24	p = 0.17 (N.S.)
Kit C + lipase	64.40 ± 5.27	–

[a] t-Test comparison.

[b] N.S. = not significant.

DISCUSSION

Although serum free glycerol may be determined for other reasons, probably the most common application is for the "blank" correction of enzymatic triglyceride measurements. In both chemical and enzymatic procedures, triglycerides are actually measured as glycerol after hydrolysis of the fatty acid esters in the triglyceride molecule. In the CDC Reference Method for triglycerides [10], serum free glycerol is removed by solvent extraction and silicic acid absorption steps before (chemical) hydrolysis of the triglycerides, thereby eliminating any significant interference from unesterified glycerol in the measurement. By contrast, most enzymatic triglyceride assays involve a single reagent mixture in which the enzymatic hydrolysis step is coupled with the analysis of glycerol, resulting in the measurement of all of the glycerol (both esterified and unesterified) in the sample. Although recent enzymatic methods have been proposed that reportedly minimize or eliminate the interference from free glycerol [5,11], most methods in routine clinical use today include serum free glycerol concentrations in the reported triglyceride value [6]. This will result in a bias relative to the reference value for the sample or reference pool, with a magnitude corresponding to the free glycerol content of the sample.

One approach to the problem of the inclusion of free glycerol in enzymatic triglyceride measurements is to perform a second analysis of the same sample, but without any lipase, to directly monitor the free glycerol content. This approach has been used by many laboratories participating in the CDC standardization program. Because of the importance of free glycerol contributions in triglyceride reference materials, CDC routinely screens reference serum samples for free glycerol levels using enzymatic methods. Such enzymatic procedures for the analysis of serum free glycerol are convenient and useful for screening purposes, but an alternative method with both high specificity and a relatively low potential for matrix influences was needed for comparative evaluations. Consequently, we examined the application of a chromatographic method to this assay.

Serum free glycerol in pig plasma has been measured by GC with flame-ionization detection by Fenton and Aherne [1], who used 1-dodecanol as the internal standard. Also, Matarese and Zamponi [3] assayed therapeutic levels of glycerol in human serum and cerebrospinal fluid samples using erythritol as the internal standard. Although good quantitative results were reported in both of those studies, an ID-GC–MS method using an internal standard with chemical and physical properties essentially identical to the analyte should afford the highest possible reliability. Björkhem *et al.* [9] followed such an approach in their analysis of serum triglycerides by ID-GC–MS, by using triglycerides labeled with deuterated glycerol as the internal standard. Glycerol has also been quantitated on human skin surfaces by using GC–MS, although no internal standard was used in that study [12].

In our analyses, glycerol (as the TMS ether) was well resolved from potential interfering compounds in serum extracts and could be measured with an overall analytical relative standard deviation of approximately 2–3%. The precision of our analyses could probably be further improved by using bracketing procedures for calibration [13] and by using [1,2,3-$^{13}C_3$]glycerol rather than [1,3-$^{13}C_2$]glycerol as the internal standard. Although the use of a standard curve is more convenient than the bracketing method and is adequate for our present application, we are evaluating the use of [1,2,3-$^{13}C_3$]glycerol as an alternative internal standard. The accuracy of the method is, of course, partly dependent on the quality of the standards and in this regard "...it is well to remember that the preparation of pure, anhydrous glycerol has always presented serious difficulties" [14]. In our analyses, freshly prepared and commercial aqueous glycerol standards provided equivalent results; also, dilutions of the same standards were used for calibrating the enzymatic and the GC–MS assays, facilitating direct comparisons between them.

Analyzing several reference serum pools for free glycerol concentrations by using three different enzymatic kits sometimes produced substantially different results, even though all three methods provided good results when pure, aqueous standards were assayed, or when standard addition studies were carried out. Thus, potential matrix-specific influences on enzymatic free glycerol assays must be considered. Comparison of the data in Table II indicates that kit C provided results that generally corresponded most closely to the ID-GC–MS values. This enzymatic method (kit C) is the one currently used at CDC for screening reference materials for free glycerol, and our results to date suggest that this kit is capable of generating serum free glycerol values that are similar to those estimated by GC–MS. The periodic comparison of free glycerol results obtained by the enzymatic and ID-GC–MS methods on selected serum pools should help to assure the reliability of these measurements in future reference material evaluation and routine method assessments.

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