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Evaluation and Improvement of Clinical Chemical Laboratory Tests for Glomerular Function

ACADEMIC DISSERTATION
To be presented, with the permission of the Faculty of Medicine of the University of Tampere, for public discussion in the main auditorium of Building B, Medical School of the University of Tampere, Medisiinarinkatu 3, Tampere, on November 9th, 2001, at 14 o’clock.

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To all of you, who have made
my life worth living
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals I – VII.


ABBREVIATIONS

$^{51}$Cr-EDTA Ethylene diamine tetra acetic acid, labelled with chromium isotope 51
$^{99m}$Tc-DTPA Diethylenetriaminopentaacetate, labelled with technetium isotope 99m

ADA American Diabetes Association
AER Albumin excretion rate
AUC Area under curve
CAP College of American Pathologists
$C_{VA}$ Analytical coefficient of variation
$C_{VG}$ Between-subject coefficient of variation
$C_{VI}$ Within-subject coefficient of variation
DCCT Diabetes Control and Complication Trial Research Group
EDTA Ethylene diamine tetra acetic acid
EQA External quality assessment
FPR False-positive rate, $1 - $specificity
FRG Federal Republic of Germany
GFR Glomerular filtration rate
GBM Glomerular basement membrane
GSC Glomerular sieving coefficient
$\text{HbA}_{1c}$ Glycated haemoglobin, glucose attached to the N-terminal valine of the beta chain
HPLC High performance liquid chromatography
IFCC International Federation of Clinical Chemistry
ISO International Organization for Standardization
NCCLS National Committee for Clinical Laboratory Standards
NIST National Institute of Standards and Technology
RIA Radio immunoassay
RID Radial immunodiffusion
ROC Receiver operating characteristic
SRM Standard Reference Material
TCA Trichloroacetic acid
TPR True-positive rate, sensitivity
WASP World Association of Societies of Pathology
INTRODUCTION

Direct determination of the glomerular filtration rate (GFR) is generally accepted as the best index of renal function (Perrone et al. 1992). Accurate methods based on measurement of e.g. inulin clearance, radiolabelled substances or iohexol, are, however, too complex and laborious for routine clinical use. Hence endogenous filtration markers offer rapid, simple and cheap estimation of the GFR in the clinical setting.

Justus Liebig gave the name creatinine to a substance he obtained by heating creatine with mineral acids (Liebig 1847). In the body creatinine is formed from both creatine and creatine phosphate. Applying the alkaline picrate method (Jaffé 1886), Folin developed its determination in urine (1904) and in deproteinised blood (1914). Since then the measurement of plasma or serum creatinine alone, or in conjunction with a 24-hour urine collection, has been the most widely used and accepted endogenous tool for clinical GFR estimation despite it’s being far from reliable. Factors limiting the sensitivity, specificity and reliability of creatinine as a measure of the GFR include the following: influence of muscle mass on the endogenous creatinine production rate, renal tubular secretion and reabsorption of creatinine, dietary intake of creatine or creatinine, and various analytical problems with the most commonly used Jaffé colorimetric assay method for creatinine.

Cystatin C is a small, basic protein synthesised by all nucleated cells (Norlund et al. 1997). It satisfies the criteria for an ideal endogenous GFR marker better than creatinine. The endogenous rate of cystatin C is constant and is not affected by lean body mass (Vinge et al. 1999), inflammation, fever (Norlund et al. 1997) or gender (Bökenkamp et al. 1998a, Finney et al. 2000). Cystatin C is freely filtered at the glomeruli and its renal plasma clearance is virtually identical with that of $^{51}$Cr-EDTA (Tenstad et al. 1996). Early investigations demonstrated that plasma cystatin C was, indeed, at least as good a marker for the GFR as plasma creatinine in the populations investigated (Grubb et al. 1985, Simonsen et al. 1985).
Richard Bright (1836) was the first physician to show clearly that albuminuria was a manifestation of kidney disease. Albumin determination in urine using a dipstick is still one of the most commonly requested laboratory tests. At the beginning of the 1980s Viberti (Viberti et al. 1982) and Mogensen (1984) showed that even a slightly elevated urine albumin concentration, not detectable with a dipstick, was predictive of clinical nephropathy in diabetics. This phenomenon, called microalbuminuria, has also been thought to be a risk factor for the development of cardiovascular disease (Haffner et al. 1990) and a generalised marker of vascular damage (Deckert et al. 1989).

In the present study the aim was to improve and to evaluate routine laboratory tests for the detection of microalbuminuria and the assessment of the glomerular filtration rate.
REVIEW OF THE LITERATURE

1. Direct measurement of the glomerular filtration rate

Glomerular filtration is the initial and generally the rate-limiting step in the renal excretory process. Estimation of the GFR is the most widely used test for renal function (Hall et al. 1976), being related to the relative mass of functional renal tissue and thus to the number of functioning nephrons. Tests, which measure the GFR directly or indirectly, are major tools in assessing the level of impairment of renal function.

The most exact technique for measuring the GFR requires the infusion of a substance, which is filtered freely and not reabsorbed, secreted or metabolised by the kidney. Inulin, a fructose polysaccharide, is considered to be an ideal substance for the purpose. This classic method entails intravenous infusion of the marker and multiple plasma samples, and urine collection of the GFR marker with a bladder catheter is therefore too cumbersome and unpractical for clinical use (Levey 1990).

In current clinical practice a GFR marker (inulin, $^{51}$Cr-EDTA, $^{99m}$Tc-DTPA or a non-radiolabelled substance such as contrast medium iohexol) is often injected as a bolus (Nosslin 1965, Aurell and Ditzel 1970, Phil and Nosslin 1974) and the GFR is determined according to plasma clearance. The number of plasma samples varies from 2 to 12, depending on whether a single or multicomartment model is chosen to calculate the GFR. A single-compartment model mathematically normalised to a multicompartment model is increasingly in use and requires only 2 – 4 plasma samples taken after the distribution phase (Bröchner-Mortensen 1972). This single-shot technique, however, cannot be used in patients with oedema, since in such case equilibration of the marker between plasma and interstitial fluid may take up to 12 hours (Garnett et al. 1967).
Although the single-shot technique is more convenient for the patient than earlier procedures based on exogenous substances, measurement of creatinine and its clearance are still the methods most commonly used to estimate the GFR.

2. Creatinine

In the body creatinine is formed by a spontaneous and irreversible conversion from creatine and creatine phosphate, which is the source of high-energy phosphate bonds for the immediate reformation of ATP during muscular contraction. Formation of creatinine is fairly constant, about 2% of the whole body creatine being transformed per day. There is a direct relationship between plasma creatinine level and muscle mass. Creatinine production is not affected by illnesses, such as sepsis and trauma, or dehydration, but may increase with increased dietary protein intake (Jacobsen et al. 1980).

Creatinine cannot be reutilised and is thus a waste product. It is freely filtered by the glomeruli and there is no significant tubular reabsorption. A small amount of creatinine is excreted by active tubular secretion, the amount increasing with raised plasma creatinine concentrations (Bauer et al. 1982).

The plasma creatinine level depends on the creatinine production rate and the rate of elimination in the glomerular filtrate. In the steady state, the rates of creatinine production and elimination are equal and can be estimated as the product of urine creatinine concentration and urine flow rate. If the production of creatinine is constant the plasma creatinine level increases when creatinine elimination via the kidneys decreases.

Measurement of creatinine in plasma or serum and urine is essential in the evaluation of renal function. Values for creatinine in plasma have been used directly to estimate the GFR. Plasma creatinine, however, is not usually measurably increased until there is at least 50% loss of renal function (Renkin 1974). Creatinine clearance calculated from measured creatinine concentrations in plasma and in timed urine collections
might thus be expected to indicate early dysfunction more sensitively (Doolan et al. 1962, Tobias et al. 1962, Bowers and Wong 1980). In practice, however, the benefits of the creatinine clearance are not so obvious. There is a considerable variation in the amount of creatinine excreted in a 24-hour collection between individuals and within the same individual (Paterson 1967, Payne 1986, Guillausseau et al. 1988). Especially in elderly patients collection of urine is difficult (Eriksson and Kallner 1989) and daily variation in creatinine clearance measurements is increased.

The reproducibility of measurements is an important aspect in the assessment of the glomerular function (Morgan et al. 1978). Based on their own data and data from literature the authors argued that plasma creatinine is more sensitive than creatinine clearance in the detection of changes in glomerular function. Rosano and Brown (1982), in a similar analysis of analytical and biological variation, also found that plasma creatinine was a more sensitive marker of change in renal function.

The concentration of plasma or serum creatinine is often considered sufficient to describe the GFR. For an accurate interpretation, reference values from healthy individuals should be collected separately for different age and gender groups to adjust with differences in muscle mass, otherwise the reference values will disguise small but significant impairment of renal function. To overcome this problem many formulae (Edwards and Whyte 1959, Jelliffe 1971, 1973, Cockcroft and Gault 1976, Schwartz & al. 1987, Levey et al. 1999) and a nomogram (Kampmann & al. 1971) have been applied with more satisfactory results.

2.1. Creatinine determination

2.1.1. Colorimetric methods

Jaffé reaction. Alkaline sodium picrate has been used as a reagent for creatinine determination ever since this reaction was first described (Jaffé 1886). Folin developed the first applications of this method for creatinine determination in urine (Folin 1904) and in deproteinised blood (Folin 1914). The reaction of picric acid under the conditions used is not specific for creatinine. Jaffé himself reported (Jaffé 1886)
that solutions of acetone and glucose reacted with the reagent and gave similar colours. Since then numerous metabolites and drugs have been shown to interfere with this creatinine determination method (Swain and Briggs 1977, Soldin et al. 1978, Bowers 1980, Bowers and Wong 1980, Spencer 1986). It is hardly surprising, therefore, that much effort has been expended in attempts to improve the specificity of the method (Table 1.)

Table 1. A summary of approaches used to improve the specificity of the Jaffé reaction with creatinine (Spencer 1986, modified)

<table>
<thead>
<tr>
<th>Adsorption of creatinine by:</th>
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<tr>
<td>- Kaolin</td>
<td>Greenwald and McGuire 1918</td>
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<tr>
<td>- Lloyd Reagent (Fuller’s Earth)</td>
<td>Gaebler1930, Owen et al. 1954, Haeckel 1981</td>
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<td>- Ion Exchange</td>
<td>Adams et al. 1962, Adams 1964</td>
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<th>Oxidation of interferents by</th>
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<tr>
<td>- Ceric Sulphate</td>
<td>Kostir and Sonka 1950</td>
</tr>
<tr>
<td>- Iodine</td>
<td>Taussky 1954</td>
</tr>
<tr>
<td>- K₄Fe(CN)₆</td>
<td>Knapp and Mayne 1987</td>
</tr>
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<th>Solvent extraction of interferents</th>
<th>Taussky 1954, 1956</th>
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<tr>
<th>pH adjustment to decolorise the Jaffé creatinine chromogen</th>
<th>Cook 1975, Heinegaard et al. 1973</th>
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<tr>
<td></td>
<td>Lolekha and Taksinamanee 1980</td>
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<th>Continuous flow dialysis</th>
<th>Chasson &amp; al. 1961</th>
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<tr>
<th>Kinetic measurements of reaction with:</th>
<th></th>
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<tbody>
<tr>
<td>- Reaction interval measurement</td>
<td>Cook 1971, Bartels and Böhmer 1971</td>
</tr>
<tr>
<td>- Modified assay conditions</td>
<td>Spencer and Price 1980</td>
</tr>
</tbody>
</table>

Efforts to develop a method both practical and accurate method separation of creatinine from non-creatinine chromogens have not been completely successful. Adsorption and extraction techniques do not lend themselves to automation and the popularity of such procedures has diminished in recent decades, largely due to the need for automated assays with large throughput.

The introduction of a dialysis step with continuous-flow automation in the early 1960s improved the specificity of the Jaffé reaction, although dialysable interfering substances like glucose, ketoacids and pyruvate still contributed to the non-creatinine
Jaffé chromogens. Other disadvantages of this technique were large sample volume and poor precision at low creatinine levels (Schwartz et al. 1987).

Nowadays the Jaffé creatinine test is usually configured to a two-point, fixed-time kinetic measurement with or without a correction algorithm (Blijenberg et al. 1994). Cook (1971) reported that the rate of colour development was initially fast, followed by a much slower phase, the slope of which was similar to that obtained when measuring aqueous creatinine solutions. He and others (Fabiny and Ertinghausen 1971, Bartels and Böhmer 1971, Larsen 1972, Bierens de Haan 1972) concluded that signals caused by ”slow and fast ” reacting interfering substances could be avoided by choosing a correct time window for measurement. In the seventies numerous applications of the kinetic creatinine method, with wide variation in the reaction conditions used, especially variations in picrate and hydroxide concentrations and measurement interval, have been published (Cook 1971, Fabiny and Ertinghausen 1971, Bartels and Bohmer 1971, Larsen 1972, Bierens de Haan 1972, Lustgarten and Wenk 1972, Schoucri and Pouliot 1977, Soldin et al. 1978, van Stekelenburg et al. 1978 Price et al. 1979, Vuorinen et al. 1987). Non-enzymatic kinetic assays are highly sensitive to reaction conditions (Spencer and Price 1980) and wide variation in these conditions has produced much conflicting data on assay precision and the extent of interference (Spencer 1986).

The advantages of the kinetic picrate method are its cheapness, rapidity and the small sample size necessary for analysis. The method also yields results which are on the average close to true creatinine concentrations, but displays significant variability in the lower range of plasma creatinine concentrations (Cottrell and Frings 1979, Hicks et al. 1979, Soldin et al. 1981).

*Alternative colorimetric methods.* Various alkaline picrate methods have dominated creatinine determinations, but alternative chemical methods have also been proposed over the years (Langley and Evans 1936, van Pilsum et al. 1956, Sullivan and Irreverre 1958, Parekh et al. 1976). They have not, however, been decisively superior to alkaline picrate, since sensitivity is not improved and specificity can be achieved only by means of protein precipitation procedures (Cooper and Biggs 1961, Parekh and Sims 1977). Nevertheless, the creatinine reaction with dinitrobenzoates (Benedict and
2.1.2. Enzymatic methods

Methods employing enzymatic breakdown of creatinine represent alternatives to the alkaline picrate type creatinine assay. In 1937, Dubos and Miller isolated bacteria which were capable of decomposing creatinine. Using crude extracts of these bacteria they developed an enzymatic procedure to estimate the creatinine concentration in plasma and urine (Miller and Dubos 1937). They treated one portion of plasma or urine with the enzyme extract and then compared the colours produced by the Jaffé reaction in the enzyme-treated and untreated samples. This difference was attributed to the true creatinine content of the sample. Since then a number of authors have studied and isolated creatinine-degrading enzymes from a variety of bacterial sources (Cook 1975). Studies of these enzymes have revealed the involvement of two enzymes, creatininase and creatinine iminohydrolase.

Creatininase. Creatininase, or creatinine amidohydrolase (E.C. 3.5.2.10), catalyses the hydrolytic conversion of creatinine to creatine. In the method described by Wahlefeld and co-workers (1972) the production of creatine was monitored with a multienzyme series of indicator reactions. Their method involved creatininase plus the following enzymes: creatine kinase, pyruvate kinase and lactate dehydrogenase. The rate of change in absorbance at 340 nm due to oxidation of NADH is directly proportional to the amount of creatinine in the sample. Endogenous creatine and pyruvate were removed during preincubation with indicator enzymes before the addition of creatininase. This method was specific, giving almost identical values with a mass fragment graphic method (Björkhem & al. 1979), but its precision was poor at low creatinine levels (Hearne and Fraser 1979). It also proved expensive and impractical for routine use by reason of the many reagents and large sample volume required.

In the second reaction scheme the transformed creatine is converted to sarcosine with creatinase (EC 3.5.3.3 creatine amidinohydrolase). Sarcosine is then oxidised with sarcosine oxidase (EC 1.5.3.1), and formaldehyde, glycine and hydrogen peroxide are
formed. The amount of the formaldehyde formed is directly proportional to the creatinine concentration in the sample and can be measured with formaldehyde dehydrogenase (EC 1.2.1.1) (Sugita et al. 1992). A more common approach is to measure the hydrogen peroxide formed using peroxidase enzymes (Guder et al. 1986):

Also other colour reagents have been used (Fossati et al. 1983, Siedel et al. 1984). Bilirubin (Witte et al. 1978) and ascorbic acid (White-Stevens 1982) may, however, interfere with peroxidase detection systems and to avoid such disturbance Guder and co-workers (1986) added potassium hexacyanoferrate and ascorbate oxidase to the assay system.

**Creatinine iminohydrolase.** Creatinine iminohydrolase, or creatinine deiminase (EC 3.5.4.21), catalyses the conversion of creatinine to methylhydantoin and ammonia. Most methods based on this approach have monitored the production of ammonia by pH change (Toffaletti et al. 1983), a specific electrode (Thompson and Rechnitz 1974, Mayerhoff and Rechnitz 1976, Ripamonti et al. 1984) or the Berthelot reaction (Tabata et al. 1983). Tabata used glutamate dehydrogenase (EC 1.4.1.4) to remove the
endogenous ammonia, but this enzyme has also been used as a part of the detection system. The reaction scheme is then as follows (Tangatelli et al. 1982):

$$\text{Creatinine} + \text{H}_2\text{O} \xrightarrow{\text{creatine iminohydrolase}} \text{N-Methylhydantoin} + \text{NH}_3$$

$$\text{NH}_3 + \alpha\text{-oxoglutarate} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{glutamate dehydrogenase}} \text{L-glutamate} + \text{NADP}^+ + \text{H}_2\text{O}$$

The reaction can be monitored by following the decrease in absorbance at 340 nm as NADPH is consumed. Endogenous ammonia is eliminated during preincubation with glutamate dehydrogenase. Especially old urine samples may contain high ammonia concentrations and the pre-incubation step then consumes substantial portion of NADPH and narrows the assay range. The $\alpha$-oxoglutarate and NADPH consumed can be restored via a reaction catalysed by isocitrate dehydrogenase (EC 1.1.1.42) (Fossati et al. 1994):

$$\text{NADP}^+ + \text{isocitrate} \xrightarrow{\text{isocitrate dehydrogenase}} \alpha\text{-oxoglutarate} \text{NADPH} + \text{CO}_2 + \text{H}^+ + \text{Mg}^{2+}$$

The activity of isocitrate dehydrogenase is strictly dependent on magnesium ion and can be completely blocked by a magnesium-complexing agent (e.g. trans-1,2-cyclohexanediamine-$N,N,N,N$-tetraacetic acid) when the creatinine assay is started with creatinine iminohydrolase.

2.2. Interferences
The unspecificity of the alkaline picric acid reaction has been known ever since it was introduced (Jaffé 1886). A number of attempts to improve the specificity have been described during the last century (Table 1). Using the kinetic procedure and choosing suitable measurement conditions many interference problems can be avoided. Bilirubin interferes with kinetic Jaffé methods (Watkins et al. 1976), but bilirubin can be oxidised to biliverdin by potassium ferrosyanide before creatinine measurement (Knapp and Mayne 1987). In spite of these improvements, however, the Jaffé
procedure still gives higher values for creatinine than reference methods, and especially in children its accuracy is poor (Schwartz et al. 1987).

Enzymatic methods are more specific than those based on the Jaffé procedure, but some interference has been reported. Bilirubin disturbs peroxide detection systems (Witte et al. 1978), but this can be avoided by adding potassium ferrosyanide to the reaction mixture (Fossati et al. 1983) or by pre-treatment of the sample with bilirubin oxidase (Artiss et al. 1884). The effect of ascorbic acid, another major interferent in peroxidase detection systems (White-Stevens 1982), can be overcome by incorporation of ascorbate oxidase in the reagent system (Guder et al. 1986).

Some drugs also hamper enzymatic approach. N-ethylglycine, a metabolite of lidocaine, interferes with assay systems containing sarcosine oxidase (Roberts et al. 1988). Bagnoud and Reymond (1993) report that patients receiving metamizol showed unusually low creatinine values when enzymatic methods containing creatinimase, creatinase, sarcosine oxidase and peroxidase enzymes were used. In like manner calcium dobesilate interferes with assays containing peroxidase enzymes (Goren et al. 1986), the effect being explained by the structural similarities to the chromogens used as indicator in the peroxidase reaction.

Creatinine assay methods based on creatinine iminohydrolase and glutamate dehydrogenase woulds appear to be less sensitive to interference. Fossati and co-workers (1994) investigated more than a hundred possible endo- and exogenous interferents and only 5-fluorocytosine proved troublesome at borderline therapeutic concentrations.

2.3. Chromatographic methods
The most accurate and precise means of measuring small organic compounds is isotope-dilution mass spectrometry. Some candidates for a Definitive Method (or Primary Reference Measurement Procedure, ISO 1994, Dybkaer 1997) also for creatinine based on this technique have been reported (Björkhem et al. 1977, Siekman 1985, Welch et al. 1986, Stöckl and Reinauer 1993). These methods are so complicated and laborious, however, that they can be used only in cases where small
numbers of samples have to be assessed accurately. Using this technique one can measure method-independent target values for control sera used in external quality control surveys. Manufacturers of creatinine kits are recommended to certify their standards with a Definitive Method.

A Reference Method (or a Secondary Reference Measurement Procedure, ISO 1994, Dybkaer 1997) is, however, needed to provide a link between the definitive and routine methods. According to the established criteria such a method must be precise, have minimal and well-defined inaccuracy as compared with the Definitive Method, and be technically accessible to a number of laboratories (NCCLS 1984).

HPLC, high-performance liquid chromatography, has been proposed as a Reference Method and a number of procedures for creatinine determination have been developed based on this technique. The first were based on ion-exchange chromatography followed by post-column alkaline picrate reaction (Brown et al. 1977, Weatherburn et al. 1978), but subsequently, direct ultraviolet-detection of creatinine became more popular (Ambrose et al. 1983, Chiou et al. 1983, Meyer et al. 1985, Zwang and Blijenberg 1992, Scott 1992). Also normal-phase (Patel and George 1981, Ziemniak et al. 1981), reverse-phase (Lim et al. 1978, Krstulovic et al. 1979, Buchanan et al. 1979, Spierto et al. 1980, Okuda et al. 1983, Achari et al. 1983, Zhiri et al. 1985, Werner et al. 1987, Lam and Tarding 1988, Linnet and Bruunshuus 1991), paired-ion (Soldin and Hill 1978, Paroni et al. 1990) and base-stable stationary phase, Al₂O₃ (Thienpoint et al. 1995) chromatographic techniques have been developed. The mobile phase varies from method to method, but in most cases has contained acidic or neutral buffer and methanol.

The specificity of these candidates as a Reference Method has been evaluated chiefly by analysing solutions and samples containing possible interfering substances. Two more elegant approaches are rechromatography of samples after enzymatic breakdown of creatinine (van Landuyt et al. 1994) and analysis of the UV spectrum by a diode-array detector after chromatography (Linnet and Bruunshuus 1991). These latter investigators also compared UV detection and enzymatic determination of creatinine after chromatography and the results were identical. Most of these HPLC methods appeared to be fairly specific, but few have been strictly validated to such an extent
that they can be regarded as Reference Methods (Rosano et al. 1990, Linnet and Bruunshuus 1991, Zwang and Blijenberg 1992, Thienpoint et al. 1995). Most HPLC methods have been developed solely for evaluation of the authors’ own routine method.

3. Cystatin C

Cystatin C is a cysteine proteinase inhibitor with widespread distribution in human biological fluids (Abrahamson et al. 1986). It contains one non-glycated polypeptide chain with 120 amino acid residues and has a molecular weight of 13 359 kDa (Grubb and Löfberg 1982). Cystatin C is synthesised as a preprotein with a signal peptide, indicating that the functions of the inhibitor are mainly extracellular (Abrahamson et al. 1987). The cystatin gene structure is known and the gene seems to be of the housekeeping type, i.e. it is steadily expressed in most human tissues (Abrahamson et al. 1990). Most nucleated cells produce Cystatin C and, since its production is not altered by inflammation, it is not an acute-phase protein (Grubb 1992).

The low molecular weight and positive charge (pI 9.3) of cystatin C mean that it passes easily through the glomerular membrane. The proximal tubular cells then reabsorb and catabolise practically all of the filtered cystatin C, with the result that its concentration in urine is very low. The concentration of is greatly increased, however, in patients with tubular disorders (Löfberg and Grubb 1979). The instability of cystatin C in urine renders its measurement as a marker of tubular disorders unreliable, while its stable production and elimination through the kidneys would strongly indicate that the plasma level might provide a good measure of the glomerular filtration rate.

3.1. Measurement of cystatin C

Since the original discovery of cystatin C in 1961 (Clausen 1961, Butler and Flynn 1961), many scientists have sought to improve assay methods for its measurement. In 1979 Löfberg and Grubb presented an enzyme-amplified single radial immunodiffusion method for quantification of cystatin C. The test proved relatively
precise, but was slow and insensitive to very low concentrations of cystatin C. Subsequently, simpler and more sensitive methods using enzyme-linked immunosorbent assay (Pergande and Jung 1993, Collé et al. 1992, Ishiguro et al. 1989, Olafsson et al. 1988, Tian et al. 1997), fluoroimmunoassay (Joronen et al. 1986) or radioimmunoassay (Poulak et al. 1983) were developed to improve the analytical reliability of cystatin C measurement. These tests were found to be precise and sensitive, but results were not always comparable and the diagnostic value of cystatin C was not clearly confirmed - perhaps by reason of the general difficulty of standardising immunological methods. The antigens and antibodies used were mostly prepared in house, with differences in the heterogeneity and purity of various isolation procedures (Pergande and Jung 1993, Finney et al. 1997). These methods were also rather slow and difficult to automate and therefore impractical in routine use.

Monitoring the formation of antigen-antibody complexes by either nephelometry or turbidimetry are the most common clinical approaches to the quantitation of specific proteins (Price et al. 1983). The sensitivities of these techniques can be increased if the light scattering properties of the immunoaggregates are increased by attaching latex particles to the antibody (Price et al. 1987). As homogenous immunoassays these techniques are easy to automate. Particle-enhanced turbidimetric (Kyhse-Andersen et al. 1994, Newman et al. 1995) and nephelometric (Finney et al. 1997) immunoassays have also been developed for cystatin C measurement. Both approaches are easily automated, rapid, precise and sufficiently sensitive for cystatin C measurement in plasma or serum. They correlate well with each other, but nephelometric results are lower than turbidimetric. One reason might be that the commercially available turbidimetric test kit contains a recombinant human cystatin C as calibrator while the calibrator of the other kit is purified cystatin C from human urine (Finney et al. 1997).

3.2. Cystatin C as a marker of the GFR
Theoretically, cystatin C has several advantages over creatinine as a marker for the GFR. The rate of production of creatinine is mainly determined by muscular mass, and is thus somewhat variable. Its elimination is complex and in addition to glomerular filtration it is also secreted via tubular cells and via the intestine (Perrone et al. 1992). A single gene, in contrast, determines the production of cystatin C. It is formed at a
constant rate by virtually all tissues in the body and is eliminated from the blood almost exclusively by glomerular filtration (Abrahamson et al. 1990, Grubb 1992).

Comparisons of the relations between cystatin C, creatinine and, the GFR are markedly influenced by the selection of the population studied. Randers and coworkers (1998) found a close correlation between cystatin C and $^{99m}$Tc-DTPA clearance ($r = 0.91$), but the diagnostic accuracy of cystatin C was no better than that of creatinine in patients with various kidney diseases with a wide range of renal function and in dialysis patients. In another study the same group found the diagnostic accuracy of cystatin C to be superior to that of creatinine in adults with normal to moderately impaired kidney function (Randers et al. 2000).

A rapidly growing body of evidence suggests that cystatin C is superior to creatinine as an endogenous marker of the GFR. Studies in patients with manifest or subclinical GFR impairment have consistently shown two key findings (Bostom and Dworkin 2000):

1) There is a significantly stronger correlation between serum levels of cystatin C and the GFR, relative to creatinine and the GFR.
2) Detection of slight impairment in the GFR is improved using plasma cystatin C levels as compared with plasma creatinine determination.

### 3.3. Limitations

Recent studies have shown that non-renal factors may also influence the plasma concentration of cystatin C. Malignant diseases, e.g., melanoma (Kos et al. 1997, 1998), asthma (Cimerman et al. 2000) and HIV (Collé et al. 1992), which increase proteolytic activity, may increase the cystatin C concentration in plasma. Cystatin C production is increased in the presence of glucocorticoids at least \textit{in vitro} (Bjarnadottir et al. 1995).

Chylomicrons might interfere with the immunoturbidimetric method. Some, but not all, rheumatoid factors may cause erroneously high readings in turbidimetry (Grubb 2000).
4. Microalbuminuria

Excretion of small amounts of albumin in the urine is normal. Microalbuminuria is defined as persistently increased albumin excretion (20-200 µg/min), undetectable by conventional test strips of about 0.2 - 0.3 g/l sensitivity (Viberti and Walker 1988, Shihabi et al. 1991). It represents the subclinical transition between normal albuminuria and proteinuria. For an individual to be identified as having microalbuminuria, an excretion rate of 20-200 µg/min must be observed in at least two out of three specimens within a six-month period (Mogensen et al. 1988, Bennett et al. 1995). Values greater than 200 µg/min are defined as macroalbuminuria or clinical albuminuria. Urinary albumin excretion is generally expressed in µg/min or mg/24h, or, to correct for variations in urine flow, as the albumin:creatinine ratio (mg/mmol, g/mol or mg/g). Accurate measurement of the albumin excretion rate requires timed urine collection, while the albumin:creatinine ratio can be estimated from a "spot" urine sample from an ambulant patient with the assumption that the subject’s urinary creatinine excretion remains constant.

4.1. Renal handling of albumin

The glomerular basement membrane (GBM) of the kidney acts as a size- and charge-selective filter for macromolecules in plasma (Brenner et al. 1978a). The permeability of GBM is indicated by the glomerular sieving coefficient, GSC (Maack et al. 1979). Molecules such as inulin which pass the GBM freely have, by definition, a GSC of one. Studies using infusions of neutral dextran have shown that fractional clearance of the dextran falls sigmoidally to zero as the size of the molecule, measured by the Stokes radius, increases (Brenner et al. 1978b). If the molecule has a negative charge, its fractional clearance is smaller than that of a neutral macromolecule of the same size. Rendering the molecule cationic increases the fractional clearance (Brenner et al. 1978b). As an anionic protein, albumin is excreted several-fold less than would be predicted for a neutral protein with the same Stokes radius of 3.6 nm (Hackbarth et al. 1980). The increased resistance is assumed to arise from electrostatic repulsion by the negatively charged heparan sulphate and sialoproteins which constitute part of the glomerular basement membrane (Kanwar 1984). The amount of protein filtered in a
given time depends on the GSC, the glomerular filtration rate and the protein concentration in plasma available for filtration (Sumpio and Hayslett 1985). Although the GSC of albumin is very low (Hackbarth et al. 1980), a considerable quantity of albumin is filtered in consequence of the high albumin concentration in plasma. Some grams of albumin reach the glomerular filtrate every 24 hours, but only a few milligrams are excreted into urine, the rest of filtered albumin being reabsorbed by tubular cells. This tubular reabsorption mechanism works normally near saturation (Park and Maack 1984) and therefore a small increase in GBM permeability leads to a clearly increased albumin excretion rate (Gosling 1995).

The pathophysiology underlying microalbuminuria in diabetic nephropathy is still not fully understood, but it is in all likelihood multifactorial. Intrarenal haemodynamic alteration, accumulation of sorbitol, and glycosylation of glomerular proteins are all thought to be involved in the pathogenesis of diabetic nephropathy (Brownlee et al. 1984, Adler et al. 1993, Hohstetter 1994).

Hypertension. It has been proposed that increased intraglomerular pressure causes endothelial damage to the vascular system of the glomerulus. This, in turn, leads to platelet aggregation and attraction of macromolecules into the endothelium and triggers mesangial proliferation (Davies et al. 1986). Mesangial cells produce more matrix, which is a typical structural abnormality in overt nephropathy. Systemic blood pressure plays an important role in the development of diabetic nephropathy. Hypertension predisposes to progression of microalbuminuria to macroalbuminuria; antihypertensive treatment markedly retards this progression (Mathiesen et al. 1991, Parving et al. 1983).

Hyperglycaemia. Protein glycation, a pathologic result of persistent hyperglycaemia may explain how elevated blood glucose induces diabetic nephropathy and other microvascular changes. Glycation of plasma proteins, and the structural proteins of the GBM or both, may cause altered glomerular permeability and constitute a link between hyperglycaemia and functional (e.g. microalbuminuria) and structural abnormalities in diabetes (Renold et al. 1978). Deposition of advanced glycated proteins induces changes in the porosity and the anionic charge of the GBM (Vlassara et al. 1986). Protein glycation reduces the net negative charge of the GBM; this leads
to less repulsion of anionic proteins like albumin, and to increased leakage of albumin into the glomerular filtrate.

Intensive insulin therapy, targeted to achieve glucose levels as close to normal as possible, leads to a reduction in the development and progression of long-term complications. The key findings of the Diabetes Control and Complications Trial were that complications such as retinopathy, nephropathy and neuropathy were very clearly reduced compared to conventional therapy (DCCT 1993). These outcomes represent convincing evidence that blood glucose significantly influences the development of diabetic microvascular complications.

4.2. Measurement of microalbuminuria

Labelled immunoassays. The first trials for measurement of low albumin concentrations in urine were made using in-house RIA methods (Keen and Chloverakis 1963, Miles and Mogensen 1970, Woo et al. 1978, Zager 1980). When the importance of microalbuminuria was realised, the reagents for the albumin RIA also became commercially available (Giampietro et al. 1987). Although RIA is still generally considered the reference method for urinary albumin assays, the $^{125}$I as a label has certain disadvantages. It is expensive, it has a relatively short shelf life and its use is possible only in laboratories authorised to handle radioactive substances. Enzyme (Harper et al. 1982, Fielding et al. 1983, Townsend 1986) and fluoroimmunoassays (Silver et al. 1986, Nisbet & al. 1993) have more stable reagents, but these methods were soon replaced by more practical nephelometric (Gatling et al. 1988, Giampietro et al. 1989) and turbidimetric (Teppo 1982, Sathianathan et al. 1986, Gosling et al. 1986, Bell et al. 1986) techniques.

Homogenous immunoassays. Urine does not contain light-scattering particles such as macromolecules and is thus eminently suitable as a sample for nephelometric measurements. The sensitivity of nephelometric assays is sufficiently high to measure even low albumin concentrations in urine. The results are comparable with those of RIA (Giampietro et al. 1992) and as a homogenous immunoassay the nephelometric procedure is easy to automate. Since, however, nephelometer is not a common instrument in clinical laboratories, many turbidimetric applications have also been
developed (Teppo 1982, Sathianathan et al. 1986, Gosling et al. 1986, Bell et al. 1986). Urine may, it is true, contain chromogens which absorb UV light and thus interfere with turbidimetric measurements (Sathianathan et al. 1986, Gosling et al. 1986), and this approach is less sensitive than labelled immunoassays and nephelometric methods, but at least all elevated urinary albumin concentrations can be measured turbidimetrically.

**Sample.** Microalbuminuria may be transiently increased for many non-renal reasons and therefore each clinical laboratory should develop its own standard for collection of urine specimens to avoid potential preanalytical sources of error. Because exercise will induce spuriously high albumin excretion, specimens should not be collected for up to 24 h following physical exertion (Viberti et al. 1982). Likewise, acute diuresis will falsely elevate the AER (Kouri et al. 1991, Shihabi et al. 1991), and false positive results may also derive from urine specimens contaminated with menstrual or seminal fluid or by the presence of a urinary tract infection (Frenchko et al. 1991). In view of the high intraindividual variability, it is recommended (Bennett et al. 1995) that urinary albumin excretion should be quantitated at least three times over a 6-month period.

A timed urinary albumin excretion rate, either a 24-hour or overnight collection, is clearly the most sensitive of assays assuming that a complete collection is made (Viberti et al. 1982, Mogensen and Christensen 1984, Kouri et al. 1991, Bennett et al. 1995). However, as precisely timed urine collections are often impractical and inconvenient for many patients, measurement of the albumin:creatinine ratio is recommended for routine practice (Viberti et al. 1994, Bennett et al. 1995, Mogensen et al. 1995, ADA 2001). This ratio minimises the effect of the variation in urine flow (Bakker 1999).

**4.3. Microalbuminuria in clinical practice**

*Diabetic nephropathy.* Persistent proteinuria and progressive renal insufficiency are the hallmarks of diabetic nephropathy. The condition is a common complication of diabetes which reduces life expectancy in 30 - 40 per cent of patients with type 1 diabetes. Renal impairment is common and also causes mortality in patients with type 2 diabetes, although death in these cases is more frequently due to cardiovascular
causes (Borch-Johnsen et al. 1985, Viberti and Walker 1988). At the stage of persistent clinical proteinuria, careful control of diabetes and hypertension can retard, but not arrest, the decline in renal function (Viberti and Walker 1988).

Several years before the appearance of proteinuria, however, subclinical elevation of albumin excretion, microalbuminuria, may be detected (Mathiesen et al. 1984). Prospective studies (Parving et al. 1982, Viberti et al. 1982, Mogensen and Christensen 1984, Mathiesen et al. 1984) have demonstrated that microalbuminuria is an important marker for the subsequent development of clinical diabetic nephropathy. Early detection of this forerunner may thus be crucial, since aggressive intervention in lowering concomitant hypertension and careful diabetic control would appear to slow down or even arrest the irreversible progression of diabetic nephropathy to renal insufficiency (Mogensen 1987, Viberti and Walker 1988).

The National Kidney Foundation in the USA has recommended that all type 1 diabetic patients over 12 years of age should have their urine tested for albumin excretion at least once a year (Bennett et al. 1995). Also patients with type 2 diabetes who are younger than 70 years with no overt renal disease should be screened for microalbuminuria. In microalbuminuric patients, blood glucose control should be improved as much as possible to delay progression to persistent proteinuria. If increased albumin excretion still persists, ACE inhibitor therapy should be started in both normotensive and hypertensive diabetic patients. The Finnish recommendations are very similar (Groop 1990, Pelkonen 1990).

*Inflammation and trauma.* In addition to its association with diabetic complications, microalbuminuria is also found in other diseases, including bacterial meningitis (Roine 1993), rheumatoid arthritis (Pedersen et al. 1995), inflammatory bowel disease (Mahmood et al. 1993), acute pancreatitis (Shearman et al. 1989) and trauma (Palister et al. 1997) and also in conjunction with surgery (Smith et al. 1994). Microalbuminuria can be detected within a few hours of acute myocardial infarction and is proportional to infarct size (Gosling et al. 1991). It is also apparent within one hour in minor ischaemic episodes such as those occurring after exercise in patients with intermittent claudication or positive exercise electrocardiogram tests (Hickey et al. 1994, Horton et al. 1994).
**Cardiovascular diseases.** Both hypertensive and diabetic patients with microalbuminuria show an increased vascular permeability to radiolabelled albumin (Parving et al. 1974, Feldt-Rasmussen 1986). Thus urinary albumin excretion is closely linked to vascular endothelial function, making microalbuminuria a possible marker of vascular diseases (Deckert et al. 1989). Endothelial dysfunction may lead to impaired insulin action as well as to capillary leakage of albumin, features possibly linked to a predisposition to cardiovascular disease (Yudkin 1996).

Attention has been drawn to the predictive power of microalbuminuria for cardiovascular mortality in both diabetic (Mogensen 1984, Patrick et al. 1990, Gall et al. 1991, Mattock et al. 1992) and non-diabetic populations (Kannel et al. 1984, Damsgaard et al. 1988, Haffner & al. 1990). Microalbuminuria may be a risk factor implicated in the development of cardiovascular disease and may therefore have a role in screening programmes (Haffner et al. 1990). The condition is, however, so frequent in many acute and chronic diseases that this non-specificity fatally limits its usefulness in this setting (Hartland and Gosling 1999).

### 5. GOAL-SETTING FOR A LABORATORY TEST

#### 5.1. Analytical accuracy

To reduce the frequency of unreliable results, laboratory medicine is continuously working to improve measurement procedures, comprehensive reference measurement systems and quality-assurance procedures. The utilisation of such a framework in a specific case requires a decision as to how good a given assay procedure should be, i.e., definition of analytical quality specifications. This decision should be based on a dialogue between the ultimate user and the laboratory.

Various criteria have been proposed for analytical quality specifications in assessing the acceptability of method performance. The requirements have been based on the state of the art (Tonks 1963), the views of expert individuals (Barnett 1968) and biological variation (Cotlove et al. 1970).
The IFCC conference on *Strategies to set Global quality specifications in Laboratory Medicine* (Kenny et al. 1999) reported consensus on a hierarchy of the models to be applied in setting analytical quality specifications (Table 2).

<table>
<thead>
<tr>
<th>Table 2. Hierarchic listing of procedures for determination of analytical quality specifications (Kenny et al. 1999)</th>
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<tbody>
<tr>
<td>1. Evaluation of the effect of analytical performance on clinical outcomes in a specific clinical setting</td>
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<tr>
<td>2. Evaluation of the effect of analytical performance on clinical decisions in general:</td>
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<tr>
<td>a. Data based on components of biological variation</td>
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<td>b. Data based on analysis of clinicians’ opinions</td>
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<td>3. Published professional recommendations</td>
</tr>
<tr>
<td>a. From national and international expert bodies</td>
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<tr>
<td>b. From expert local groups or individuals</td>
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<td>4. Performance goals set by</td>
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<td>a. Regulatory bodies</td>
</tr>
<tr>
<td>b. Organizers of External Quality Assessment (EQA) schemes</td>
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<tr>
<td>5. Goals based on the current state of the art</td>
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<tr>
<td>a. As demonstrated by data from EQA or Proficiency Testing scheme</td>
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<tr>
<td>b. As found in current publications on methodology</td>
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In theory, quality specifications should be derived objectively from an analysis of medical needs (Hyltoft Petersen and Hörder 1992, Klee 1993). Thus, assessment of the effect of analytical quality on clinical decision-making in special clinical situations, test by test, is probably the ideal approach. A major disadvantage of this strategy is that only a few tests are used in single well-defined clinical situations with standard well-accepted medical strategies directly related to the test result (Fraser 1999). Another significant drawback is that any quality specifications calculated depend on assumptions regarding how test results are used by clinicians (Lytken Larsen et al. 1990, Fraser and Hyltoft Petersen 1993a).

The next-best strategy (Kenny et al. 1999, Fraser and Hyltoft Petersen 1999) is the generation of quality specifications based on the general practice of clinicians in applying test results. The two major clinical settings in which results are used are the monitoring of individual patients and diagnosis using reference intervals. Well-
documented models suggest that applicable quality specifications for precision and trueness might best be based on the components of biological variation, namely, within-subject (CV\(_I\)) and between-subject (CV\(_G\)) variation (Fraser and Hyltoft Petersen 1993b, Stöckl et al. 1995).

Cotlove, Harris and Williams (1970) and Young, Harris and Cotlove (1971) recommended that tolerable analytical variability should be less than one half of the within-subject biological variation (CV\(_I\)). Harris (1979) demonstrated that if analytical variation, CV\(_A\) < 0.5 CV\(_I\), then the amount of variability added was about 10% (in reality 11.8%). More strictly, *desirable performance* is defined as CV\(_A\) < 0.5 CV\(_I\), while users of quality specifications based on biology might consider that *optimum performance* could be defined by CV\(_A\) < 0.25 CV\(_I\). More stringent quality specifications generated using this formula should be used for those analytes for which desirable performance standards are easily achieved with current technology and methodology. *Minimum performance* is defined by CV\(_A\) < 0.75 CV\(_I\) and less stringent quality specifications generated using this formula should be used for those analytes for which desirable performance standards are not attainable with current technology and methodology (Fraser et al. 1997). Here, it is assumed that no bias exists.

In addition, trueness of a measurement should be considered. Gowans and associates (1988) showed that for this to be achieved, the bias (B\(_A\)) should be < 0.250(CV\(_I^2\) + CV\(_G^2\))\(^{1/2}\). Analogously to precision, this quality specification for trueness can be termed *desirable trueness*. *Optimum trueness* is defined as (B\(_A\)) < 0.125(CV\(_I^2\) + CV\(_G^2\))\(^{1/2}\) and *minimum trueness* as (B\(_A\)) < 0.375(CV\(_I^2\) + CV\(_G^2\))\(^{1/2}\) (Fraser et al 1997). Fulfilment of these quality specifications would allow all laboratories to use the same reference intervals for their patients.

The strategy based on biological variation has advantages in that this approach includes statistical rigor and systematic definition of permissible laboratory error (Kaplan 1999). The within-subject variation seems to be independent of analytical methodology, age of subjects or whether they are in health or have a stable chronic disease (Fraser 1999). Also data on the components of biological variation are easily available for many analytes (Sebastian-Gambaro et al. 1997, Ricós et al. 1999).
A strategy based on the biological variation characteristic of healthy individuals has in fact some weaknesses. A goal involving a fixed percentage of CV_A from CV_I and CV_G is too simplistic (Kaplan 1999). The use of a single decision limit may be of no relevance in the actual medical decision process (Werner 1995). Sometimes this tool has resulted in error limits which might be expensive to achieve without yielding concomitant medical utility (Doumas 1997).

In spite of these weaknesses, however, many professionals consider the strategy based on biological variation to be the most practical. Expert groups from the College of American Pathologists (CAP, Elevitch 1977) and the World Association of Societies of Pathology (WASP, Elevich 1979) considered that this should be basis for analytical goal setting, and a working group from the European Group for the Evaluation of Reagents and Analytical Systems in Clinical Chemistry (Fraser et al. 1992) supported the conception that quality specifications are best thus based. A Working Group from the European External Quality Assessment Scheme Organizers Group reinvestigated all published approaches and upheld this view (Stöckl et al. 1995). A further European Working Group set quality specifications for Reference Methods, again based on biological variation (Thienpoint et al. 1995). The common professional consensus is thus that calculations involving biological variation offer the most satisfactory basis for quality specifications. Reasonable compromises (from desirable to minimum specifications) are then to be considered, if medical application in diagnostic testing or monitoring of patients does not fit into the calculated theoretical frames.

5.2. Reference intervals
The components of the human organism are subject to variations caused by physiological processes, genetic differences, diseases and environmental factors. Rational interpretation of laboratory results calls for a knowledge of the variation of these components in the individual under study or in one or more adequately defined sets or reference individuals. Reference intervals are used clinically, together with additional information, as guidelines concerning the state of patients.

Both the IFCC and the NCCLS have issued recommendations on procedures for the establishment of reference values and their treatment, and presentation of observed
values in relation to reference data (Solberg 1987a, Solberg 1987b, PetitClerc and Solberg 1987, Solberg and PetitClerc 1988, NCCLS 1995). In order to be relevant, the population used for the derivation of any reference intervals should represent the population in which the test will be clinically employed (Gräsbeck 1984). The validity of any reference interval is also dependent on collection of specimens under defined conditions, and control and assessment of analytical variation in the production of reference values (PetitClerc and Solberg 1987).

Reference intervals should be expressed as fractiles, most commonly the central 0.95 fraction of the reference distribution. Reference limits are accordingly estimates of the 0.025 and 0.975 fractiles (Solberg 1987b). In addition, the confidence interval (CI) of a given fractile is helpful in ascertaining the accuracy of a given limit. Typically 90% CIs are used. If the distribution is not univariate the reference values should be divided into homogeneous subgroups (Solberg and Gräsbeck 1989), provided the resulting subgroups are not too small for the calculation of reliable reference limits (Harris and Boyd 1990).

Regression analysis is particularly useful when covariate-dependency, e.g. age-dependency, is present and when the reference groups are so small that the resulting subgroups are not large enough for calculation of valid reference limits. The requisite size of reference sample groups is smaller, but on the other hand calculations are considerably more complicated (Irjala et al. 1990, Virtanen et al. 1998a,b).

5.3. Diagnostic accuracy
The diagnostic process involves assessment of whether a given disease is present or absent. Sensitivity and specificity have been the key descriptors of the performance of a given test in detecting a given disorder (Läärä and Aro 1988). Unfortunately, sensitivity and specificity usually vary inversely over the range of theoretically possible cut-off values, which complicates the task of describing the diagnostic accuracies of tests.

Receiver operating characteristic (ROC) analysis approaches this problem by plotting the true positive rate ( = sensitivity) versus the false positive rate ( = 1 - specificity)
for all possible cut-off scores of the test (Lusted 1971, McNeil et al. 1975, Swets 1988, Zweig and Campbell 1993, Forsström 1995). At each possible cut-off score, the true positive rate is plotted as a function of the false and the points are connected. The area under the ROC curve (AUC) can be used to describe the diagnostic accuracy of the test. The higher the sensitivity and specificity at each cut-off score, the more closely the curve will approach the upper left corner of the graph and the greater will be the area under the curve.

The ROC plot has the following advantages: It is simple, graphical and easily understood. It is a comprehensive representation of diagnostic accuracy, i.e., the discriminating ability of the test. It does not require selection of a particular cut-off level, because all possible decision thresholds are included. It is independent of the prevalence of a disease (presuming that the original data were produced with a representative patient population). It provides a direct visual comparison between tests on a common scale, in contrast to dot diagrams and frequency histograms (Zweig and Campbel 1993). The ROC plot has become popular in recent years in evaluating the discriminatory power of a test (Swets 1988, Henderson 1993, Zweig and Campbell 1993) and scientific journals (Anonymous 1993) and authorities (NCCLS 1995b) recommend it for this purpose.

The ROC plot has also a number of obvious disadvantages. Generation of plots and calculation of parameters are cumbersome without computer software. Though fortunately, flexible softwares have recently become commercially available (Vida 1993, Zweig and Campbell 1993, Kairisto and Poola 1995). Actual decision classification criteria for positive and negative cases are usually not displayed in the plot. The prerequisite for the estimation of decision limits is that the disease under consideration have clear-cut definitions, clinical, pathological or laboratory criteria other than the test to be evaluated. In the final assessment of each laboratory test, the "clinical cost" (i.e., the clinical consequences) of positive or negative misdiagnoses should be known before the optimal decision limit can be estimated (Sunderman 1975).
AIMS OF THE STUDY

Routine clinical laboratory methods were developed for the determination of glomerular function.
In detail the aims were:

1) to develop a Reference Method for the evaluation of new routine creatinine methods

2) to develop an application for cystatin C determination and assess its usefulness in clinical practice

3) to develop practical methods for the measurement of urinary albumin
MATERIALS AND METHODS

1. Samples
Plasma, serum and urine specimens obtained for routine monitoring purposes were used in all studies. Only for the determination for reference values of microalbuminuria extra samples were collected from 42 volunteers. Urine samples were refrigerated and measured within two weeks. Plasma and serum samples of the diabetic and control patients were collected as previously described (Wirta and Pasternack 1995) and stored at –20°C or -70°C (V).

2. New methods
Creatinine:
High-pressure liquid chromatography was developed to represent a Reference Method using a Varian 5000 chromatograph (Varian Instruments, Walnut Creek, CA, USA), an MSI 660 autosampler (Kontron AG, Zurich, Switzerland) and a Chromatopac C-1B recorder (Shimadzu Corp., Kyoto, Japan) (I).

An enzymatic test kit (E. Merck, Darmstadt, Germany) was applied to a Hitachi 704 Analyzer (Boehringer Mannheim, Mannheim, Germany) (I).

An enzymatic test kit (Randox Laboratories Ltd, Ardmore, Crumlin, UK) was also applied to a Hitachi 704 Analyzer (Boehringer Mannheim, Mannheim, Germany) (Randox Laboratories Ltd, Ardmore, Crumlin, UK) (II).

Cystatin C:
An application of the particle-enhanced turbidimetric immunoassay (Dako, Glostrup, Denmark) was developed for a Hitachi 704 Analyzer (Boehringer Mannheim, Mannheim, Germany) (III, IV, V).

Urinary Albumin:
A nephelometric method was developed using a Transcon 102 FN nephelometer (Orion Analytica, Espoo, Finland) (VI).
A turbidimetric method was evolved using a Kone Progress analyser (Kone Oy, Instrument Division, Espoo, Finland) (VII).

3. Reference methods

$^{51}$Cr-EDTA clearance was used as a Reference Method for cystatin C (III, V).

Pharmacia albumin RIA (Pharmacia, Uppsala, Sweden) was used as Reference Method for the nephelometric measurement of urinary albumin (VI).

4. Other comparison (routine) methods

Creatinine:
Jaffé reaction with a continuous-flow system (SMA 12/60 Analyzer, Technicon, NY, USA) was used as a comparison for the developed HPLC method (I).

The kinetic alkaline picrate method with a Hitachi 704 or 717 Analyzer (Boehringer Mannheim GmbH, Mannheim, Germany) was used as a comparison for the new enzymatic method (II) and for cystatin C (III, IV).

The enzymatic dry slide method (Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA) was used as a comparison for the new enzymatic method (II).

Cystatin C:
The cystatin C concentrations in plasma or serum were compared with creatinine and creatinine clearance measured by the kinetic alkaline picrate method (II, III) or with the HPLC method (V).

Urinary Albumin:
LC-Partigen®-Albumin immunodiffusion plates (Behringwerke AG, Mahrburg, Germany) were also used as a comparison for the nephelometric method for urinary albumin (VI).
5. Other methods

Bilirubin determinations (II) were made with a Hitachi 704 Analyzer with commercially available reagents based on a diazo reaction (Boehringer Mannheim GmbH, Mannheim, Germany).

Blood glucose concentrations (V) were measured with a Hitachi 704 Analyzer by the dehydrogenase method of Merck (Cat no 13886, Darmstadt, Germany) in blood haemolysate, obtained by diluting capillary blood 1:21 with haemolysis solution.

HbA1c (V) was measured by liquid chromatography (Pharmacia, Uppsala, Sweden) using a Mono S column (Code No. 17-1040-01, Pharmacia Biotech, Uppsala, Sweden).

Urinary protein (V) was measured colorimetrically using a pyrogallol red method (Harmoinen et al. 1987). A 24-hour urinary sample was collected and the excretion rate for total protein calculated.

Absorption spectra of urinary samples and albumin-antialbumin complexes were scanned using a Perkin-Elmer 550 SE UV/VIS Spectrophotometer and a Perin-Elmer R 100 Recorder (The Perkin-Elmer Corporation, Oak Brook, IL, USA) (VII).

6. Statistical methods

The data were evaluated by standard parametric tests using the Microsoft Excel programme (Microsoft Corporation, Incline Village, Nev., USA) or the STATISTICA for Windows programme package (Statsoft Inc., Tulsa OK, USA). Plasma concentrations of cystatin C and creatinine were inversely related to the GFR. This curvilinear relationship was linearised using the reciprocals of the concentrations measured. Comparison of correlation was performed after z-transformation. \( P \) values below 0.05 were considered statistically significant.

Maximum efficiency, sensitivity, specificity and positive and negative predictive values were calculated and comparisons of the area under the ROC curve were made using GraphROC\(^\text{R}\) software (Kairisto and Poola, Turku, Finland).
The dependency of results on age or sex was tested by Kruskal-Wallis analysis of variance or the Mann-Whitney U test. If subgroups differed statistically ($P<0.05$) they were treated separately. After regrouping of similar subpopulations, non-parametric 95% reference intervals were calculated using the GraphROC software (III). Reference intervals for urinary albumin (VI) were calculated after logarithmic transformation (mean ± 2SD).

RESULTS

1. Creatinine (I, II)

Chromatographic method (I). The isocratic chromatographic method developed here was rapid. With a 2 ml/min flow rate of lithium acetate buffer the retention time of creatinine was only one minute. The sensitivity of the method was as good as 1 µmol/l. The method was linear at least up to 2000 µmol/l: $y_{\text{peak height}} = 244x + 1.3$ ($r = 0.999$).

Analytical recovery was determined by adding given amounts of creatinine to serum samples. Recovery was essentially complete, varying from 97% to 102%. The accuracy of the chromatographic method was tested by measuring lyophilised Certified Human Sera (SRM 909b, Level 1 and 2 from NIST). The assigned concentration of creatinine for Level 1 was $56.18 \pm 0.55$ µmol/l (mean ± SD) and for level 2 $467.4 \pm 5.3$ µmol/l. We measured these sera in five different series and obtained the following results: Level 1 mean 55.0 µmol/l (SD 1.41) and Level 2 mean 464.0 µmol/l (SD 6.04) (author’s unpublished results).

The precision of the method was evaluated by repeated analyses of three serum samples with low (mean 31 µmol/l), normal (mean 114 µmol/l) and high (mean 361 µmol/l) creatinine concentrations. Within-assay imprecision varied from 1.7% to 2.9% ($n = 25$) and between-assay imprecision from 2.4% to 3.9% ($n = 20$), respectively.

The specificity of the method was tested by measuring serum samples containing substances known to interfere with Jaffé methods, structurally related compounds of
creatinine, and some common drugs. Although some of these substances were absorbed at the measurement wavelength of 234 nm, only toxic concentrations of phenytoin could interfere with the procedure.

*Enzymatic methods.* The first enzymatic method (Creatinine PAP, E. Merck) tested correlated well with the chromatographic method. Serum: \[ y_{\text{Enz}} = 0.99x_{\text{HPLC}} + 6.7 \ \mu\text{mol/l} \] and urine \[ y_{\text{Enz}} = 0.99x_{\text{HPLC}} + 0.01 \ \text{mmol/l} \] (I). Its precision was also excellent, CV of the within-assay measurement varying from 0.50% to 0.93% and that of between assays from 1.92% to 2.92%, depending on the creatinine concentration (Harmoinen et al. 1991). In spite of the addition of potassium hexacyanoferrate to the reagents, bilirubin concentrations > 80 \( \mu \text{mol/l} \) were seen to interfere with this method.

Bilirubin hampers measurement of hydrogen peroxide. The second enzymatic creatinine method (Randox Enzymatic Creatinine Test; Randox Laboratories, Ardmore, Crumlin, UK) evaluated contained only two enzymes, creatinine iminohydrolase and glutamate dehydrogenase. The method proved insensitive to bilirubin interference (II). Metamizol, which interferes with some enzymatic creatinine methods (Bagnoud and Reymond 1993) had likewise no influence on this approach. The method correlated very well with chromatography (\( r = 1.00; y_{\text{Enz}} = 0.98x_{\text{HPLC}} + 1.4; n = 72 \)), also with the Jaffé (\( r = 0.99; y_{\text{Enz}} = 1.00x_{\text{Jaffé}} - 14.1 \)) and dry slide (\( r = 1.00; y_{\text{Enz}} = 0.93x_{\text{Kodak}} - 4.1 \)) methods, but these techniques gave slightly higher creatinine values than the HPLC or the Randox enzymatic method.

The precision of the iminohydrolase method was excellent, within-assay imprecision varying from 0.79% to 1.54% and that between assays from 1.25 to 1.97%.

2. Cystatin C (III, IV, V)

*Latex-enhanced turbidimetric method.* The application developed for a Hitachi 704 Analyzer was practical and rapid. Cystatin C could be measured together with other common clinical chemical analytes such as creatinine. The total analysis time was only ten minutes and 180 samples could be measured within an hour.
The precision of the application was assessed by measuring two control sera 20 times in a series and between series over a time period of 20 months. At a level of 1.5 mg/l the within-assay coefficient of variation (CV) was 2.6% and that between assays 6.6%. At the level of 5.8 mg/l the CVs within and between assays CVs were 0.9% and 3.2%, respectively.

Plasma or serum concentrations of creatinine and cystatin C were inversely related to the GFR. This curvilinear relationship was linearised by using the reciprocals of the concentrations measured. The reciprocal concentrations of cystatin C correlated well with the GFR measured using the $^{51}$Cr-EDTA clearance in paediatric ($r = 0.89$) (IV) and in type 2 diabetic ($r = 0.77$) patients (V).

Reference intervals (III). Plasma cystatin C concentrations in boys and girls did not differ from each other ($P = 0.926$) and the respective values could therefore be combined. The highest concentrations of cystatin C in plasma were found immediately after birth, with a rapid decrease during the following week (III, Fig. 2). Pre-term infants had significantly higher cystatin C concentrations in plasma than full-term ($P = 0.0145$). After the first week the decrease continued more slowly during the following three years. Above three years of age the cystatin C concentration was independent of age. In contrast, after the first week of life plasma creatinine increased gradually with age until adulthood ($r = 0.82$).

Diagnostic accuracy. ROC analysis showed that in paediatric patients the diagnostic accuracy of cystatin C was significantly superior ($P = 0.037$) to that of creatinine in discriminating between subjects with normal renal function and those with reduced GFR measured by the $^{51}$Cr-EDTA clearance (IV). Also in patients with type 2 diabetes the diagnostic accuracy of cystatin C was superior to that of creatinine ($P = 0.047$) and even better than that of creatinine clearance ($P = 0.001$) (V).

The highest diagnostic efficiency in discriminating patients with reduced GFR from those with normal was reached in adults and children when the upper cut-off limit was about 1.3 mg/l. When the limit was set at 1.31 mg/l the diagnostic efficiency was 98% in paediatric patients (IV) and at 1.32 mg/l likewise 98% in patients with type 2 diabetes (V).
3. Urinary albumin (VI, VII)

*Nephelometry (VI).* The incubation time for the proposed method was only fifteen minutes and the method was clearly faster than the comparison methods, RIA and radial immunodiffusion (RID). The sensitivity of the nephelometric method was about ten times better than that of RID and closely comparable to that of RIA.

The precision of the method was evaluated by repeated analyses of three urine samples with low (2.5 mg/l), high normal (15 mg/l) and high (70 mg/l) albumin concentrations. CVs of within-assay imprecision varied from 1.3% to 1.8% (*n* = 15) and between assays from 2.2% to 4.1% (*n* = 8), respectively.

The nephelometric method developed here was compared with RID by measuring 51 patient samples with normal and elevated albumin concentrations (range about 20 – 1000 mg/l). The correlation was good: \( y_{\text{Neph}} = 0.97x_{\text{RID}} + 2.1 \) (\( r = 0.99 \)). Comparison with RIA gave a correlation: \( y_{\text{Neph}} = 0.99x_{\text{RIA}} + 1.6 \) (\( r = 0.99; n = 40 \)).

Logarithmic transformation was used in calculation of the preliminary reference values. The control group was composed of 42 healthy volunteers from the laboratory staff, aged 25 – 63 years. The excretions of the men and the women did not differ statistically and the respective values could therefore be combined. The calculated reference interval for 24-hours excretion was 2.6 – 16.6 mg.

*Turbidimetry (VII).* The turbidimetric method was even faster than the nephelometric, the incubation time being only two minutes. It was also practical and user-friendly, measurement being possible with common laboratory photometers. Calculated theoretically the sensitivity of the turbidimetric method here was 1.3 mg/l, but in practice it varied from sample to sample so that albumin values less than 5 mg/l could not be measured reliably by this means.

The difficulties caused by high blank values could be reduced by changing the measurement wavelength from the commonly used 340 nm to 450nm, where the blank
values were ten times lower than those at 340 nm. In fact the turbidity caused by albumin-antialbumin complexes was also diminished, albeit only moderately.

The precision of our turbidimetric method was assessed by measuring replicates of three urine samples with normal, slightly elevated and high albumin concentrations. The CVs of within-assay imprecision varied from 2.4% to 8.7% ( n = 15 ) and those of between assay imprecision from 2.9% to 13.0% ( n = 15 ).

The turbidimetric method was compared with the nephelometric by assaying the same 86 urine samples by both methods. The correlation was good: yTurb = 0.96xNeph + 0.6 ( r = 0.99 ).

DISCUSSION

1. Creatinine

In clinical routine, creatinine has in most cases been measured by colorimetric methods based on the Jaffé reaction. This reaction, however, is particularly unspecific and the numerous attempts made to improve its specificity (Table 1.) have had only limited success. Reference system is thus needed to ascertain the accuracy of routine methods and to guide the development of new methods.

The chromatographic method evolved fulfils most of the criteria for a Reference Method. It appeared to be highly specific. More than forty endogenous and exogenous substances were checked for interference and only extremely high concentrations of phenytoin might interfere with this method. During the past decade very few of the hundreds of patient samples measured have contained extra peaks interfering with the creatinine peak.

The sensitivity of the method described suffices to measure also low creatinine levels, while the precision, though not as good as it should be according the NCCLS criteria, is well comparable to that of other HPLC methods (Ambrose et al. 1983, Paroni et al. 1990, Scott 1992 ). The procedure is very simple, no complicated instrumentation is needed and it is thus within the technical reach of a number of laboratories.
The first enzymatic method (Creatinine PAP, E. Merck) tested here correlated well with the chromatographic method (I). Its precision was also excellent. This method used the following enzymes: creatininase, creatinase, sarcosine oxidase and hydrogen peroxidase. Sera contain some substances, which interfere with the measurement of hydrogen peroxide and sarcosine (James and Price 1984). This creatinine test was also negatively biased slightly more than recommended (Ricós et al. 1999) when serum samples were measured, whereas when creatinine was measured in urine no bias could be seen. In spite of the addition of potassium hexacyanoferrate to the reagents, bilirubin was seen to interfere, reducing its value as a routine test.

Bilirubin hampers the measurements of hydrogen peroxide (Witte et al. 1978). The second enzymatic creatinine method evaluated contained only two enzymes, creatinine iminohydrolase and glutamate dehydrogenase, and was therefore insensitive to bilirubin interference (II). Metamizol, which interferes with some enzymatic creatinine methods (Bagnoud and Reymond 1993) had likewise no influence in this case. The method correlated very well with our chromatographic method and was only slightly biased. Common reference values can therefore be used, when creatinine is measured using this enzymatic test or our chromatographic method.

The within-subject coefficient of variation $CV_1$ for plasma or creatinine is 4.3% (Ricós et al. 1999); the analytical imprecision of a routine method should therefore be less than 2.2%. Both enzymatic methods fulfilled this criterion.

2. Cystatin C

Early studies of cystatin C were made by an enzyme-amplified single radial immunodiffusion method (Löfberg and Grubb 1979). The procedure was, however, extremely slow and cumbersome and had a relatively high coefficient of variation, which reduced the usefulness of this approach in routine use. The development of an automated particle-enhanced immunoturbidimetric method (Kyhse-Andersen et al. 1994, Newman et al. 1995), significantly improved the possibility of using plasma cystatin C as a GRF marker in routine clinical work.
(CV) of cystatin C is about 13% (Keevil et al. 1998). The precision of our application for cystatin C measurement varied from 0.9 to 2.6% (within-run CV), which is quite sufficient for a routine test.

There has hitherto been no commonly accepted reference method for cystatin C. The two commercially available tests also have dissimilar reference materials. The immunoturbidimetric method (Dako, Glostrup, Denmark) uses a recombinant human cystatin C as a standard, while the nephelometric method (Dade Behring, Marburg, Germany) has a calibrator purified from human urine. Users of enzyme immunoassays have, however, their own ways of calibrating the method (Pergrande and Jung 1993, Tian et al. 1997, Kos et al. 1999). Assessment of the accuracy of the cystatin C measurement is thus as yet not possible.

Laboratories have sought to overcome this problem by determining their own reference intervals. Laboratories using the same turbidimetric method have obtained very similar reference intervals (Helin et al. 1998, Bökenkamp et al. 1998, Bökenkamp et al. 1999, III) an exception being that children between one and three years of age also have slightly higher cystatin C concentrations in plasma than the older ones (III). If the commercially available nephelometric method is used, the reference intervals are clearly lower (Randers et al. 1999, Finney et al. 2000).

Many studies using unselected patient material have suggested that plasma cystatin C is as good a marker of the GFR as plasma creatinine (Grubb et al. 1985, Randers et al. 1998, Chantrel et al. 2000) or even superior to creatinine (Kyhse-Andersen et al. 1994, Newman et al. 1995, Tian et al. 1997, Coll et al. 2000).

Since the production rate of creatinine is determined mainly by muscular mass, the rate and also the plasma concentration of creatinine varies considerably, especially in children. It was observed here that in children the reciprocal of cystatin C correlated strongly with $^{51}$Cr-EDTA clearance and the diagnostic accuracy of cystatin C was better than that of creatinine (IV). The present results were quite similar to those previously published (Helin et al. 1998), whereas Bökenkamp and colleagues (1998) found no statistically significant difference between these tests.
Available evidence indicates that plasma cystatin C is a better marker for the GFR than plasma creatinine, especially when the rate undergoes an initial small decrease. Diagnostic accuracy with cystatin C is better than that with creatinine in patients with manifest or subclinical GFR impairment due to essential hypertension (Seco et al. 1999), type 2 diabetes (V), nondiabetic nephropathy (Hayashi et al. 1999, Herget-Rosenthal et al. 1999), renal transplantation (Plebani et al. 1998, Risch et al. 1999), hepatic cirrhosis (Woitas et al. 2000) or rheumatoid arthritis (Mangge et al. 2000). In diabetic patients the diagnostic accuracy of cystatin C seemed to be even better than that of creatinine clearance (V).

Recent studies have shown, however, that also non-renal factors such as asthma, HIV and malignant diseases may influence the plasma concentration of cystatin C (Collé et al. 1992 Kos et al. 1997, 1998, Cimerman et al. 2000). Therefore, when an elevated cystatin C concentration is found these factors should also be borne in mind and appropriate studies undertaken to verify the cause of the elevated cystatin C level.

In situations, where the GFR has been accurately measured by an invasive clearance method, creatinine is preferable to use in following changes, because its biological and analytical variation are smaller and its determination is cheaper than that of cystatin C, even when measured with a specific enzymatic test.

It should be noted that some kidney diseases might affect differently the filtration of cystatin C, a positively charged 13 kDa protein, and that of creatinine, an uncharged 113 Da molecule. Indeed, although plasma cystatin C is considered to be a better marker than creatinine in patients with renal transplants (Risch et al. 1999, Le Bricon et al. 1999), transplanted and untransplanted patients with the same inulin clearance have been reported to display different levels of cystatin C (Bökenkamp et al. 1999). Combined use of cystatin C and creatinine would thus probably yield optimal, when more accurate, invasive methods cannot be used.

3. Urinary albumin
Today screening for microalbuminuria plays an essential role in the follow-up of diabetic patients, and accordingly concerns a large number of cases. For this reason
simple routine methods are needed for its measurement. To be reliable, such methods for microalbuminuria should be sufficiently sensitive, about 2-10 mg/l of albumin depending on use (Hutchinson and Paterson 1988). The excretion of urinary albumin varies markedly (Mogensen 1987, Cohen et al. 1987, Chachati et al. 1987, Howey et al. 1987, 1989) and thus the precision criterion for a viable method is not so critical (Ricós et al. 1999). The sensitivity of the nephelometric method developed here (VI) proved adequate to measure all physiological albumin concentrations in urine and was comparable to that of the reference method (Gatling et al. 1988, Gianpietro et al. 1992). Its precision was at least as good as that of the RIA (Gianpietro et al. 1992) and it correlated well both with the immunodiffusion and with the radioimmunossay methods. The method was thus suitable for all mode of albumin measurements in urine.

Immunoturbidimetry has been suggested for large-scale screening for microalbuminuria (Teppo 1982, Watts et al. 1986, Sathianathan et al. 1986, Pejacovic et al. 1987, Bakker 1988, VII). Although a close correlation has been repeatedly found between values measured by immunoturbidimetry and those by RIA (Watts et al. 1986, Sathianathan et al. 1986, Pejacovic et al. 1987), discrepancy has been found at low albumin concentrations. Urine contains an abundance of substances which absorb UV light, and specificity can be improved by changing the measurement wavelength (VII). Although the detection limit was found here to be 1.3 mg/l, in practice it might be at least five times higher since the amount of interfering substances in urine varies greatly. More sensitive methods are therefore preferable when low normal albumin concentrations are measured in experimental protocols and physiology studies.
SUMMARY

When a new routine method or assay measurement procedure has to be created, it should be compared to a Reference Method. If this is impossible for any reason, method-specific reference intervals are needed. Quality specifications are best based on the biological variation of the analyte. The diagnostic accuracy of a new analytical procedure has to be compared with that of its predecessor(s), before being accepted for routine use.

These principles were applied in the development of new methods for the assessment of the glomerular filtration rate (GFR), the most important parameter of renal function. The plasma or serum concentration of creatinine measured by the Jaffé picrate method is widely used as an indirect estimate of the GFR. This procedure is, however, unspecific and especially in children may yield erroneous results. A simple, isocratic HPLC method was developed here and two creatinine measurement procedures based on different indicator enzymes were evaluated using the HPLC method as a reference. Both appeared to be more specific than the Jaffé method, but high bilirubin concentrations still interfered with enzymatic creatinine measurement if hydrogen peroxidase was used as the indicator enzyme involved.

Recent studies have shown that plasma or serum cystatin C is a better marker for the GFR than serum creatinine, particularly for the detection of an initial decrease in rate. In patients with type 2 diabetes, the cystatin C concentration in plasma was better than plasma creatinine and even better than creatinine clearance in discriminating subjects with normal renal function from those with a reduced GFR. Also in paediatric patients the diagnostic accuracy of cystatin C was superior to that of creatinine. Cystatin C measurement also helps clinicians to assess the GFR in pre-pubertal children, because the reference interval of cystatin C is independent of age after the first three years, in contrast to the creatinine concentration, which is dependent on both the age and the size of subjects.

During the last two decades microalbuminuria has found its place as an indicator of “incipient” nephropathy of diabetes, but also as a generalised marker of vascular
damage. The nephelometric assay procedure for urinary albumin developed here correlated well with the reference method, the radioimmunoassay. Its sensitivity was good enough to detect also physiological albumin concentrations in urine. Turbidimetry is a viable means of detecting and quantitating elevated albumin concentrations in urine, as in screening diabetic patients for microalbuminuria. Change of the measurement wavelength from 340nm to 450nm improved the specificity of the turbidimetric method. If accurate results are needed also at normal levels of albumin, a good nephelometric method or labelled immunoassay is needed.
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