Establishing a reference system in clinical enzymology

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ABSTRACT

Even if the "recommended method" approach led to significant improvements in clinical enzymology, it is now clear that the goal of a single, universally used method for measuring the catalytic concentration of a given enzyme will never be achieved. Over the last 25 years, the recommended standard procedures have been increasingly corrupted to be adapted to the use in clinical laboratories. This fact makes impossible the definition of common reference intervals, creates confusion among patients and practitioners, and causes problems to the External Quality Assessment Scheme organisers. For these reasons, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), by means of its Working Group "Calibration in clinical enzymology", recently launched a project for the establishment of a reference system (RS) in clinical enzymology. The RS is based on three hinges: a) reference methods (RM), extensively evaluated and carefully described, b) certified reference materials (CRM), and c) a network of reference laboratories (NRL) operating in a highly controlled manner. In RM resides the accuracy of the RS. The original IFCC recommended procedures have some characteristics that are impractical to be transferred in routine: temperature (30°C), need of sample blank, excessively prolonged reaction times, limited linearity. Moreover, they are not described in a detailed manner leaving some room for interpretation. The procedures have been therefore slightly modified to optimise them at 37°C and avoid sample blanking, also re-evaluating incubation times and linearity. This has led to the definition of very detailed standard operating procedures (SOP) for ALT, AST, CK, GGT, LDH, and AMY (ALP and lipase under development). CRM are used to control the operation of the NRL and, if commutable, they can be used directly (at least by the manufacturers) to transfer the accuracy of the RM to the field procedures. Partially purified materials prepared years ago by the Community Bureau of Reference (BCR) have been recertified by the NRL for ALT, CK, GGT, and LDH activities. NRL consists of a small group of laboratories (n=13, world-wide located) trained to perform manual procedures, with self-made reagents, on carefully calibrated instrumentation and following the above-mentioned SOP. The NRL is also able to perform "split sample" comparisons on fresh patient samples between RM and routine methods, thus directly certifying commercial systems. This RS constitutes the structure of the traceability chain to which the routine procedures can be linked via an appropriate calibration process, provided that they have a comparable specificity (i.e. they are measuring the same quantity).

BACKGROUND

The task of achieving interlaboratory agreement of enzyme activity measurements remains one of the most problematic efforts directed toward standardization in clinical chemistry. The catalytic activity of an enzyme is a property measured by the catalyzed

rate of reaction, produced in a specific assay system, and is not an amount of substance. If the system of reaction is changed, the magnitude of the measured activity will also change, so that the obtained result is method-dependent. Therefore, the numerical results of catalytic-activity measurements depend entirely on the experimental conditions under which the measurements are made. In particular, the catalytic activity is affected by a large range of variables: pH and nature of the buffer, temperature, presence of activators and inhibitors, substrate nature and concentration. Consequently, two methods that measure the same enzyme using different analytical conditions may produce different results for a given sample. In the standardization of enzyme assays, therefore, a standard method, which defines the conditions under which a given enzyme activity is measured, occupies the role filled by standard materials of known mass concentration with regard to non-enzymic analytes. Due to this fact, it is extremely important to select and define 'recommended' methods for each enzyme determination, carefully designed and investigated to prevent any source of errors.

Over the last 17 years, recommended methods have actually been defined by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), which provide guidelines on reaction conditions, such as substrate concentration and reaction temperature, for a number of the most measured enzymes (1-7).

The widespread use of these selected procedures has been for long time the main goal of national and international scientific bodies concerned with clinical enzymology (8,9). However, even if the recommended-method approach has led to significant improvements in clinical enzymology, for instance, by hastening the elimination of some unsatisfactory methods, it is now clear that the goal of a single, universally used method for measuring the catalytic concentration of a given enzyme will never be achieved, even in those countries, such as the Scandinavian region, in which a highly uniform standard of laboratory practice prevails (10). To illustrate this, although a recommended method exists, at least 21 different substrates are currently distributed for the measurement of catalytic activity of α-amylase (AMY) (11).

The main reason is that the recommended methods are generally not appropriate for direct routine use in the clinical laboratories and do not respond to the continuous development and improvement of the analytical methodology.

The original IFCC recommended procedures have some characteristics that are impractical to be transferred in routine: temperature (30°C), need of sample blank, excessively prolonged reaction times, limited linearity. Moreover, they are not described in a detailed manner leaving some room for interpretation. So that, over the last 10-15 years, the recommended standard procedures have been increasingly corrupted to be adapted to the practical laboratory use and, in some cases, technological advances, such as the recent rapid development of liquid, ready-to-use reagents, have even created reagents with specifications different from the recommended methods. Moreover, modern analyzers are available with only the fixed calibrated temperature of 37°C, instead of the original IFCC-recommended 30°C, furtherly hampering the advancement of standardization through methodology. On the other hand, the use of calibrators in the routine main frame analysers has become quite common, even for the determination of enzyme activities, thus replacing the use of the theoretical factor based on the molar extinction coefficient of the indicator molecule and potentially influencing the accuracy of results.

This situation frequently results in a lack of comparability of results from different analytical systems, with far-reaching and serious consequences. For instance, this fact makes impossible the definition of common reference intervals, creates confusion among patients and practitioners when results are clinically interpreted, and causes problems to the organisers of the External Quality Assessment Schemes (EQAS) to ascertain whether the wide variation in results observed within a given method group corresponds to a poor analytical performance within that group or to heterogeneity in the assays included in the group (12).

The objective of all the present efforts to standardize the measurements of catalytic concentrations of enzymes should therefore be to achieve comparable test results for human serum samples, independent of the measurement test kit and instrument, and also independent of the laboratory where the procedure is carried out.

A REFERENCE SYSTEM FOR ENZYME MEASUREMENTS

In order to achieve this goal, an approach is needed that will provide for reliable transfer of the measurement values of a uppermost recommended reference method to those methods which are routinely used in laboratories. Such a structure is presented by the concept of the *Reference System* (Figure I). This system also requires reference materials as an intermediate to transfer the values of the reference method to the routine laboratory methods. The reference procedure and the certified reference materials are the grounds of the metrological traceability chain. The reference procedure is used to assign a certified value to a reference material. It should be noted that reference procedures do not need to be calibrated because, as said before, the enzymes are defined in terms of the response of the reference procedure itself. Once the reference material is certified, this material and the manufacturer's standing procedure can be used in industry to assign values to commercial calibrators. Finally, the clinical laboratory uses routine methods with validated calibrators, both from commercial sources, to measure human specimens. In this way, the obtained value will be traceable to the reference measurement procedure.

In addition, the use of validated calibrators will clearly compensate for any variation of routine procedure if this variation affects both the calibrator and patient specimens to the same degree. For instance, this approach may correct for undetected systematic errors introduced by differences in temperature setting and control of analyzers, and volumes dispensed by the instruments. Variation in wavelengths or bandwidths can also be circumvented. It should however be noted that, of course, this approach cannot compensate for poor precision and lack of analytical specificity of analytical systems.

The need to have and to use stable, well-characterized enzyme materials to obtain better enzyme values is not new. Review of the literature on enzyme standardization for the past three decades demonstrates that many investigators have contributed to the slowly but progressively evolving body of knowledge that now supports the systematic use of enzyme reference materials in conjunction with enzyme reference procedures to create an enzyme reference system (13-16). The insight about the unifying power of enzyme reference materials that Stromme and Eldjarn wrote in 1970 (17) has since been shared by and added to by many other investigators. For example, the general conclusion from the 'Richmond Project' reported in 1986 by Greg Miller et al. in US is one that unequivocally supports the incorporation of suitable enzyme calibration materials into

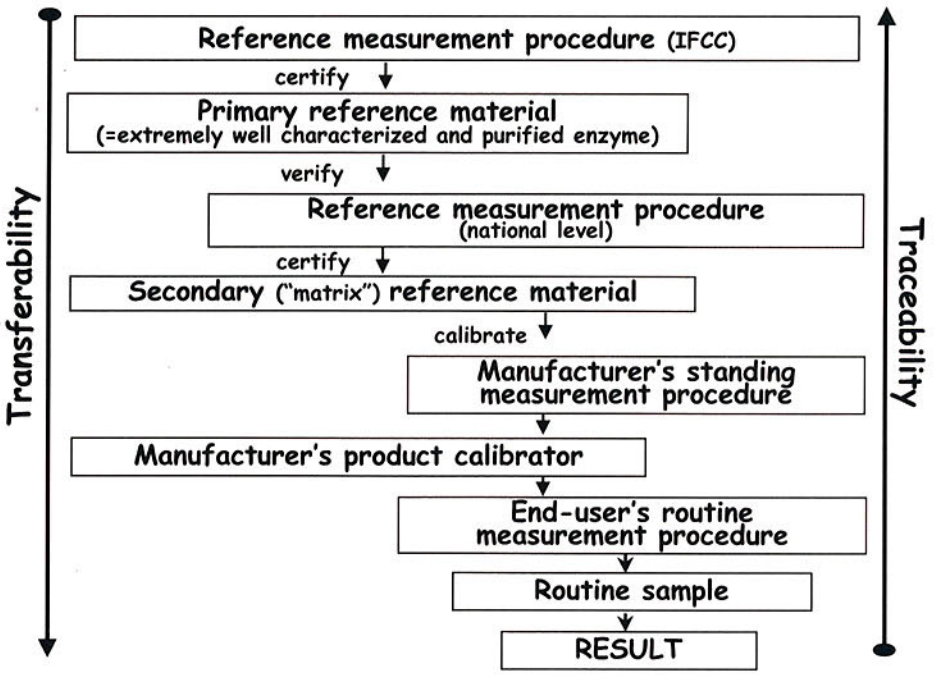

Figure I
The Reference System for enzyme measurement

enzyme-standardization programs (18). In 1995, a multicenter study was performed concerning alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) with widely used reagent kits and with IFCC recommended methods (19). Results clearly indicated that calibration with the corresponding reference material dramatically improved the intermethod consistency for most of the tested measurement procedures. It was, therefore, possible to transfer accuracy from the method used for the certification of the reference material to several routine procedures by using the reference material as calibrator (20).

These publications, taken together, speak clearly to the need for an increased emphasis on the use of enzyme reference materials. From the practical point of view, two types of refence materials are identified (Figure I). The primary reference materials are intended only for control of reference procedure and may be unsuitable for use directly with a routine clinical laboratory method. In fact, the limited number of different primary materials, their concentration range, and method production (for instance, enzyme purification or lyophilization procedure to provide long-term stability) might hinder their use for direct routine method calibration. Accuracy transfer to field methods uses secondary reference materials just produced to meet the unique calibration needs of a commercial assay system.

In short, the enzyme standardization must rest upon a hierarchical reference system that a every level fully exploits the interdependency of enzyme procedures and enzyme materials. This reference system is based on three hinges: 1. a reference measurement procedure, extensively evaluated and carefully described, 2. certified reference materials with well-defined characteristics, and 3. a network of reference laboratories, operating in a highly controlled manner as technically active body of the reference system. These three components build upon each other.

As pointed out before, the main objective of the system is the establishment of the comparability of clinical laboratory measurements through the assignment of accurate values to calibrators and control materials. But, as we will see later, the reference system may also permit to directly evaluate the accuracy of analytical systems for routine use and to evaluate the performance of routine laboratories on an objective basis within the mandatory quality assurance schemes.

THE INTERNATIONAL COOPERATION

Since a reference system requires intensive research activities, the IFCC created in 1997 a Working Group on Calibrators in Clinical Enzymology (WG-CCE) to promote the interlaboratory compatibility of enzyme results through the creation of a project for the establishment of a reference system in clinical enzymology (21). Both from the medical and from the economic point of view, a worldwide reference system is indeed desirable. The decision was comunicated to the industries, whose consensus was taken to mean that they agree to include the future outcome of the project, that is, the certified reference materials and the establishment of the network of reference laboratories, in their standardization processes. In fact, improvement in analytical quality cannot be expected if the industries are not deeply involved in efforts to improve the quality of the measuring systems they supply.

Let us now examine each element of the new enzyme reference system to see how it may promote and mantain accuracy and comparability of results.

Reference Measurement Procedures

In the *Reference Measurement Procedure* resides the accuracy of the reference system. The term "reference procedure" instead of "reference method" is to be preferred because it includes the specific and exact description of the individual obligatory working steps. The main function of the reference procedure should be its use as a "golden standard" for the validation of other methods that can be more easily adapted to the automated instrumentation and other routine conditions. For this purpose a suitable method must be developed internationally.

From a practical point of view, new reference measurement procedures have recently

been designed on the basis of the currently existing IFCC recommended methods. The original IFCC procedures were slightly modified to optimise them at the measuring temperature of 37°C and avoid sample blanking, also re-evaluating incubation times and linearity (22). This led to the definition of very detailed standard operating procedures for the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), GGT, lactate dehydrogenase (LDH), and AMY (with protocol for ALP and lipase under discussion). This is perhaps the first time that the exact methodological and procedural details of the enzyme methods finally used are unambiguously spelled out.

In order to achieve low levels of uncertainty much concern was devoted to the careful control of all metrological aspects related to gravimetry, volumetry, and fotometry. In particular, a "General Requirements" document, containing minimal performance criteria for the function of the measuring apparatus, the actual measurement, etc., was developed and circulated in the reference laboratories.

Network of Reference Laboratories

In the *Network of Reference Laboratories*, the second hinge of the reference system, the reference procedures were developed, validated, and tested for transferability (23). In fact, even when a reference procedure is described in detail, problems may nevertheless be encountered when it is transferred to a different laboratory. The main criterion used to select laboratories for the network was the expertise in the field of methods in enzymology, demonstrated by the publication of scientific papers, together with the capability to do continuous work under specified conditions of analytical performance and to provide results in a reasonable time. Participation of industrial laboratories was also welcomed.

Under the patronage of IFCC and of the Institute for Reference Materials and Methods (IRMM), located in Belgium, a group of 13 laboratories (including manufacturer's laboratories), worldwide located, was selected to provide the necessary skill and equipment for performing measurements following the above-mentioned standard operating procedures using self-made reagents.

The first objective of this international group of reference laboratories was to demonstrate the satisfactory comparability of their individual results. Agreement between the results obtained from different reference laboratories is indeed the most valuable guarantee for achieving accuracy. A meaningful protocol to assure photometric accuracy of instrumentation and to check pipette and diluter calibration and temperature equilibration of reaction cuvettes was prepared. The results showed very good control for the wavelength accuracy in all laboratories with very small deviations from the target absorbance values and a good accuracy for temperature in the cuvettes with no cases with temperature bias higher than ±0.1°C.

In a preliminary experiment for several enzyme calibrator and control materials in a ring trial, an astonishingly good agreement between results from individual laboratories

Table 1
Within-laboratory reproducibility using reference procedures for five enzymes in the two Italian laboratories, member of the IFCC network

Enzyme	Mean acitvity, U/L	CV, %
Lab. A		
ALT	115.4	0.93
AST	121.9	1.26
CK	377.7	0.66
GGT	143.7	1.23
LDH	389.5	0.43
Lab. B		
ALT	118.9	0.69
AST	126.2	0.97
CK	374.9	0.55
GGT	142.5	0.50
LDH	389.1	1.36

within narrow limits of uncertainty (less than ±3%) was achieved.

According to the protocol, the performance of a participating laboratory was judged from: 1. its within-laboratory reproducibility; 2. its deviation from other laboratories; and 3. the ability to provide measurements in due time. Table 1 shows, as an example, the results obtained in the two Italian laboratories, member of the network, for five of the evaluated enzymes on the same control material with regard to the reproducibility. From these results, it is also possible to appreciate the very small differences between the mean of the activities obtained in the two laboratories on the same samples.

Once established and verified the high analytical performance of the reference laboratories included in the group, the primary actions of the network were identified (24). In particular, the following services could be offered (Figure II):

1. the certification of reference materials in cooperation with the IRMM;
2. the target-setting for national or commercial external quality assessments and internal quality controls;
3. the validation of commercial methods or systems, employed for routine determination of enzymes in patient specimens.

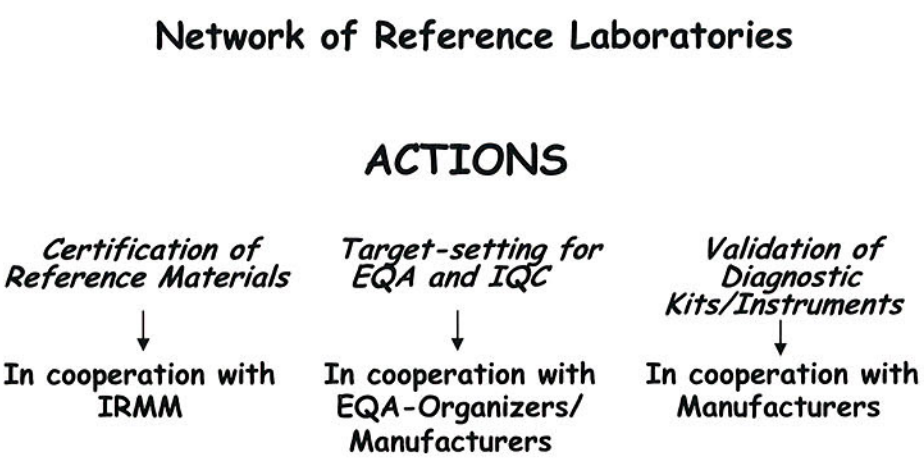

Figure II
Actions and services offered by the IFCC Network of Reference Laboratories
IRMM, Institute for Reference Materials and Methods; EQA, External Quality Assessment; IQC, Internal Quality Control

Reference Materials

The major objective for the network of reference laboratories is to certify the catalytic activity of enzymes in appropriate *Reference Materials* (25). As already stated, the initial value assignment to these materials is the crucial step for the enzyme reference system when it is a question of transferring accuracy from the high metrological level of reference procedures to routine clinical chemical measurements (26). Furthermore, these reference materials, once certified, will also be needed by the network for the process of continuous self-assessment and internal accuracy control.

On the basis of the experience of the IFCC network of reference laboratories applying the new IFCC 37°C reference procedures, IFCC and IRMM have co-operated to certify the already existing reference materials for GGT, LDH, ALT, and CK, prepared by the Community Bureau of Reference (BCR) of the European Community (Table 2). BCR has been very active during the last ten years in preparing reference materials for enzymes (27). As well documented, these reference material preparations have great stability at

Table 2
The Community Bureau of Reference (BCR) enzyme reference materials certified by the IFCC lab network

Enzyme	Code	Origin	Form	Stability (at -20°C)
GGT	CRM 319	Pig kidney	Light subunit	25 years
LDH	CRM 404	Human erythrocytes	LD1 isoenzyme	>50 years
ALT	CRM 426	Plg heart	-	>50 years
CK	CRM 608	Human heart	MB isoenzyme	>10 years

-20°C, with a deterioration rate between 0.01% and 0.04% a year, depending on the enzyme (Table 2). The collaborative study was performed by all the 13 laboratories of the network. Each participant performed the reference procedure on two different occasions in triplicate. The values assigned to the four reference materials carried a 95% uncertainty interval of ±1.5% of the mean. Details of this study are now in publication by the laboratories of the network.

These monoenzyme BCR reference materials are however available in limited amounts and are expensive to produce and to purchase. So that, they cannot be used routinely to calibrate enzyme assays. They can be only used as verifier or *primary* reference material, which is the principal reason for BCR to manufacture them. Consequently, other materials are needed to transfer accuracy and insure comparability of results in clinical enzymology: the so-called *secondary* reference materials.

With regard to these materials, their "commutability" is the central issue and the main criterion to be satisfied to transfer accuracy to the routine methods using them (28). Commutability has been defined as "the ability of an enzyme material to show interassay activity changes similar to those of the same enzyme in human serum" (29). In other words, the numerical ratio between the activity results determined by the routine and reference methods found for the reference material must be the same as the average ratio found for a large number of patients' samples. Of course, general-purpose reference materials intended for use with multiple methods must be validated for each system (30).

Commutability of reference materials and calibrators can be affected by many factors (Table 3). It is well known that catalytic properties depend on the source of material. More precisely, catalytic properties of an enzyme analyte may differ from one species to another, also from one tissue to another in the same species (31). Commutability of an enzyme material may also depend on the purification procedure and on the matrix chosen. Matrix-modified material may lack the same chemical reactivity as autentic clinical specimens in many of the routine analytical systems used in clinical laboratories (32). Procedures such as lyophilization result in physical changes in serum specimens, while addition of stabilizers or other additives further alters the properties of the specimen (33).

Commutability of enzyme-containing materials and samples between methods is so fundamental to the use of enzyme reference materials that it should not be accepted uncritically, or without the most rigorous experimental verification. In fact, any significant difference in the behavior of the materials and the clinical specimens in measurement systems will result in measurement errors. Commutability, or the convertibility of results between methods, thus requires the experimental demonstration of the same constant ratio between results given by the methods for the reference material and for all unknown samples.

Recently, commutability of two candidate reference materials was evaluated for two methods used for the assay of AST catalytic activity (IFCC reference procedure and a routine method incorporating also pyridoxal phosphate) (34). Both methods were performed at 37°C, the only difference being the manual performance of the reference procedure and the automation of the routine method on a Dade Behring Mega analyzer. As can be seen in Figure III, a constant interassay relationship was observed for the series of patients' serum specimens, distributed throughout all the measuring range. The same observation was made for cRM 2, demonstrating that the material is commutable for the two defined assays and can be used to calibrate the routine method in terms of the reference procedure. Conversely, the other candidate reference material, cRM 1, was not commutable for the two methods, indicating that this material cannot be used for AST accuracy transfer in this case. This example indicates clearly that the commutability cannot be affirmed without experimental assessment even for apparently similar methods

Table 3
Factors involved in commutability of reference materials for enzymes

- Nonhuman origin(s) of the enzyme
- Isoenzyme pattern
- Matrix of the solution
- Procedures that result in physical changes, e.g. lyophilization
- Addition of preservatives, antimicrobial agents, stabilizers, or other additives

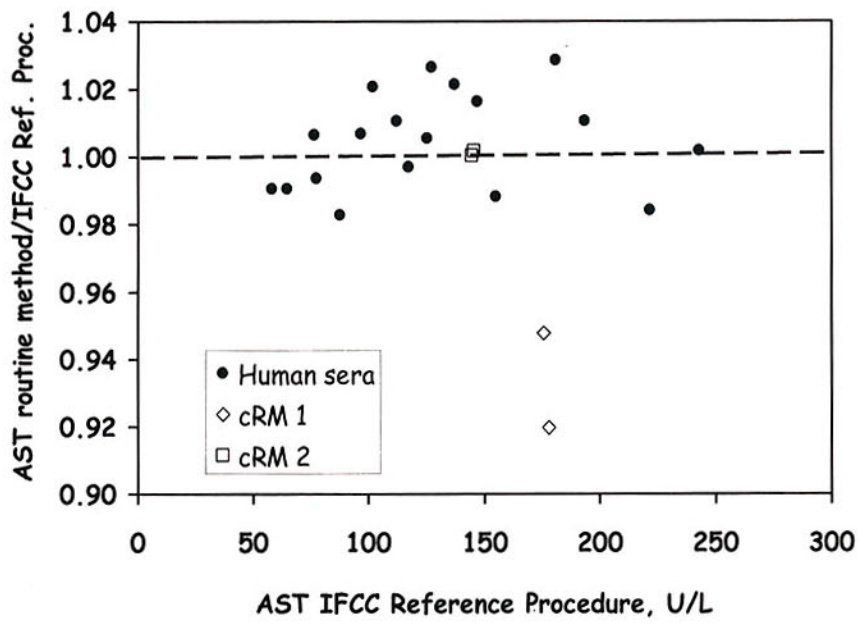

Figure III
Commutability study of two candidate Reference Materials (cRM) for aspartate aminotransferase (AST) measurement using the IFCC reference procedure and a routine automated method
The measurement of cRM was done in duplicate

of measurement.

In addition to the need of commutability with the human specimens of the different reference and calibrator materials involved, the concept of the reference system is valid only if the reference procedure and corresponding routine methods have identical, or at least closely similar, specificities for the measured enzyme (20). This requires, among more general factors, similar selectivities toward individual isoenzymes or molecular forms of enzymes. Some examples may illustrate this point. It will not be possible to calibrate methods for transaminases which do not incorporate pyridoxal phosphate in terms of one that does, such as the IFCC reference procedure, because no constant proportion of preformed holoenzyme to reactivatable apoenzyme can be found in a series of patients' specimens (35-37). AMY is another example to illustrate the different selectivity of some assays for pancreatic or salivary isoenzymes. In short, the idea of relating methods to each other through the use of the enzyme reference system should not run the risk to be dismissed as impossible simply because of a choice of suboptimal routine methods or as a result of attempts to correlate methods which clearly employ different measurement principles.

ADDITIONAL SERVICES OFFERED BY THE NETWORK OF REFERENCE LABORATORIES

In addition to the certification of reference materials, the network of reference laboratories may also be regarded as a concerted means of supporting EQAS by setting up reference procedures for their control materials (Figure II). The target concentrations in the EQAS samples are usually defined as method-dependent values. However, it has now become clear that there is a need to replace method-dependent values by the reference procedure values, thus providing a new objective target for the evaluation of participants' results (23).

The network could also assist industrial manufacturers of internal quality control samples in setting up reference procedure-based target values in these control samples.

Finally, the network is also able to assist commercial companies in validation of routine measurement procedures by performing "split sample" comparisons between reference procedures and routine methods on a panel of fresh patient sera, thus directly certifying commercial systems (24). This action is based upon direct comparison of a routine analytical system with the reference procedure, on a number of properly selected, native human sera. As a result of this operation, appropriate values are generated for the calibration material of the routine analytical system, which often contains a more or less complicated matrix, independently of the possible lack of commutability of this material.

Furthermore, the calibrator material could be stored under ideal conditions for re-use as reference material by the manufacturer for production of new batches of calibrators, reagents, etc. (38).

In conclusion, the time has come to provide compatible numerical enzyme results from all laboratories. This presentation is intended to show that by consistent application of metrological concepts, that is, the reference procedures and the reference materials in a reference system, it is possible to improve accuracy and thus conparability of the enzyme analysis results. The reference system approach can give the clinical laboratory and medical community a universal means of creating and ensuring comparability without requiring disruptive changes in the existing working methods or in an individual's preference for one or another analytical system.

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