



WHO MONICA PROJECT
MANUAL OF OPERATIONS



STANDARDIZATION OF LIPID MEASUREMENTS

CONTENTS

	<u>Page</u>
List of Abbreviations.....	2
1.0 Obligatory and voluntary biochemical measurements.....	3
2.0 Sample collection and initial processing.....	3
3.0 Storage of plasma/serum samples.....	4
4.0 Recommended analytical procedures for total cholesterol and high-density lipoprotein cholesterol measurement.....	5
5.0 Calibration of methods.....	8
6.0 Units of reporting.....	9
7.0 Internal quality control (IQC).....	10
8.0 External quality assessments (EQA).....	13
9.0 Recording and reporting Monica and quality control results.....	15
10.0 Actions if performance with required accuracy and/or precision cannot be achieved in a laboratory.....	15
BIBLIOGRAPHY.....	16
TABLE 1 Acceptability criteria for performance in EQA.....	19
TABLE 2 Monica - Time Estimates for Sample Processing (from WHO-LW report).....	20
TABLE 3 Monica - Proposed Methods for Lipid Analysis (from WHO-LW report).....	21
APPENDIX I Recommended sequence of specimens in a run.....	22
APPENDIX II Calculation of the pool mean and total (overall) standard deviation.....	23
APPENDIX III Example of Quality Control Chart.....	25

CVD Unit

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LIST OF ABBREVIATIONS

Apo-B	Apoprotein B
CDC	Centers for Disease Control, Atlanta, Georgia 30333, USA
EDTA	Ethylene diamine tetra acetic acid
EQA	External quality assessment
G	Gravity
HDL	High density lipoproteins
HDL-C	High density lipoprotein cholesterol
IKEM	Institute for Clinical and Experimental Medicine, Prague, Czechoslovakia
IQC	Internal quality control
LDL	Low density lipoproteins
LRC	Lipid Research Clinics
Monica	Monica Project
OD	Optical density
QA	Quality assurance
R.p.m.	Rotations per minute
RV	Reference value
TC	Total cholesterol
TG	Triglycerides (Triacylglycerols)
VLDL	Very low density lipoproteins
WHO	World Health Organization
WHO-HQ	WHO Headquarters
WHO-KRIS	WHO-Kaunas-Rotterdam Intervention Study
WHO-LW	WHO Lipid Workshop (Prague, 26-28 January 1982)
WHO-RLRC	World Health Organization Regional Lipid Reference Centre (WHO Collaborating Centre for Blood Lipid Research In Atherosclerosis and Ischaemic Heart Disease at the Institute for Clinical and Experimental Medicine (IKEM), 14622 Prague - 4, Czechoslovakia

1.0 Obligatory and voluntary biochemical measurements

1.1 Total cholesterol (TC) is the minimum obligatory parameter to be measured in blood (serum, plasma) samples of the Monica study subjects in all cooperating centres (see Monica Protocol, WHO/MNC/82.1). However, wherever possible, samples should also be analysed for HDL-C and further parameters on a voluntary basis. Laboratory directors who attended WHO/LW agreed upon HDL-C measurements in Monica study centres.

1.2 HDL-cholesterol (HDL-C) is strongly recommended and will be measured by many centres (who must standardize their technique on the Reference Centre). Other voluntary complementary measurements might include, e.g.: triacylglycerols (triglycerides), lipoprotein typing and composition (electrophoresis, ultracentrifugation, ultrafiltration and nephelometry, chromatography of lipoproteins, etc.), apoprotein (12) concentrations (immuno-electrophoretic, radial-diffusion and/or nephelometric methods), glucose, uric acid, urea, electrolytes, creatinine, thyroxine, haemoglobin, carboxyhaemoglobin, and in certain situations, trace elements. If myocardial infarction is suspected, the asparate amino transferase, alanine amino transferase, lactate dehydrogenase may be measured, e.g.

2.0 Sample collection and initial processing

2.1 Centres should collect Monica blood samples in comparable seasonal periods (i.e. during the same months in subsequent years) to prevent effects of seasonal variation on the levels of measured serum/plasma parameters. Centres collecting Monica blood samples in the course of the whole year should randomize blood taking for sex and age groups to obtain each year approximately the same number of subjects of different categories in comparable time periods (months).

Medical staff should keep detailed records on circumstances which may affect lipid levels and/or methods (certain drugs, alcohol consumption, pregnancy, etc.). Because the Monica survey is concerned with risk factor levels in the community, however they have been modified, lipid affecting medication should not be interrupted before blood taking.

It is recognized that it is not feasible to obtain fasting samples in all cooperating centres and subjects. Since circulating levels of serum/plasma TC and HDL-C are relatively stable with respect to immediate dietary history, use of non-fasting samples can be admitted in this study for measurement of TC and HDL-C [34]. Centres must standardize their procedures so that they are consistent as to time of day and fasting status from one survey to the next. The time of venipuncture should be recorded.

Subjects should be asked before venipuncture about their food intake during the previous 12-16 hours and adequate records made if a non-fasting sample is taken and analysed (see also 4.5.6).

2.1.2 Blood should be drawn from the subject or patient in a sitting position by venipuncture. Prolonged venous occlusion can cause changes in the apparent concentrations of blood constituents (3). Use of tourniquet should therefore be avoided. If a good flow cannot be obtained in some subjects and the tourniquet has to be used, it is released prior to withdrawal of blood.

Standardization of the position (sitting position is recommended) is necessary since plasma volume changes occur when a standing subject assumes a recumbent position [20, 3].

3.2 Serum should be used in preference to plasma.

It is realised that the group of laboratories standardized with CDC using LRC methods will continue using these methods for the Monica (Table 3, page 21). It follows that the group will continue using EDTA plasma prepared according to the LRC prescriptions [20].

2.2.1 Either 10 ml vacuum tubes without anticoagulant or syringes and glass tubes may be used to collect blood. Glass tubes should be equipped with stoppers made from material inert to the blood components. When vacuum tubes are used, the type with stoppers not lubricated with glycerol should be selected (glycerol causes interference with TG assays by enzymatic methods).

2.2.2 The use of vacuum tubes containing EDTA is recommended if plasma is used (group of laboratories following LRC methods).

2.3 For serum preparation, blood samples are allowed to clot at not more than 20°C for up to one hour before centrifugation whenever possible. There is evidence (personal communication from the Helsinki Centre) that this period could be prolonged by up to three hours.

Blood specimens should be centrifuged at a temperature of no more than 20°C (warning: Prolonged running of a non-refrigerated centrifuge may result in considerable warming of the inner centrifuge compartment and centrifuged samples) at a minimum of 1500 G for at least 10 minutes to separate serum from the clot. If a refrigerated centrifuge is not available, it may be necessary to cool blood samples (for instance, in a refrigerator or on wet ice) before centrifugation. With a refrigerated centrifuge, centrifugation should be done preferably at 4°C. Blood samples must not be frozen during processing (this would cause haemolysis).

2.4 For plasma preparation (groups following LRC method) the tube(s) filled with blood must be immediately stoppered (if vacuum tubes are not used) and inverted about 10 times gently to ensure prompt and thorough mixing of blood sample(s) with EDTA. Mixing should not be vigorous. According to LRC recommendations the blood samples are then cooled on wet ice. Within 3 hours (and preferably within one hour) the tubes should be centrifuged at 4°C in a refrigerated centrifuge at 1500 G for 30 minutes. If a refrigerated centrifuge is not available within 3 hours of collection, the samples may be centrifuged at room temperature within 1 hour of collection, and the plasma stored at 4°C.

2.5 After centrifugation, the serum/plasma should be promptly separated from clot or cells (transfer to a clean tube). The white cell layer (buffy coat) is not transferred with plasma.

2.6 Haemolytic serum/plasma samples should be discarded and new samples should be retaken from the subjects and analysed.

3.0 Storage of serum/plasma samples

3.1 It is recommended to estimate the TC and HDL-C levels on the day of sample collection. If analyses cannot be performed within 4 days the serum or plasma samples have to be immediately stored at -20°C or lower in tightly closed glass vials [1].

3.2 Samples can be stored for up to four days at 4°C [1, 3, 5].

3.3 Precipitation of Apo-B containing (LDL + VLDL) lipoproteins (see 4.5) should preferably be done on fresh (non-frozen) serum aliquots on the day of blood collection. Should it be impossible to perform precipitation on fresh samples, the serum or plasma for HDL-C determination should be immediately frozen at -20°C or lower [1, 3]. Precipitation should then be performed within 14 days. The sample should be thawed only once and well mixed. It should be recognized, however, that freezing may introduce a systematic error of up to 4% in HDL-C measurements [3].

3.4 Storage of serum/plasma samples for other purposes than immediate analysis: if samples are to be stored for voluntary analysis, laboratories must select adequate storage conditions at which measured parameter concentrations would not change.

4.0 Recommended analytical procedures for TC and HDL-C measurement

4.1 It is recommended to use an enzymatic cholesterol method with practically 100% cholesterol ester hydrolysis. Advantages include non-corrosive reagents, easy and fast operational steps, specificity, possibility to use automated methods as well as manual methods with inexpensive instruments, unification of methodology in the project, etc.

However, it is realised that a group of laboratories standardized with CDC using LRC methods based on extraction into isopropanol, will continue using their methods for the Monica (Table 3, page 21) for the purpose of research continuity.

4.2 Use of a number of commercial brands of enzymatic reagents for cholesterol analysis might diminish comparability of results between laboratories due to non-homogeneity of production lots or due to loss of kit enzyme activities on storage, for example. Information collected at the WHO-LW showed that most of the participating laboratories have worked or are going to work with the same commercial brand which is widely used in Europe. This is fortunate for the project. Suitability of selected enzymatic methods will be checked by means of the EQA.

4.3 It is possible that a few laboratories taking part in the Monica would be unable to comply for local reasons with the recommendation to use an enzymatic method. These laboratories probably will continue to use the so-called "direct", "one-step", "Liebermann-Burchard" method(s) in manual or automated version(s), etc. The WHO-RLRC will circulate a questionnaire concerning analytical and associate procedures to be used in the different centres, with the aim of obtaining full information namely on the use of other than LRC or enzymatic methods. It is the policy of Monica to accept in exceptional cases participants not able to use the recommended methods. In such cases particular attention to methods for ensuring comparability would be necessary if performance within allowable EQA limits is unattainable with use of primary pure standards. The laboratories concerned may have to calibrate with serum calibrators provided by the WHO-RLRC.

4.4 Every participating laboratory should provide a full detailed description of their entire procedure of sample collection and analysis to WHO-HQ, Cardiovascular Diseases unit, and to Dr D. Grafnetter (WHO-RLRC) at the latest six months before analysis of Monica samples should start in the centre concerned.

- 4.5 Isolation of the HDL-fraction:
- 4.5.1 Laboratories are recommended to use the "Phosphotungstate-Mg²⁺" (PT) precipitation method [2, 10, 11, 15, 31, 32, 33] for isolation of HDL from LDL + VLDL. In this method final concentrations after mixing serum (or plasma) with precipitation reagent(s) are: 3.6 g/litre for phosphotungstic acid and 0.045 M for Mg²⁺ [21, 24]. According to the quoted papers, precipitation of LDL+VLDL is complete if pH of the final mixture (serum/plasma + PT reagents) remains below pH 7.6.
- 4.5.1.1 Some laboratories, e.g. those with the LRC methodology, may need to continue with other precipitation techniques (Mg²⁺-dextran, Mn²⁺-heparin, e.g.). Acceptance of these methods is the same policy case as mentioned under 4.3.
- 4.5.2 Preparation of PT method [31, 32] reagents for precipitation at pH below 7.6 (for alternative concentrations and use of PT reagents see section 4.5.7):
- 4.5.2.1 Sodium phosphotungstate, 40.0 g/l, pH 7.4. Dissolve four grams of phosphotungstic acid (reagent grade) in about 60 ml distilled water. Add gradually under mixing and using pH meter as much of 1 mol/l NaOH as to reach pH 7.4 (usually somewhat less than 16 ml). Then make the volume up to 100 ml.
- 4.5.2.2 Magnesium chloride, 2.0 mol/l: Dissolve 40.6 grams of MgCl₂·6H₂O (analytical grade) in about 80 ml of distilled water and make the volume up to 100 ml.
- 4.5.2.3 Reagents should be kept at 4°C between use and can be used as long as 6 months, provided they show no bacterial growth.
- 4.5.3 Use of PT reagents described under 4.5.2.1 and 4.5.2.2:
- 4.5.3.1 Add 100 µl of the phosphotungstate solution (4.5.2.1) to 1.0 ml of sample and mix (preferably by vortexing). Add 25 µl of magnesium chloride solution (4.5.2.2) and again mix well. Let each precipitated sample stand for ten minutes at room temperature and then centrifuge. For centrifugation conditions see section 4.7.0.
- 4.5.3.2 Volumes of sample and reagents could be reduced (e.g. halved) for the precipitation step.
- 4.5.3.3 It is also permissible to use pre-mixed reagent. This is prepared and used as follows: phosphotungstate and magnesium chloride solutions are mixed on the day of use in the ratio of 100 : 25 (e.g. 4 ml + 1 ml). Then 125 µl of the pre-mixed reagent are added to 1.0 ml of sample and the whole is mixed well.
- 4.5.4 Centrifugation after precipitation: Since centrifugation at +4°C on one hand, and +20°C on the other hand can lead to certain differences in HDL-C levels in some reconstituted lyophilized quality control materials (observations made in the WHO-RLRC) but not in fresh sera, it is recommended (for all but the LRC methods laboratories) to centrifuge "HDL samples" after PT precipitation of Apo B containing lipoproteins at room temperature (not below +15°C or above +25°C) at 2000 G for 30 minutes.
- 4.5.5 After centrifugation the supernatant should be immediately transferred to a clean and dry tube.
- 4.5.6 With study subject samples only clear (non-turbid) supernatants should be used for subsequent cholesterol analysis. With the PT method (if TG are elevated) the supernatant may occasionally show some turbidity. In that case the precipitation step should be repeated with the serum/plasma sample diluted 1:1 with 0.9% sodium chloride solution in distilled water (saline, physiological solution). This

usually results in obtaining a clear supernatant (but precision of the cholesterol assay is decreased; remember that result must be multiplied by two if the 1:1 diluted sample is used - see also section 4.6.4). Should dilution of serum/plasma sample not give a clear supernatant after precipitation and centrifugation, do not proceed. Any such irregularity and/or difficulty in obtaining clear supernatant should be recorded in the form for recording results.

4.5.6.1 In some lyophilized quality control materials slight turbidity of supernatants may be unavoidable. The WHO-RLRC will distribute instruction for use of pools with individual shipments of EQA sets.

4.5.7 Alternative concentrations and use of PT method reagents:

Special (adjustable or constriction) pipettes are necessary to deliver the unusual volumes (e.g. 25 μ l or 125 μ l) of the above "classical" PT method reagents. The following modified reagent concentrations (33) and their use (see sections 4.5.7.1, 4.5.7.2 and 4.5.7.3) yield the same result but enable the use of normal precision pipettes.

4.5.7.1 Sodium phosphotungstate, 48.0 g/l, pH 7.4: dissolve 4.8 grams of phosphotungstic acid (reagent grade) in about 50 ml distilled water. Add gradually under mixing and using pH meter as much of 1 mol/l NaOH as necessary to reach pH 7.4. Then make the volume up to 100 ml.

4.5.7.2 Magnesium chloride, 3.0 mol/l: dissolve 60.9 grams of $MgCl_2 \cdot 6H_2O$ (analytical grade) in about 80 ml of distilled water and make the volume up to 100 ml.

4.5.7.3 Use of the modified PT method reagents:

Precipitation reagent is prepared by mixing 5 parts of phosphotungstate (4.5.7.1) with one part of magnesium chloride (4.5.7.2) solution (e.g. 5 ml + 1 ml) on the day of use. Then 100 μ l of the premixed reagent are added to 1.0 ml of serum/plasma sample and the whole is mixed well. Further steps (centrifugation, etc.) are the same as those starting at section 4.5.4. However, multiplication (dilution) factor (4.6.3) will be 1.10 in this case (not 1.125).

4.5.8 Some laboratories have used a commercial PT precipitation kit and followed its working instructions, and they may wish to continue doing so. This is possible if results obtained with the use of such a kit are found compatible with the EQA criteria.

4.6.0 Determination of cholesterol in the HDL containing supernatant

4.6.1 Determination should be done preferably shortly after precipitation of LDL+VLDL and the centrifugation step (on the same day). If it is necessary to store supernatant(s) analysis is recommended within 4 days if stored at +4°C. Prolonged storage requires at least -20°C. Storage is recommended in small volume glass tubes (vials) with leak-proof stoppers to prevent volume and concentration changes (evaporation, freezing out). After storage and before analyses the samples brought to room temperature should be gently mixed.

4.6.2 Cholesterol should be determined in the HDL-containing supernatants by the same method as used in the laboratory concerned for TC estimation. Should method sensitivity be low (optical density readings in unreliably low range) it might be necessary to use a photometric cuvette with greater optical length and/or to increase the supernatant sample volume. Reliability of the method must be particularly checked in such cases [30].

- 4.6.3 To obtain the serum/plasma HDL-C concentration, the determined concentration of cholesterol in the supernatant must be multiplied by the dilution factor (e.g. in case of 1 ml non-diluted sample + 125 μ l of precipitation reagents, the dilution factor is 1.125).
- 4.6.4 When serum/plasma sample was diluted 1:1 before us (see section 4.5.6) one must multiply still by two to obtain final serum/plasma HDL-C concentration.
- 4.6.5 Duplicate or single TC and HDL-C measurements: If possible, all Monica serum/plasma samples should be analyzed in duplicate. For analysis of cholesterol by extraction chemical methods two extracts would be required (do not perform two analysis on one extract). For HDL-C analysis duplicate LDL+VLDL precipitations would be required. Mean results are reported if analysis are performed in duplicate.
- 4.6.6 For IQC and EQA samples all analyses are performed in duplicate (but mean values are not reported, both values are recorded and reported).
- 4.6.7 The IQC rules shown below (see section 7.7.0) give criteria for each single measurement result on control samples, from which the maximal allowable difference between side-by-side duplicates is in fact also defined. Daily use of IQC information is of prime importance for maintaining good precision, since EQA information is only retrospective and primarily devoted to following interlaboratory comparability.

It is recommended in addition that laboratories analyzing not only control but also Monica project samples in duplicate should reanalyse the samples with duplicates differing more than 0.40 mmol/l for TC (15.0 mg/dl), or 0.20 mmol/l (8.0 mg/dl) for HDL-C. Should duplicate results differ that much rather frequently, the method should be revised and every effort be made to improve precision.

5.0 Calibration of methods

Recommendation: Primary standards and/or secondary serum/plasma calibrators are used at least in duplicate for calibration.

- 5.1.0 Each participating laboratory is responsible for its own analytical primary standards and/or secondary serum calibrators.
- 5.1.1 It is assumed that all participating laboratories will implement the best and most appropriate pure substances and method reagents. Cholesterol substance used for preparation of standards should be of more than 99% purity.
- 5.1.2 Secondary (serum) calibrators should preferably be prepared and/or labelled with correct TC and/or HDL-C concentrations in the WHORLRC and/or CDC. It is understood, however, that staffing and budgetary restraints may be a limiting factor in such an undertaking.

- 5.1.3 The WHO-RLRC will distribute during the pre-standardization period a set of at least three cholesterol standards by use of which linearity should be tested over the working range. Preliminary agreements consider shipment of a commercial cholesterol standards pack (6 standards with concentrations of 50-400 mg%) to each laboratory. Such set should enable control of linearity and at the same time calibration of several enzymatic and/or extraction cholesterol methods. Some cholesterol methods (the so called "direct" Libermann-Burchard chemical methods) can not be calibrated by these "water soluble" standards (falsely high results might be obtained without special arrangements), but linearity response of these methods can be judged on the basis of these standards.
- 5.1.4 Each standard (calibrator) should be run at least in duplicate.
- 5.1.5 Each laboratory should perform tests on linearity over the usual working range (0.5 - 10.0 mmol/l), even if the WHO-RLRC or other body were not able to supply them free of charge with appropriate control material for this purpose.
- 5.1.6 It is essential that linearity should be checked repeatedly during the prestandardization period. During the study linearity should be checked regularly, and particularly if greater than allowable imprecision and/or inaccuracy (allowable limits on accuracy and precision - see 7.8.5 and 8.3.8) is detected. Ideally linearity should be checked with at least three standards in each run in which Monica samples are analysed. It is understood, however, that this will not always be possible in all laboratories and that it may not be necessary to use standards to judge linearity where adequate data can be obtained from IQC and EQA samples (see 7.3.2). Laboratories are free to check on linearity by any reasonable method guaranteeing acceptable results within the limits of accuracy and precision, therefore.

It is understood that linearity enabling use of a mean calculation factor (which asks for calibration line passing through the X,Y intercept after subtraction of reagent blank) may not be attainable in some of participating laboratories (e.g. because of limitations in the photometer or fluorometer available for the assay). Non-linearity need not necessarily mean wrong results. Linearity must be checked, and every effort should be made to attain it. If unattainable the laboratory should derive results mathematically from regression equation or graphically from standard curves covering the working range of the method. (Enzymatic cholesterol methods can usually provide linearity up to 11.5 mmol/l. When higher serum/plasma cholesterol concentration is expected analysis should be repeated after appropriate dilution of the sample according to the instructions of the manufacturer of the enzymatic kit).

- 5.1.7 More detailed information on checking on linearity, calibration, and QA can be found in the literature [4, 14, 19, 20, e.g.].

6.0 Units of reporting

Recommendation: All results should ideally be reported to 2 decimals in the International SI Units [23].

However, the older units (e.g. mg/dl,) are still used in some laboratories because continuity of methods is desirable until certain related projects are finished. In the Monica project results for TC and HDL-C will therefore be accepted both in mmol/l and md/dl since units can easily be converted by computer at statistical evaluation.

- 6.1 Results obtained in mmol/l should be calculated and reported to two decimals.
- 6.2 Results obtained in mg/dl should be calculated to one decimal, but TC concentrations should then be reported rounded off to whole numbers and HDL-C concentrations as calculated, i.e. to one decimal.
- 6.3 The conversion factor for cholesterol to mg/dl to mmol/l is: 0.025864.
Example: (250 mg/dl) x (0.025864) = 6.466 = 6.47 mmol/l.

7.0 Internal (intralaboratory) Quality Control (IQC)

Recommendation: at least two serum control pools are used for IQC. The preparation, checking, and use of these pools are described under 7.3.0, 7.7.0 and 7.8.0.

- 7.1.0 Performance of methods within IQC limits is a prerequisite for successful participation in EQA programme and for reliable analysis of Monica samples.
- 7.2.0 The following recommendations on how to prepare IQC pools and to proceed with IQC represent the views of the WHO-LW on the desirable methods of quality control. It is understood however that some laboratories will not be in a position to comply with all of these recommendations, and others will already have instituted adequate but different quality control procedures, which they are unable to change. In these cases each participating laboratory should inform the WHO-RLRC centre of their quality control and standardization methods. In other words, other than the recommended internal quality control system is permissible as long as there is adequate indication that analytical results as judged by IQC and EQA are within the IQC and EQA control limits described below. It follows that use of self-made and/or commercial frozen or lyophilized internal quality control materials is allowed. If lyophilized material is used for HDL-C, it should be material specifically designed for this purpose.
- 7.3 Establishment of pools: Each laboratory should establish at least two control serum pools for internal quality control. Each pool should last through the whole of one phase of the Monica and in any cases should be sufficient to last for one year of normal operations. The pools under 7.3.1 and 7.3.2 are the only two pools that are strictly required for Monica.
- 7.3.1 TC + HDL-C pool: This pool should be prepared from non-turbid human serum containing "normal" TC and TG concentrations. The pool should be distributed in suitable aliquots into tightly closed glass vials and ideally kept frozen at -60°C or below.* Should it be impossible to use a self-made or commercial frozen serum pool, a lyophilized pool could be used. In the latter case, there should be guarantee that the pool was prepared only from human sera, without use of additives or enrichments which could affect TC and HDL-C determinations, and it should be suitable for the HDL-C determination. The TC + HDL-C pool would be used both for TC and HDL-C method control, including the precipitation step in the latter case (the quality control specimen should be treated in exactly the same way as the test samples). Lyophilized pools do not require -60°C for storage. Even 4 to 6°C may be satisfactory for a defined expiration period.

* CDC laboratories found -20°C unsatisfactory for long term storage (personal communication). However, a number of Monica laboratories may have to store at -20°C only. In such cases the TC+HDL-C pool should be periodically checked for HDL-C concentration stability in runs containing EQA samples.

- 7.3.2 Low total cholesterol pool (LTC pool): This pool should contain 1.30-1.60 mmol/l cholesterol and it should be used for control in the low TC range. It could be prepared from a human serum pool diluted to the appropriate cholesterol concentration with 0.15 M NaCl. Alternatively, animal (bovine, horse) serum pool slightly diluted to the desired CH concentration level could be used.
- 7.3.2.1 Ideally, the appropriately (4-5 times) diluted TC + HDL-C pool (under 7.3.1) should be used as a LTC pool with the advantage that both pools could be used also for linearity checking (plot of absorbance against concentrations or dilution factors should result in a line passing through the X, Y intercept). This checking will work only with enzymatic and/or extraction cholesterol methods. In the "direct" chemical cholesterol methods the concomitant dilution of interference (by bilirubin, tryptophane, proteins, etc.), as well as changed viscosity in automated versions, may result in non-proportionality of absorbances to dilution.
- 7.4 Some laboratories may elect to establish a further (or several more) pool(s) for TC and/or HDL-C measurements, e.g. one with the cholesterol concentration in the upper part of the working range. The HDL-C pool(s) should have a low TC concentration (under 1.5 mmol/l).
- 7.5 Useful information concerning the preparation of self-made pools can be found in the literature [13, 14, 16, 17, 18, 22, 26, 27, 28, 29].
- 7.6 Use of IQC material to ensure analytical precision for TC and HDL-C analysis:
- 7.6.1 Once opened, an ampoule (vial) of quality control materials should not be used for more than one day's operations. Accurate reconstitution of lyophilized materials must be ensured (to prevent introduction of errors not related to the method itself). If a lyophilized pool is used for HDL-C control (precipitation included) each laboratory concerned should standardize the time period between reconstitution and analysis.
- 7.6.2 Each analytical run should begin with calibration standard(s). This could be the primary standard(s) and /or secondary serum calibrator(s) (see example in Appendix I).
- 7.6.3 IQC samples should follow. All quality control samples should be analysed in side-by-side duplicates. The results obtained from quality control samples following the standard(s) are used to indicate whether the run is in control and whether the analysis of study samples can be begun (criteria see 7.8.1).
- 7.6.4 When a new pool is introduced both the new and the old pool should be in use together (overlap period) for at least the number of runs (analyses) required to establish the starting mean (see 7.6.6), to ensure adequate calibration of the new pool and method control.
- 7.6.5 Control of drift in a run:
- In each analytical run (or part of analytical run) containing Monica samples a quality control material should be placed on every tenth position among the samples (to monitor within run stability). This can be other than the LTC+HDL-C or LTC pool material and need not necessarily be of human origin nor accurately calibrated because it serves only for monitoring within-run reproducibility (see Appendix I).

7.6.6 Before a pool is introduced, its starting concentration mean must be established. This is done as follows: at least 40 values are collected on at least 20 pool vials randomly distributed in independent analytical runs over at least 20 days (do not use 40 times in one run). Finally, the whole set of values should be examined for potential outliers [19]. If any value differs from the overall mean (\bar{X}) by a number greater than $3.3 s_t$ (i.e. if any $X_i > \bar{X} + 3.3 s_t$), the pair of results in which it occurs is discarded and \bar{X} recalculated. Only one such outlier is permissible. Should there be more outliers, the method should be revised and complemented with further runs and checks on outliers as above.

(s_t is calculated only for the purpose of checking on outliers; it is not needed for IQC in Monica, common control limits are stated below.)

Total (overall) standard deviation (s_t) is calculated on the basis of individual repeated measurements as follows:

$$s_t = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n - 1}} = \sqrt{\frac{X_i - \left(\frac{\sum X_i^2}{n}\right)}{n - 1}}$$

when X_i are individual measurements results

\bar{X} is the mean of all measurements ($\frac{\sum X_i}{n}$)

n = the number of measurements

$\sum X_i$ = the sum of the measurements results

Relative standard deviation (coefficient of variation-C.V.) is calculated:

$$\text{C.V.} = \frac{s_t}{\bar{X}} \times 100$$

For an example of mean and s_t calculation see Appendix II.

7.7 IQC criteria for acceptability of the run: Once the acceptable pool mean (\bar{X}) is obtained, the precision control limits are defined as follows (for an example of the quality control chart for visual follow up of method performance see Appendix III):

Warning limits for TC (levels above 2.60 mmol/l or 100 mg/dl) analysis: $\pm 3\%$ from the mean ($\pm 0.03 \bar{X}$).

Example: at a 5.17 mmol/l (200 mg/dl) level:

- the lower warning limit = 5.02 mmol/l (194 mg/dl)

- the upper warning limit = 5.33 mmol/l (206 mg/dl)

Maximum allowable limits for TC (levels above 2.6 mmol/l (100 mg/dl) measurement: $\pm 4.5\%$ from the mean ($\pm 0.045 \bar{X}$)

Example: at a 5.17 mmol/l (200 mg/dl) level:

- the lower allowable limit = 4.94 mmol/l (191 mg/dl)

- the upper allowable limit = 5.41 mmol/l (209 mg/dl)

Warning limits for HDL-C: $\pm 6\%$ from the mean ($\pm 0.06 \bar{X}$)

Maximum allowable limits for HDL-C: $\pm 9\%$ from the mean ($\pm 0.09 \bar{X}$).

7.7.1 HDL-C limits are tentative until completion of the Monica pre-checking period.

7.7.2 Ideally in runs containing Monica samples no single quality control pool value (not the mean of a duplicate) should surpass the warning limits. Should this occur and the result be still within maximum allowable limits, the run results can be used but it is a strong signal that method(s) should be checked (see standards, calibrator(s), reagents, OD reading instrument(s), pipettes, dispensers, quality control pool expiration dates (see 7.3.0) eventual microbial growth, possible calculation errors, etc.). Best quality volumetric glass, dispensers, and measurement instruments are recommended.

7.7.3 In no case should single control value(s) fall beyond the maximum allowable limits. If this occurs, the run must be stopped, results cannot be used, the cause of trouble must be eliminated, and the method must be brought under control before Monica samples are started.

8.0 External quality assessment (EQA)

Recommendation: the WHO-RLRC is responsible for sending the EQA samples.

8.1 EQA is complementary to the IQC and its main purpose will be to check on accuracy (assessment of bias), although it will also supply laboratories with evaluations concerning overall, between-day (between-run) and within-run variability. (Regular use of IQC is a prerequisite for successful participation in the EQA programme).

8.2 EQA will be carried out from the WHO-RLRC (Chief: Dr D. Grafnetter, Institute for Clinical and Experimental Medicine (IKEM) Videnska 800, 146 22 Prague, Czechoslovakia).

8.3 EQA assistance to participating laboratories:

8.3.1 1982 is considered as a pre-checking (pre-calibration, pre-standardization) period. Each laboratory assigned to analyse Monica samples (all addresses should be made available to the WHO-RLRC by WHO/HQ, see also 4.4) should utilize before the start of the proper EQA with "blind" samples (concentration unknown to the laboratories) one or two self-evaluation sets (concentrations known) provided by the WHO-RLRC. Self-evaluation sets contain vials with control sera selected from lyophilized pools. They are provided with instructions for use and with the list of reference TC, HDL-C, TG (and eventually also Apoprotein AI, AII and B) concentrations. Self-evaluation sets enable a daily check on methods and/or their calibration. They should be used during the pre-checking period together with methods definitively selected for Monica.

After the "open" period, a "blind" EQA system will start to evaluate laboratory performances. Its principles and methods were explained in detail to the laboratory directors who attended the WHO-LW. Others who are interested or who join the project later may obtain information from the literature [6, 7, 8, 9]. It is necessary to mention here only that the system has been based on repeated analysis of control sera shipped at regular intervals as lyophilizates to laboratories ("sets" composed of 14-21 samples). This system of "blind" EQA sets will be used throughout the Monica study.

8.3.2 Sets should be sent periodically to participating laboratories with clear instructions for use, as well as with relevant information, reporting forms, and eventually also questionnaires. Samples contained in the shipments may originate from commercial pools, as well as from pools prepared in the WHO-RLRC or elsewhere. It will be the responsibility of the WHO-RLRC to check, eventually in cooperation with CDC or other bodies, on the suitability of pools.

- 8.3.3 Control samples should be used in the laboratories according to attached instructions (reconstitution, sequence of samples, etc.). Each sample should be analysed in side-by-side duplicate on the day of reconstitution. If more measurements are performed, only the first two should be reported to the WHO-RLRC. The rest may serve for internal purposes, e.g. analysis of drift.
- 8.3.4 The decision on how many samples (or sets) should be distributed to individual laboratories will be made on individual basis, basing on the questionnaires completed and results reported by the laboratories. In most cases 4-5 EQA sets per year will be distributed to each laboratory. Each set should contain about 20 samples sufficient for about 2 months.
- 8.3.5 Some laboratories may wish to analyse Monica period samples (each period covers 2400 samples) in batches separated by intervals of days or weeks, while others may spread their analyses over year(s). Frequency of EQA sets should be adjusted to their needs.

Laboratories will be requested to complete analysis of individual sets and report results at the latest within 2 months. Failure to do so may mean that a further set will not be received, since only receipt of results will be a signal to the WHO-RLRC to indicate that a further set should be sent. Failure of a laboratory to undertake a sufficient EQA control will cause elimination of its TC and HDL-C results from Monica study evaluations.

- 8.3.6 Based upon the EQA results received, the WHO-RLRC will supply the participating laboratory (and WHO-HQ if needed) as soon as possible with information on the analysis of the EQA set, i.e. on the calculated overall, between-run and within-run standard deviation, and on bias, from which acceptability of results for the different pools and methods can be judged. Performance acceptability criteria are shown in Table 1 (page 19) and discussed under 8.3.8.
- 8.3.7 Reference values (RV) will be obtained in the WHO-RLRC as before by the modified Abell et al. method [18], in cooperation with CDC. CDC will be requested to analyse the EQA pools and make results available to the WHO-RLRC, to ensure comparability between WHO-CDC and WHO-RLRC evaluations. As a secondary check in obtaining the RVs a CHOD-PAP enzymatic cholesterol method with practically 100% cholesterol-ester hydrolysis may be used by WHO-RLRC. For LDL+VLDL precipitation in the HDL-C reference value estimation the described PT reagents and procedures will be used (see sections 4.5, 4.6).
- 8.3.8 Performance acceptability criteria in EQA (limits on maximum allowable inaccuracy and imprecision) (see Table 1, page 19):

Total cholesterol: for any of the control pools, calculated bias (based on the EQA set mean and on RV) should not be greater than 5%. At the same time s_t should be smaller than that shown for individual reference values in Table 1, page 19.

HDL-cholesterol: tentative limits (for 1982-1983) subject to possible modification: for any control pool, calculated bias (based on the EQA set mean and RV) should not be greater than 7.5%. At the same time s_t should be smaller than that shown for individual reference values in Table 1, page 19.

- 8.4 Laboratories should not begin analysis of Monica samples before they are able to show that their analysis of at least two successive blind EQA sets are within the limits of acceptability (see 8.3.8) for every control pool.
- 8.4.1 If EQA results suggest trouble with a method a decision will have to be made on the admissibility of data from the laboratory. It is strongly recommended that a statistical centre be appointed by WHO to solve such problems with the laboratory concerned, using QA information including also IQC data. Laboratories should keep good records on their IQC data to be able to present them upon request.
- 8.4.2 However, a situation might arise when a laboratory might demonstrate greater than allowable but fairly stable analytical bias with acceptable precision (small s_t). In such a case the WHO Monica appointed statistical centre (which should receive evaluation copies from WHO-RLRC through WHO-HQ) should consider the case and advise the laboratory and WHO-RLRC immediately of the course to be followed (Results might perhaps be corrected mathematically provided that the bias was practically the same at different concentration levels in successive tests, and that s_t did not surpass allowable limits). Once s_t is above the allowable limit no allowance for bias consideration can be made, and the laboratory is definitely requested to revise and correct the method before proceeding with the Monica study samples.
- 8.5 To help overcome problems of retrospectivity in EQA, the WHO-RLRC will include in sets, if possible, a pool with nominated RVs to be made known to participating laboratories for continual self-evaluation.
- 9.0 Recording and reporting Monica and quality control results
- 9.1 Monica results reported to WHO/HQ and/or a statistical centre elsewhere will be analyzed under the auspices of WHO. WHO staff will design forms for data reporting suitable for computer analysis.
- 9.2 Laboratories should at all times indicate whether they report single measurements results or means.
- 9.3 WHO-RLRC will provide laboratories with reporting forms prepared for EQA sets.
- 9.3.1 Laboratories will be identified in the EQA system by code numbers which will not be made publicly available without written consent from the respective laboratories. However the code will be open for WHO/HQ and for the Monica appointed statistical centre (who are also bound to keep it confidential).
- 10.0 Actions if performance with required accuracy and/or precision cannot be achieved in a laboratory
- Initial action should be to make every effort to bring the method(s) into required performance limits, with use of information obtained and with special samples for method calibration, then to advise the WHO-RLRC or CDC about the problem.
- If all efforts fail, WHO may assist in nominating and supporting a visit from a consultant to the Laboratory concerned.

BIBLIOGRAPHY

1. Albers, J.J., Warnick, G.R., Johnson, N., Bachorik, P.S., Muesing, R., Lippel, K. & Williams, J.D. (1980) Quality Control of Plasma High-density Lipoprotein Cholesterol Measurement Methods. The Lipid Research Clinics Programme Prevalence Study. Circulation, Part II, Volume 62, No. 4, November 1980. American Heart Association Monograph Number 73. pp IV-9 to IV-18.
2. Assmann, G., Schriewer, H. Schopohl, B. & Funke H. (1979) Investigation of the Specificity of the HDL-Cholesterol Test. In: Greten, H., Lang, P.D. & Schettler (Eds.) Lipoproteins and Coronary Heart Disease. New Aspects in the Diagnosis and Therapy of Disorders of Lipid Metabolism. International Symposium, Vienna, May 12-13, 1979. Gerhard Witzstrock Publishing House, Baden Baden, 1980, pages 43-45
3. Bachorik, P.S. (1979) Factors That Affect HDL-Cholesterol Measurements: Sample Preparation and Storage at Refrigerator and Freezer Temperatures. In: Lippel, K (ed) : Report of the High Density Lipoprotein Methodology Workshop. San Francisco, California, 12-14 March 1979. US Department of Health, Education, and Welfare, NIH publication no. 79-1661, August 1979: pp 164-177
4. Boerma, G.J.M (1979) Studies in Standardization - Serum Cholesterol Analysis Performed for Epidemiological Investigations. Erasmus University, Rotterdam.
5. Girault, A., Loiseau, D. & Girault, M. (1980) Quantitative Determination of Apolipoprotein A in Human Serum by Laser Nephelometry. Medical Laboratory, Volume 9, Medical Diagnostics "E.v. Behring", pages 11-20. Die Medizinische Verlagsgesellschaft mbH, 3550 Marburg, West-Germany, POB 1732
6. Grafnetter, D. (1977) World Health Organization (WHO) Coordinated Quality Control in the Lipid Laboratory. Giorn. Arterioscl., Vol. 2, 113-128
7. Grafnetter, D. (1978) External Quality Control Carried out by the WHO European Lipid Reference Center in Prague. In: Carlson, L.A. et al. International Conference on Atherosclerosis, Raven Press, New York, 1978, pages 379-386
8. Grafnetter, D. (1979) International Standardization of Lipid Methods, Experience and Trends. In: Proceedings of the 3rd Dresdner Lipid Symposium, 14-16 May 1979, pp 362-367
9. Grafnetter, D. (1980) WHO Lipid Reference Programme. A Quality Control Substudy in Socialist Countries with Use of the Precilip and Precilip E.L. Samples. Paper presented at the symposium on the "Lipid Methods and Their Quality Control", Prague, 8 September 1980. Boehringer Mannheim, GmbH, Mannheim, West-Germany
10. Greten, H., Lang, P.D. & Schettler (Eds.) (1980) Lipoproteins and Coronary Heart Disease. New Aspects in the Diagnosis and Therapy of Disorders of Lipid Metabolism. International Symposium, Vienna, May 12-13, 1979. Gerhard Witzstrock Publishing House, Baden Baden.
11. Grove, T.H. (1979) Effect of Reagent pH on Determination of High-density Lipoprotein Cholesterol by Precipitation with Sodium Phosphotungstate- Magnesium. Clinical Chemistry, Vol. 25, No. 4, 560-564
12. Kostner, G.M. (1980) Lipoproteins and Atherosclerosis: Influence of the Diet. Medical Laboratory, Volume 9, Medical Diagnostics "E.v. Behring", pages 1-10. Die Medizinische Verlagsgesellschaft mbH, 3550 Marburg, West-Germany, POB 1732

13. Kuchmak, M., Taylor, L. & Olansky, A.S. (1981) Low Lipid Level Reference Sera with Human Serum Matrix. Clinica Chimica Acta, 116, 125-130
14. Lippel, K (ed) : Report of the High Density Lipoprotein Methodology Workshop. San Francisco, California, 12-14 March 1979. US Department of Health, Education, and Welfare, NIH publication no. 79-1661, August 1979
15. Seigler L. & Wu, W.T. (1981) Separation of Serum High-Density Lipoprotein for Cholesterol Determination: Ultracentrifugation vs. Precipitation with Sodium Phosphotungstate and Magnesium Chloride. Clinical Chemistry, Vol. 27, No. 6, 838-841
16. Tyroler, H.A. (Ed) Epidemiology of Plasma High-Density Lipoprotein Cholesterol Levels. The Lipid Research Clinics Program Prevalence Study. Circulation, Part II, Volume 62, No. 4, November 1980. American Heart Association Monograph Number 73.
17. US Department of Health and Human Services, Centers for Disease Control: CDC Survey of High Density Lipoprotein Cholesterol Measurement: A Report. CDC Clinical Chemistry Division, 1980
18. US Department of Health and Human Services, Centers for Disease Control: Manual of Instructions and Protocols for Laboratory Quality Control in the Lipid Research Clinics Program. March 1978
19. US Department of Health and Human Services, Centers for Disease Control - National Heart Lung and Blood Institute High Density Lipoprotein Cholesterol Standardization Program. CDC, 15/10/80
20. US National Heart and Lung Institute: Lipid Research Clinics Program Manual of Laboratory Operations, Volume 1: Lipid and Lipoprotein Analysis. DHEW Publication No. (NIH) 75-628, May 1974
21. Warnick, G. R. & Albers, J.J. (1979) HDL Cholesterol Quantitation: Comparison of Six Precipitation Methods and Ultracentrifugation. In: Lippel, K (ed) : Report of the High Density Lipoprotein Methodology Workshop. San Francisco, California, 12-14 March 1979. US Department of Health, Education, and Welfare, NIH publication no. 79-1661, August 1979: pp 53-69
22. Warnick, G.R, Mayfield, C. & Albers, J.J. (1981) Evaluation of Quality-Control Materials for High-Density-Lipoprotein Cholesterol Quantitation. Clinical Chemistry, Vol. 27. No. 1, 116-123
23. World Health Organization (1977) The SI for the Health Professions, WHO, Geneva
24. Draeger, B., Wahlefeld, A. (1979) Development of the Test for Determination of HDL-Cholesterol Precipitation. In: Greten, H., Lang, P.D. & Schettler (Eds.) Lipoproteins and Coronary Heart Disease. New Aspects in the Diagnosis and Therapy of Disorders of Lipid Metabolism. International Symposium, Vienna, May 12-13, 1979. Gerhard Witzstrock Publishing House, Baden Baden, 1980, pages 38-42
25. Williams, J.H., Taylor, L., Kuchmak, M. & Witter, R.F. (1970) Preparation of hypercholesterolemic and/or hypertriglyceridemic sera for lipid determinations. Clin. Chim. Acta 28, 247-253
26. Kuchmak, M., Williams, J.H. Taylor, L. & Witter, R.F. (1973) Alcoholic precipitation and 1000xcentrifugation preparation of hypercholesterolemic and hypertriglyceridemic sera as lipid determination control. US Patent No. 3,764,556

27. Kuchmak, M., Taylor, L. & Williams, J.H. (1981) Preparation of reference sera with desired levels of cholesterol and triglyceride. *Clin. Chim. Acta* 114, 127-135
28. Proksch, G.J. & Bonderman, D.P. (1976) Preparation of optically clear lyophilized human serum for use in preparing control material. *Clin. Chem.* 22, 456-460
29. Proksch, G.J. & Bonderman, D.P. (1979) Development of a stable lipoprotein diluent for use of reconstituting lyophilized human serum for the preparation of clear hyperlipidemic quality-control materials. *Clin. Chem.* 25, 1377-1380
30. van Kampen, E.J., Zijlstra, W.G. (1965) General requirements for Hb determination and standardization. In: Hemoglobin and its derivatives. *Advances in Clinical Chemistry*, Part 8. Acad. Press, New York.
31. Burstein, M., Scholnick, H.R. & Morfin, R. (1970). Rapid method for isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lip. Res.*, 11:583
32. Lopes-Virella, M.F., Stone, P., Ellis, S. & Caldwell, J.A. (1977) Cholesterol determination in high density lipoproteins separated by three different methods. *Clin. Chem.*, 23:882-885
33. Test-Combination HDL-Cholesterol Precipitant No. 400971, Boehringer Mannheim, GmbH.
34. Henderson, L.O., Saritelli, A.L., LaGarde, E., Herbert, P.N. & Shulman, R.S. (1980) Minimal within-day variation of high density lipoprotein cholesterol and apolipoprotein cholesterol and apolipoprotein A-I levels in normal subjects. *Journal of Lipid Research*, Vol. 21, 7, 953-955

TABLE 1

Performance acceptability criteria in EQA (limits on maximum allowable inaccuracy and imprecision)

Individual standard deviations can be obtained for any RV concentration by interpolation.

Reference value (RV)	Maximum allowable overall standard deviation (s_t)		Maximum allowable deviation of set pool mean from RV (bias)		
	mmol/l	mg/dl	mmol/l	mg/dl	%
TOTAL CHOLESTEROL	2.5846	100	0.1681	6.5	±5% (±0.05 RV)
	5.1728	200	0.1940	7.5	±5% (±0.05 RV)
	7.7592	300	0.2198	8.5	±5% (±0.05 RV)
	10.3456	400	0.2457	9.5	±5% (±0.05 RV)
HDL-CHOLESTEROL	0.5173	20	0.0336	1.3	±7.5% (±0.075 RV)
	1.0346	40	0.0672	2.6	±7.5% (±0.075 RV)
	1.5518	60	0.1009	3.9	±7.5% (±0.075 RV)
	2.0691	80	0.1345	5.2	±7.5% (±0.075 RV)

TABLE 2
MONICA - Time Estimates for Sample Processing

	Time until serum/ plasma separation*	Time until HDL-separation	Time until analysis
Australia	Plasma 6 hours	Same week	4° up to 1 week; frozen if longer; promptly after thawing
Belgium	1 hour	Same day	1 day
People's Rep. of China	1 hour	Same day	1 day
Czechoslovakia	4 hours	Same day	3 days
Denmark	1 (2) hours	Same day	Same day or frozen
Fed. Rep. Germany:			
Heidelberg	Plasma 6 hours	Same day	1 day
Münich	1-2 hours	Same day	3 days
Finland	1 hour	2-4 days (mail)	4 days
German Dem. Rep.	Plasma 1 hour	Same day	1 day
Greece	2 hours	Same day	1 day
Hungary	1-2 hours	Same day	1 day
Italy	1 hour	3 days	3 days
New Zealand	1-2 hours	Up to 1 month (-20°)	Frozen then promptly after thawing
UK - North Ireland	1 hour	Same day	1 day
UK - Scotland	Plasma 6 hours (1 hour?)	4 days (-20°)	4 days
USSR	2(-3) hours	Same day	1 day
Yugoslavia	2 hours	Same day	1 day

*Serum unless otherwise specified

TABLE 3

MONICA - Proposed Methods for Lipid Analysis

	Cholesterol method	Technique	HDL method	Serum/ plasma
Australia	LRC	AALII	HEP/0.092 M Mn	P (EDTA)
Belgium	Enz	AALII	PT	S
People's Rep of China	Chem (Enz?)	Manual	PT	S
Czechoslovakia	Enz	Manual	PT	S
Denmark	Enz?	Manual (SMAC?)	Dx SO ₄ (PT)	S
Fed. Rep. Germany:				
Heidelberg	Enz	Hitachi 706 (discrete)	PT	P
Münich	Enz	Manual	PT	S
Finland	Enz	Olli 3000 discrete	Dx SO ₄ (PT?)	S
German Dem. Rep.	Enz (micro)	Manual (paper fluorescence)	PT	P (capillary heparin)
Greece	Enz	Manual	PT	S
Hungary	Enz	Manual	PT	S
Italy	Enz	Gemsac	PT	S
New Zealand	LRC	AALII	PT	S
UK - North Ireland	Enz	Cobas-Bio	PT	S
UK - Scotland	Enz	AALII	Dx SO ₄ (PT?)	P (EDTA)
USSR	Chem (Enz)	Olli 3000 or Fp-9 (discrete)	PT	S
Yugoslavia	Enz	Manual	PT	S

Recommended sequence of N specimens in a run

For total cholesterol

For HDL-cholesterol

- | | |
|---|---|
| 1) Reagent blank | 1) Reagent blank |
| 2) Reagent blank | 2) Reagent blank |
| 3) Standard 1 (low, 2.60 mmol/l, e.g.) | 3) Standard 1 (very low, 0.65 mmol/l, e.g.) |
| 4) Standard 1 (low, 2.60 mmol/l, e.g.) | 4) Standard 1 (very low, 0.65 mmol/l, e.g.) |
| 5) Standard 2 (medium, 5.20 mmol/l, e.g.) | 5) Standard 2 (low, 1.30 mmol/l, e.g.) |
| 6) Standard 2 (medium, 5.20 mmol/l, e.g.) | 6) Standard 2 (low, 1.30 mmol/l, e.g.) |
| 7) Standard 3 (elevated, 7.80 mmol/l, e.g.) | 7) Standard 3 (low, 2.60 mmol/l, e.g.) |
| 8) Standard 3 (elevated, 7.80 mmol/l, e.g.) | 8) Standard 3 (low, 2.60 mmol/l, e.g.) |
| 9) TC+HDL-C pool, non-precipitated | 9) TC+HDL-C pool supernatant |
| 10) TC+HDL-C pool, non-precipitated | 10) TC+HDL-C pool supernatant |
| 11) LTC pool, non-precipitated | 11) LTC-pool, non-precipitated |
| 12) LTC pool, non-precipitated | 12) LTC-pool, non-precipitated |

- 13) IQC pool for monitoring drift
14) Patient sample
15) Patient sample
16) Patient sample
etc
..
..
23) IQC pool for monitoring drift
24) Patient sample
.. Patient sample
.. Patient sample
.. Patient sample

- N-1) TC+HDL-C pool - at end of run - TC + HDL-C pool
N) TC+HDL-C pool - at end of run - TC + HDL-C pool

-
- HDL-cholesterols should be preferably analysed in separate runs (or parts of runs) to avoid between sample transfer contamination(s) depending on the instrumentation ("carry over").
 - Whenever there could be expected significant carry-over (with older photometers, e.g.) it will be necessary to wash out the photometer cuvette first (notably between "high" standard(s) and first IQC samples) with a part of the next sample/reagent mixture and take the reading on the rest of it.

Appendix II

Calculation of the pool mean (\bar{X}) and standard deviation (s_t).
Cholesterol in mmol/l

Date	Pool vial number	Determination 1		Determination 2	
		x	x ²	x	x ²
	1	6.31	39.8161	6.41	41.0881
	2	6.47	41.8609	6.21	38.5641
	3	6.26	39.1876	6.21	38.5641
	4	6.21	38.5641	6.41	41.0881
	5	6.41	41.0881	6.31	39.8161
	6	6.36	40.4496	6.36	40.4496
	7	6.21	38.5641	6.26	39.1876
	8	6.26	39.1876	6.36	40.4496
	9	6.21	38.5641	6.26	39.1876
	10	6.23	38.8129	6.44	41.4736
	11	6.44	41.4736	6.23	38.8129
	12	6.26	39.1876	6.36	40.4496
	13	6.21	38.5641	6.26	39.1876
	14	6.26	39.1876	6.21	38.5641
	15	6.21	38.5641	6.36	40.4496
	16	6.31	39.8161	6.41	41.0881
	17	6.41	41.0881	6.21	38.5641
	18	6.21	38.5641	6.26	39.1876
	19	6.21	38.5641	6.47	41.8609
	20	6.41	41.0881	6.31	39.8161
	Σx	125.86	-	126.31	-
	Σx^2		792.1926		797.8491

Appendix II (continued)

Calculation of the mean: $\bar{X} = \frac{\sum x}{n} = \frac{252.17}{40} = 6.30$

Calculation of the standard deviation:

$$\begin{aligned}\sum x &= (125.86 + 126.31) = 252.17 \\ (\sum x)^2 &= 63589.7089 \\ \sum x^2 &= (792.1926 + 797.8491) = 1590.0417 \\ n &= 40 \\ n - 1 &= 39\end{aligned}$$

$$\begin{aligned}s_t &= \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}} = \sqrt{\frac{1590.0417 - \frac{63589.7089}{40}}{39}} = \\ &= \sqrt{\frac{1590.0417 - 1589.74273}{39}} = \sqrt{\frac{0.2989775}{39}} = \underline{0.087556}\end{aligned}$$

Calculation of the coefficient of variation:

$$C.V._t = \frac{s_t \times 100}{\bar{X}} = 1.389 = \underline{1.4 (\%)}$$

