Evaluation of Commercial Heparin Preparations for Use in the Heparin–Mn$^{2+}$ Method for Measuring Cholesterol in High-Density Lipoprotein

Cathy Mayfield, G. Russell Warnick, and John J. Albers

Commercial heparin preparations (18 lots) from seven manufacturers were compared in the heparin–Mn$^{2+}$ procedure for high-density-lipoprotein cholesterol quantitation. With normotriglyceridemic samples, 16 heparin lots, isolated from porcine intestinal mucosa, gave mean values for supernatant cholesterol that did not differ statistically; all were within 7 mg/L. Two heparin preparations from bovine lung gave results that were slightly (16 mg/L, average) but significantly ($p < 0.005$) lower. With hypertriglyceridemic samples, we observed greater variation in supernatant cholesterol among the heparin preparations, which was ascribable to variable sedimentation by centrifugation of very-low-density and low-density lipoproteins precipitated by heparin–Mn$^{2+}$ treatment. If the precipitated lipoproteins were completely removed by an ultrafiltration procedure, we saw no significant difference among the heparin preparations for results with hypertriglyceridemic samples.

Additional Keyphrases: heparin from pig intestine and bovine lung · variation, source of · hyperlipoproteinemia

The inverse relationship between HDL$^1$ concentration and cardiovascular disease risk has been emphasized in recent epidemiological studies (1–3). As a result, clinical laboratories have experienced increased demand for HDL measurement and there is a subsequent need for reliable and convenient quantitation procedures. HDL, defined as the lipoproteins with hydrated density between 1.063 and 1.21 g/mL, may be isolated by ultracentrifugation (4). Because lipoproteins are generally measured in terms of their cholesterol content, the cholesterol in this fraction, or in practice the cholesterol in the $d > 1.063$ fraction, has been used as a reference for HDL quantitation to which other methods have been compared. Because ultracentrifugal separation is not practicable for routine analysis, chemical precipitation methods have generally been used (reviewed in ref. 5). A common procedure for HDL quantitation involves precipitating the apoB-associated lipoproteins, primarily VLDL and LDL, with heparin and Mn$^{2+}$, then measuring the cholesterol remaining in the supernetant fluid (6–9). This procedure is demonstrably accurate in relation to the ultracentrifugation procedure (9–12).

Heparin, a multi-sulfated mucopolysaccharide (13), is composed of partly sulfated units of glucuronic acid and amino sugars, joined by 1→4 bonds. The interaction of the negatively charged sulfate groups with positively charged lipoprotein amino groups facilitates complex formation (14). However, heparin is polydisperse, and differences in source or purification techniques produce variability in commercial heparin preparations. Heparin isolated from porcine intestinal mucosa has been reported to differ from bovine lung heparin in molecular size and composition (15, 16). Whether this reported variability in commercial heparin is a source of error in HDL quantitation has not been evaluated.

In this study, we compared 18 heparin preparations from seven manufacturers and two tissue sources to determine whether variability in commercial heparin preparations might be a significant source of error in HDL quantitation by the heparin–Mn$^{2+}$ procedure.

Materials and Methods

Heparin Preparations

We compared 18 sodium heparin lots from seven manufacturers (Table 1) in the heparin–Mn$^{2+}$ procedure for HDL quantitation. The two Upjohn lots were purified from bovine lung; the remainder were purified from porcine intestinal mucosa. Preparations from Abbott Laboratories, Invenex Pharmaceuticals, Organon Canada, The Upjohn Company, and Riker Laboratories lots 34402, 68032, 68762, 68763, and 68761 were contributed by other laboratories.2 Heparin from Invenex Pharmaceuticals, received from another laboratory after the comparisons were begun, was tested on a subset of the samples. Calbiochem kindly provided lot 60238, and Riker Laboratories provided their lots 79311, 80726, 80906, 79767, and 80908. We purchased Riker lot 68006. Lot 65C-0061 was purchased from Sigma Chemical Co. and had been stored undesiccated in a refrigerator for about three years. Riker lot 34402 was prepared by the manufacturer about three years ago. Working heparin solutions were prepared by diluting the

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2 Heparin preparations were supplied by the following persons: Carl Breckenridge, Lipid Research Clinic, University of Toronto, Toronto, Ontario, Canada; Allen DeShong, Burien Medical Laboratory, Seattle, WA; Mauro Nava, Lipid Research Clinic, Baylor College of Medicine, Houston, TX; Paula Steiner, Lipid Research Clinic, University of Cincinnati, Cincinnati, OH; Stewart Weidman, Lipid Research Clinic, Washington University, St. Louis, MO; Donald Wiebe, Lipid Research Clinic, Iowa City, IA.

Northwest Lipid Research Clinic, Harborview Medical Center, 326 Ninth Ave., Seattle, WA 98104; and Department of Medicine, University of Washington, Seattle, WA 98195.

1 Nonstandard abbreviations used: HDL, LDL, VLDL: high-, low-, and very-low-density lipoproteins; and apoB: the major apolipoprotein of VLDL and LDL.

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preparations to 5000 USP kilounits/L with 0.15 mol/L NaCl solution. Mn$^{2+}$ solutions of 1.0 and 2.0 mol/L were prepared from reagent-grade MnCl$_2$·4H$_2$O.

Samples

Plasma samples were obtained from normolipidemic and hypertriglyceridemic subjects according to Lipid Research Clinic protocol (8). Blood was collected from subjects after a 12–14 h fast into tubes containing 1.5 g of disodium ethylenediaminetetraacetate per liter. Without delay, the samples were cooled to 4°C and the cells removed by centrifugation within 2 to 4 h. Samples were stored at 4°C.

Heparin–Mn$^{2+}$ Precipitation

The apoB-associated lipoproteins were precipitated by heparin–Mn$^{2+}$ treatment, with Mn$^{2+}$ at 46 mmol/L (6–8) or at 92 mmol/L (9) final concentration. To 2.0 mL of plasma were added sequentially 80 μL of heparin solution (each of 18 lots at 5000 USP kilounits/L) and 100 μL of either 1.0 or 2.0 mol/L MnCl$_2$ solution. Generally, samples were precipitated in duplicate for each heparin lot. Samples were mixed thoroughly with a vortex-type mixer after each addition and then placed in crushed ice for 30 min. Subsequently, precipitated lipoproteins were sedimented by centrifugation (1500 × g, 4°C, 30 min).

After this centrifugation, the supernates were carefully examined for turbidity (indicating incomplete sedimentation of the LDL/VLDL precipitate). Clear supernates were collected by pipetting. Turbid supernates were cleared by a previously described ultrafiltration procedure (17). In brief, a 0.22-μm filter, 25 mm in diameter, was placed on the lower portion of a 25-mm Swinnex holder (Millipore Corp., Bedford, MA 01730). A silicone gasket, fitted with 22-mm AP 15 and AP 20 depth prefilters, was placed on top of the filter, and the upper portion of the Swinnex holder was tightened over the filter assembly. The turbid supernate was forced through the filter with moderate pressure from a 5-mL syringe. The clear filtrates were collected for analysis.

Heparin Titration

A donor plasma pool (total cholesterol 2210 mg/L, triglycerides 680 mg/L) was precipitated with each of six representative lots of heparin over a range of final heparin concentrations from 46 to 367 USP kilounits/L at a constant final Mn$^{2+}$ concentration of 92 mmol/L. The precipitates were sedimented by centrifugation, and the clear supernates were collected for analysis by pipeting.

Lipid and Apoprotein Analysis

Cholesterol and triglycerides were measured by semi-automated, continuous-flow procedures for the AutoAnalyzer II, following Lipid Research Clinical protocol (8) as described previously (9–17). In the heparin comparison experiments, all supernates obtained from a single sample were analyzed together in one analytical run. Within each set of 17 supernates (precipitation with each of 17 heparin lots at either 46 or 92 mmol/L), supernates were analyzed consecutively but in random order. Similarly, samples were precipitated in duplicate at each of two Mn$^{2+}$ concentrations, and all four sets were arranged in random order to minimize the systematic effects of scale expansion and baseline drift. Under these conditions, CVS of about 1% were obtained for cholesterol measurement in the heparin–Mn$^{2+}$ supernates (17).

Incomplete precipitation of LDL/VLDL in heparin–Mn$^{2+}$ supernates was assessed by measuring apoB-associated cholesterol by a radial immunodiffusion procedure (18) described previously (9, 17), in which supernates were applied to wells in agarose gels containing antibody for specific apoB. Results were determined from precipitation ring diameters in relation
to LDL standards and reported as apoB-associated cholesterol.

Results and Discussion

Eight normotriglyceridemic plasma samples were precipitated by the heparin–Mn²⁺ procedure with each of 17 different heparin preparations. Supernatant cholesterol concentrations resulting from duplicate precipitations at each of two Mn²⁺ concentrations are shown in Table 2. Supernatant cholesterol concentration averaged about 25–45 mg/L lower when final Mn²⁺ concentration was 92 mmol/L than when it was 46 mmol/L, which is in agreement with previous reports (9, 11, 19). Also, at the higher Mn²⁺ concentration, supernatant cholesterol was slightly (18–28 mg/L) but significantly (p < 0.005, Student’s paired t-test) lower with bovine lung heparin preparations than with porcine intestinal mucosa heparins. With Mn²⁺ concentration at 46 mmol/L, the bovine lung heparin gave results 4–11 mg/L lower than the intestinal heparin. Values with lung heparin were significantly lower (p < 0.005) than the highest intestinal heparin values (see Table 2). Results for supernatant cholesterol were not significantly different among the various intestinal heparin preparations or between the two bovine lung preparations.

An additional heparin lot from intestinal mucosa (Invenex 1557624, Table 1), received after the earlier studies had been completed, was compared with Riker lot 68006 in duplicate determinations on 10 plasma samples. With the Invenex heparin, the mean supernatant cholesterol concentration was 503 mg/L; with the Riker preparation it was 508 mg/L. The difference, 5 mg/L, was not statistically significant.

These results suggest that all of the heparin preparations from intestinal mucosa are essentially equivalent in heparin–Mn²⁺ precipitation of normotriglyceridemic samples. Even with the two preparations known to be at least three years old, results were not different from those obtained with the other lots. However, the heparin batches from bovine lung consistently gave mean values for supernatant cholesterol that were slightly lower than those with the intestinal heparin preparations. Inspection of results for individual plasma samples showed that the difference in supernatant cholesterol between bovine lung and intestinal mucosa heparin preparations was greater in samples with higher HDL cholesterol concentrations. We saw very little difference in samples with low HDL cholesterol concentrations.

Measurement in supernates of apoB, the major protein of VLDL and LDL, assesses incomplete precipitation of these lipoproteins. It has been previously demonstrated that supernates of samples precipitated with intestinal mucosa heparin and Mn²⁺ at 46 mmol/L contain measurable apoB (9, 19). In this study a subset of 10 samples precipitated with Riker lot 68006 contained an average of 3 mg of apoB-associated cholesterol per liter, as measured by radial immunodiffusion assay. The same samples precipitated with heparin from bovine lung were all negative for apoB. With Mn²⁺ at 92 mmol/L, apoB was not detectable in supernates obtained with either type of heparin, which is in agreement with previous reports that at the higher Mn²⁺ concentration virtually all of the apoB-containing lipoproteins were precipitated (9, 19). Preliminary experiments suggest that the amount of supernatant apoB present after precipitation with heparin of intestinal origin and heparin–Mn²⁺ is a function of the time delay between blood collection and heparin–Mn²⁺ treatment. Samples precipitated immediately after collection contained substantially less apoB than did samples precipitated several days later (11). These results suggest that lung heparins produce on the average lower supernatant apoB as well as lower cholesterol concentrations. However, if samples are precipitated soon after collection, supernatant apoB is negligible with both intestinal and lung heparins.

Precipitation was performed with six representative heparin preparations over a range of final heparin concentrations from 46 to 367 USP kilounits/mL and a constant final Mn²⁺ concentration.
centration of 92 mmol/L. Values for supernatant cholesterol are shown in Figure 1. In agreement with the data in Table 2, the bovine lung heparin, lot 109 EM, gave lower values for supernatant cholesterol at each concentration than did the intestinal preparations, which were all similar in precipitation. The difference was particularly apparent at the highest heparin concentrations. Values for supernatant cholesterol decreased slightly with increasing intestinal heparin concentration. As compared to values for supernatant cholesterol at the usual final heparin concentration (18 USP kilounits/L), results were about 10 mg/L higher with heparin at half the usual concentration and 10 mg/L lower with heparin at twice the usual concentration. The results suggest that with intestinal heparin preparations, lipoprotein precipitation is affected only slightly by small differences in final heparin concentration. Thus, the small variations in weight concentration among different heparin preparations are not likely to significantly affect supernatant cholesterol concentrations.

A plasma pool with a moderately above-normal triglyceride concentration (1530 mg/L) was precipitated with each of 17 heparin preparations and Mn2+ at 46 and 92 mmol/L. With Mn2+ at 92 mmol/L, supernatant solutions were all clear and cholesterol concentrations similar, within experimental error (Table 3). However, with Mn2+ at 46 mmol/L, seven of the 17 lots of heparin produced turbid supernatants, which had substantially higher cholesterol concentrations than did the clear supernatants. This supernatant turbidity has been demonstrated to be a result of incomplete sedimentation of the precipitated apoB-associated lipoproteins, primarily VLDL and LDL, and therefore results in overestimation of HDL (17). Sedimentation is incomplete when the density of the heparin-Mn2+ lipoprotein precipitate is nearly that of the solution, precluding separation by low-speed centrifugation. The material producing turbidity can be removed by centrifugation at higher forces (9) or by ultrafiltration (17). The complete sedimentation with Mn2+ at 92 mmol/L is in agreement with previous reports of improved sedimentation of the precipitated apoB-containing lipoproteins at the higher Mn2+ concentration (9, 19). We did not establish why sedimentation was incomplete with some of the heparin preparations.

We further investigated this variability in supernatant cholesterol due to incomplete sedimentation by precipitating hypertriglyceridemic samples with the various heparin preparations and Mn2+ at 46 or 92 mmol/L (Table 4). The turbid supernates obtained with some of the heparin preparations were cleared by ultrafiltration. Mean results for HDL cholesterol were similar with all of the heparin preparations. These data demonstrate that, if unsedimented lipoproteins are removed, there is no difference in lipoprotein precipitation, even with hypertriglyceridemic samples. Of particular note in these samples, no difference was observed in mean supernatant cholesterol values between bovine lung and porcine intestinal heparin preparations. However, this was probably because of the generally low HDL cholesterol concentrations in these samples. The one sample in this group with the highest HDL cholesterol value (44 mg/L) did have lower supernatant cholesterol values with bovine lung heparin.

In summary, these data demonstrate that the heparin preparations derived from porcine intestinal mucosa produce essentially equivalent supernatant cholesterol results in the heparin-Mn2+ technique for HDL cholesterol quantitation, even though heparin concentrations varied as much as 15% by weight. The heparin preparations evaluated here are not
inclusive, but they are representative of those used by laboratories for lipoprotein precipitation. The fact that even a twofold increase or decrease in intestinal heparin concentration produced only a relatively small change in supernatant cholesterol, suggests that the variation in weight concentration among commercial preparations is not a significant source of error. Incomplete sedimentation of precipitated lipoproteins is a potential source of variability, especially with lipemic samples. However, turbid supernates should be detected and the precipitate completely removed by a technique such as ultracentrifugation, which will eliminate this source of variation.

With intestinal-mucosal heparin, the heparin–Mn2+ procedure has been previously demonstrated to provide results in good agreement with an ultracentrifugation method (9, 11). The two preparations from bovine lung gave slightly lower results, particularly with Mn2+ at 92 mmol/L. Whether this systematic difference is unique to the two lots evaluated here or characteristic of all bovine lung heparin preparations was not established. However, the fact that heparin from bovine lung has been reported to be chemically different from intestinal-mucosa heparin (15, 16) suggests the systematic difference may be characteristic.

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References