

Evaluation of urinary metanephrine and normetanephrine enzyme immunoassay (ELISA) kits by comparison with isotope dilution mass spectrometry

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Determination of urinary 3-O-methylated catecholamines (metanephrines) is generally considered a principal test for the clinical chemical diagnosis of pheochromocytoma and is currently performed predominantly with chromatographic techniques such as gas-liquid chromatography and HPLC. Enzyme immunoassays based on microtiter plate technology have recently been developed for the quantitative determination of urinary metanephrine (M) and normetanephrine (NM). We compared the results for urinary M and NM determined by these ELISA methods with those obtained by a recently developed isotope dilution mass spectrometric method. From this comparative study we can conclude that the investigated ELISA methods are applicable in the quantification of urinary M and thus can be successfully used to establish the diagnosis of pheochromocytoma. These relatively simple methods can be executed in any clinical laboratory and in time may replace the present, more complicated, chromatographic techniques.

INDEXING TERMS: gas chromatography • mass spectrometry • pheochromocytoma

Several laboratory tests have been proposed for the detection and follow-up of patients with catecholamine-producing, mostly benign or occasionally malignant tumors called pheochromocytomas: determinations of the catecholamines themselves or one or more of their various metabolites in either plasma or urine. Recently Lenders et

al. [1] reported that measurements in plasma of the 3-O-methylated metabolites normetanephrine (NM) and metanephrine (M) are more sensitive for that purpose than are the usually performed determinations of either plasma catecholamines or urinary total metanephrines.³ In their comparison, however, they used HPLC with electrochemical detection for the measurement of plasma catecholamines and metanephrines, whereas an insensitive colorimetric method [2] was used for urinary total metanephrines. In studies in which more specific and sensitive methods were used for measurements of urinary metanephrines, significantly higher sensitivities were reported [3]. Thus, determination of plasma metanephrines probably does not produce better results in terms of sensitivity and specificity in the diagnosis of pheochromocytoma than urinary metanephrines.

Pheochromocytomas usually produce free catecholamines, which very likely before entering the circulation are already converted into metanephrines. Eisenhofer et al. [4] showed that after intravenous infusion of ³H-labeled catecholamine precursors, steady-state plasma concentrations of ³H-labeled free metanephrines were <6% of steady-state concentrations of the precursor amines, whereas in pheochromocytoma patients plasma concentrations of free metanephrines were ~50% of their precursor amines [1]. Moreover, Eisenhofer et al. [5] demonstrated that >90% of circulating free M is formed by metabolism of adrenaline within the adrenals and thus <10% is formed by metabolism of adrenaline after its release into the circulation. Also, formation of metanephrines within pheochromocytoma tissue is supported by

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³ Nonstandard abbreviations: NM, normetanephrine; M, metanephrine; GC, gas chromatography; MS, mass spectrometry; and VMA, vanillylmandelic acid.

findings of high concentrations of metanephrines within tumor tissue [6, 7] and a high concentration of free NM in the plasma draining a tumor [8]. Deactivation of circulating 3-O-methylated catecholamines followed by sulfoconjugation and excretion suggests high specificity and sensitivity for urinary total metanephrines, compared with oxidatively deaminated catecholamine metabolites. In addition, unlike urinary total catecholamines, urinary total M concentrations are not significantly influenced by diet [9].

For the reasons stated above, one of the most sensitive and specific methods for the clinical chemical diagnosis and follow-up of patients with pheochromocytoma remains the assay of urinary metanephrines, provided that their analysis is carried out by modern chromatographic techniques [3, 10, 11]. As urinary M and NM are in majority present as sulfoconjugates [12], a deconjugation (hydrolysis) step to determine each of them as the sum of their free and conjugated fractions generally precedes their analysis. The determination of urinary M and NM separately has been reported to be useful to distinguish between tumors located in the adrenal medulla (secreting mainly adrenaline, to be converted into M) and extraadrenal tumors (secreting mainly noradrenaline, to be converted into NM) [13]. Moreover, measuring total metanephrines (sum of M and NM) is less sensitive than measuring M and NM separately [14].

Although chromatographic techniques such as HPLC, gas chromatography (GC), and GC-mass spectrometry (MS) allow the measurement of urinary metanephrines with good precision and accuracy [3], the necessary know-how and the time-consuming analytical procedures (and the restricted availability of some types of equipment, e.g., GC-MS) impede an intensive use of these methods, resulting in relatively few laboratories actually carrying out these assays. In recent years more laboratories introduced M assays, since commercial HPLC equipment along with reagents in kit form as "turn-key instrumentation" became available. Nevertheless, in past years, development of immunoassays for these analytes was also attempted. Thus, papers describing RIA techniques for urinary M and NM were published some years ago [15, 16]. Again, the use of radioactive substances consti-

tutes a disadvantage, not offering an attractive substitute for existing chromatographic methods. Recently, however, enzyme immunoassay kits, based on microtiter plate technology, for the quantitative determination of urinary M and NM have become commercially available. These kits for the first time offer an opportunity to replace chromatographic techniques for methods accessible to common routine clinical laboratories. The principles on which these kits are based will be described here.

We have evaluated these M and NM kits by assaying several urine samples obtained from healthy persons and patients with and without pheochromocytoma by applying the ELISA methods as well as a GC-MS method previously developed in our laboratory, which was recently published [17].

Materials and Methods

CHEMICALS

Details on chemicals used in the GC-MS determinations of NM and M are mentioned in ref. 12. All chemicals used for the ELISAs are provided by the manufacturer.

ELISA KITS FOR DETERMINATION OF METANEPHRINE AND NORMETANEPHRINE

These new kits are available either as RIA or ELISA versions. Although according to the supplier the performance of the RIA and ELISA kits are comparable, we prefer the ELISA version, as no handling of radioactive substances is required. Thus only the ELISA kits were evaluated in this study. The kits are based on the following principle. Since the metanephrines as such do not possess sufficient immunogenic properties, making it difficult to raise specific antibodies against them, use is being made of their N-acetylated derivatives. By reaction with Bolton-Hunter reagent [3-(*p*-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester], both metanephrines are readily converted to their N-acylhemisuccinates, with excellent immunogenic properties (see Fig. 1). Specific antibodies against these derivatives can be produced in rabbits by coupling the latter to bovine serum albumin as hapten, whereas as tracers (in the ELISA version) these hemisuccinates coupled to biotin are used. In short, the procedure is as follows. Urinary M (or NM) is first

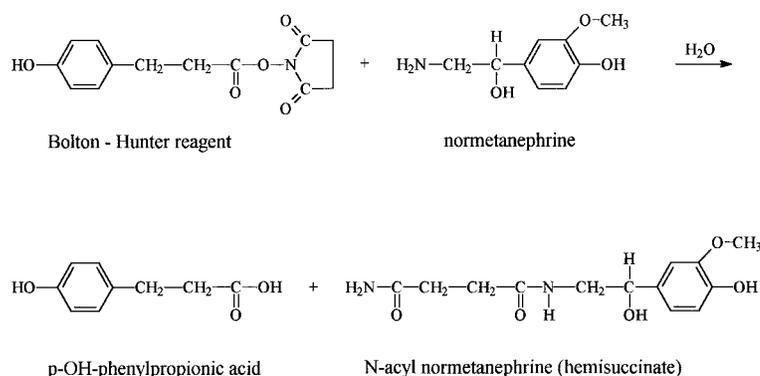


Fig. 1. Reaction between the Bolton-Hunter reagent and normetanephrine.

converted to its N-acyl derivative by means of the Bolton–Hunter reagent and then the product transferred to microtiter plates coated with goat anti-rabbit IgG. After addition of a fixed amount of the appropriate tracer and (rabbit) antibody, subsequent incubation overnight, and washing, anti-biotin–alkaline phosphatase conjugate is added. After incubation for some time, plates are washed, followed by addition of alkaline phosphatase substrate (*p*-nitrophenylphosphate) and measurement of the released *p*-nitrophenol spectrophotometrically after a fixed incubation time. Maximal enzyme activity is observed when no acylated M (or NM) is present in urine, because in that case all tracer molecules (acylated M or NM coupled to biotin) are bound to the walls of the microtiter plate, which consists of a coating of M-antibody (the goat anti-rabbit IgG:M rabbit-antibody complex) after the first incubation step. On the other hand acylated M (or NM) molecules, if present in urine, compete with tracer molecules in binding to the antibody, resulting in less binding of biotin-containing tracer to the wall and consequently less binding of anti-biotin–alkaline phosphatase conjugate after the final incubation, leading to reduced enzyme activity. Thus, lowest activity is reached at highest urinary M concentration.

The test kits were supplied by Immuno Biological Laboratories (IBL), Hamburg, Germany [cat. no. RE 591 71 (AMICYL-Test[®] normetanephrine-ELISA) and cat. no. RE 591 81 (AMICYL-Test[®] metanephrine-ELISA)]. They contain all necessary materials and reagents (in total 16 components, among which is one microtiter plate) to carry out 96 assays of either M or NM, including calibration samples. The contents of the kits can be divided in such a way that three separate runs can be performed, each comprising 32 determinations, equivalent to 4 microtiter strips of 8 wells each. Both kits are practically similar in composition and differ only with respect to the tracer (M-biotin or NM-biotin), antiserum (specific for M or NM), and calibrator solutions (M or NM). Included in all kits is a detailed prescription of the analytical procedure to be followed. The kits should be stored in the dark at 2–8 °C. To carry out tests, only appropriate pipettes, a vortex-type mixer, a heating block (set at 90 °C), a temperature-controlled water bath (37 °C), a microtiter washer, and a microtiter plate reader, able to measure at 405 nm, are further required.

ANALYTICAL PROCEDURE FOLLOWED FOR ELISA KITS

For a detailed description of the analytical procedure (both for M and NM) we refer to the booklets (edited in both English and German) supplied by the manufacturer and included in each test kit. Here we present a shortened version describing the different analytical steps in less detail.

Hydrolysis and derivatization. Since urinary metanephrines are predominantly present as sulfated conjugates, a hydrolysis step is necessary, unless one wants to determine

free (unconjugated) metanephrines. In reagent tubes, 200 μ L of 0.1 mol/L HCl solution was added to 50 μ L of patient urines, 7 calibrators (present in the kits), and 2 control samples, and all mixtures were heated for 1 h at 90 °C. After cooling, the acylation reaction (with Bolton–Hunter reagent) was carried out for 30 min at 37 °C.

Incubation of acylated samples with tracer and antibody. Twenty microliters of the acylated samples was pipetted in duplicate into the appropriate wells, followed by addition of 50 μ L of M (or NM)-biotin tracer solution and 50 μ L of antiserum solution (specific for M or NM), and allowed to stand at 2–8 °C overnight (at least for 14 h).

Incubation with enzyme conjugate. After three washing cycles, 150 μ L of enzyme conjugate solution (alkaline phosphatase–anti-biotin conjugate) was pipetted into the wells and the plate was shaken for 2 h at room temperature.

Enzyme reaction. After three washing cycles 200 μ L of substrate solution (*p*-nitrophenylphosphate) was pipetted into each well, followed by incubation for 20 min at room temperature while shaking. The reaction was stopped by addition of 50 μ L of a 1 mol/L NaOH + 0.25 mol/L EDTA solution. Within 60 min extinction coefficients were measured in the wells by means of a microtiter plate reader at a wavelength of 405 nm.

Calculation of results. Calculations were performed with the computer program “Easy Fit” distributed by SLT/Tecan Labinstruments, Grödig, Austria, which converts the absorbance readings acquired by the microtiter plate reader into concentrations (μ mol/L) of M or NM by taking into account absorbance values of seven calibrators (ranging from 0 to 12.7 μ mol/L for M and from 0 to 40.9 μ mol/L for NM). Both for the calibrators and for the patient samples the mean of the obtained duplicate absorbance readings was taken and absorbance as percentage of the absorbance value of the zero calibrator was calculated. To obtain a calibration curve, the absorbance percentages (*y*-values) of the calibrators were plotted against the logarithms of the concentrations of the calibrators (*x*-axis, see Fig. 2). From these calibration curves, concentrations, and the calculated absorbance percentages of the patient samples, M or NM concentrations in these samples were derived.

The microtiter equipment (washer and reader) used in the present investigation was from Dynatech Labs., Chantilly, VA. The reader (type MR 400) was linked to an IBM-type AT compatible computer.

CROSS-REACTIVITY MEASUREMENTS IN ELISA KITS

For these measurements, calibrator solutions in water of M or NM (depending on the investigated ELISA kit) were used, to which a known amount of calibrator, containing the potentially cross-reactive substance to be investigated,

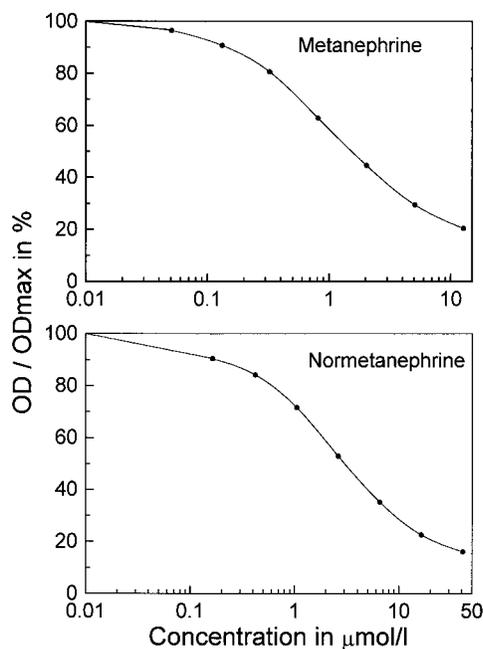


Fig. 2. Typical calibration curves observed for M (top) and NM (bottom).

The *abscissa* represents (N)M concentrations of the calibrators, the ordinate the percentage of measured absorbance (OD) of the calibrators, in comparison with the absorbance of the zero calibrator.

was added. The following procedure was used. A 10 000-fold concentration excess of this substance, in comparison with calibration M or NM concentrations needed for a 50% reduction in absorbance, was added in the application of the test procedure instead of urine to see whether or not it could achieve a >50% reduction in absorbance. If so, a 3000-fold concentration excess was added and again checked, to determine if a >50% absorbance reduction could be observed. Likewise, if necessary, a 1000-, 300-, or 100-fold concentration was tried. In this way cross-reactivity could be estimated: An observed 50% absorbance reduction occurring at an added 100-fold concentration excess is equivalent to a cross-reactivity of 1%. No drugs commonly used in treating hypertensive or other patients suspected of having a pheochromocytoma were investigated, because of the observed low cross-reactivity percentages of the tried endogenous compounds with high structural resemblance to metanephrines. Therefore significant cross-reactivity of such substances is highly improbable.

GC-MS MEASUREMENTS

For the assessment of urinary metanephrines with isotope dilution MS our previously described procedure [17] was used. Essentially the procedure was as follows: To a 300- μ L urine sample, deuterated analogs of 3-methoxytyramine, M, and NM were added as internal standards. After evaporation to dryness in a stream of nitrogen, 300 μ L of a derivatization mixture consisting of acetonitrile, dimethylformamide, and pentafluoropropionic anhydride was added.

Mixtures were derivatized at 80 °C for 15 min. After cooling, samples were extracted and washed with a mixture of heptane and water. Heptane layers were evaporated to dryness and redissolved in 50 μ L of ethyl acetate:pentafluoropropionic anhydride (250:1, by vol). Volumes of 1–5 μ L were injected into a gas chromatograph–mass spectrometer combination. Samples were monitored in the ammonia–chemical ionization mode. Quantification was done by use of calibration curves.

SAMPLE COLLECTION AND PRESERVATION

Urine samples were collected from 47 apparently healthy persons (ages 3–27 years, median 8.8) and 10 patients previously diagnosed as having pheochromocytoma [on the basis of high urinary vanillylmandelic acid (VMA) excretion]. Some patient urine samples were collected after surgical treatment. Samples were collected in 2-L brown polypropylene bottles (Sarsted, Nuembrecht, Germany) containing ~250 mg each of Na₂S₂O₅ and EDTA as preservatives. Samples were acidified to pH 4 with acetic acid before freezing at –20 °C. Urinary creatinine concentrations used to quantify excretion in terms of creatinine were measured by a picric acid method on a SMA-2 analyzer (Bayer, Tarrytown, NY). For clinical purposes, concentration values (originally obtained in nmol/L or μ mol/L) were either expressed in nmol (or μ mol) excreted in 24 h (when 24-h urine portions were collected) or in μ mol/mol creatinine (when no 24-h portions were collected).

STATISTICS

Results of comparisons between measurements by ELISA and GC-MS were obtained by method comparison statistics according to Passing and Bablok [18] [correlation coefficients calculated on the basis of usual least-squares (Pearson) linear regression analysis].

Results

PERFORMANCE OF THE TWO ELISA TEST KITS

Precision. Intraassay and interassay variation of the two test kits was investigated by assaying a number of urine samples from controls 10 times within a series of measurements and in 10 different runs. For M the CVs within a series of measurements (for samples with mean values of 0.424 and 0.303 μ mol/L) varied from 6.42% to 9.47% and the CVs regarding interassay precision (for samples with mean values of 0.366 and 1.541 μ mol/L) from 10.1% to 14.3%. For NM these values were 6.76% to 9.22% (mean values 1.479 and 4.958 μ mol/L) and 9.19% to 12.5% (mean values 1.087 and 4.728 μ mol/L) respectively.

Linearity. Three different pathological urine samples were assayed undiluted and after twofold, fourfold, eightfold, 16-fold, and 32-fold dilution. The results, both for M and NM in μ mol/L, are shown in Table 1, indicating a satisfactory linearity of both assays.

Table 1. Linearity of ELISAs for M and NM.

	Dilution						
	1/1	1/2	1/4	1/8	1/16	1/32	1/64
<i>M</i>							
Urine 1	9.36	9.41	10.3	11.0	10.8		
Urine 2	11.8	12.1	13.3	12.5	11.9	12.0	12.0
Urine 3	12.5	11.4	12.8	11.4	12.1	13.3	
<i>NM</i>							
Urine 1	10.0	8.05	9.19	10.4	10.4	11.0	
Urine 2	11.2	11.8	11.7	11.1	12.3		
Urine 3	11.4	12.0	10.4	11.4	11.9	10.8	

Obtained values in $\mu\text{mol/L}$ (multiplied with the dilution factor).

Recovery measurements. Four different urine samples in the case of NM and three in the case of M (all samples from control persons) were determined as such and after supplementing with various amounts of NM and M respectively. From the obtained concentrations, recovery percentages were calculated. For NM, recovery ranged from 91% to 117% and for M from 74% to 120%.

Cross-reactivity. Several compounds with chemical structures related to M and NM were investigated as to their possible interference in the two test kits. As seen in Table 2, both test kits showed minimal cross-reactivity with other substances.

COMPARISON OF ELISA WITH GC-MS

To check the accuracy of the ELISA test kits by comparison with GC-MS, two series of urine samples, one comprising samples of a group of 47 apparently healthy control children or young adults, and another comprising samples of a group of 10 adult patients from whom urine

was collected because of previously established pheochromocytoma, were investigated. Results obtained by applying the ELISA and GC-MS procedure were compared and graphically displayed in Fig. 3, in which the two upper panels comprise only the results for the controls and the two lower panels the combined results of controls and patients. The obtained parameters from the statistical analyses are given in Table 3. In the control group ($n = 47$) the M concentrations obtained from the ELISA method showed a tendency to be slightly higher than those obtained from GC-MS, whereas the reverse held for the NM results; correlation coefficients were 0.911 and 0.928, respectively. The combined results of patients and controls ($n = 56$) showed correlation coefficients of 0.993 for M and of 0.988 for NM; the slopes of the linear regression curves were 0.984 and 0.988, respectively. From Fig. 3 and Table 3 one can conclude that an acceptable correlation between both methods exists. Passing–Bablok regression analysis, applied to the results obtained from the control group and also the combined control/patient group, further revealed that in the regression lines no significant deviation from zero for intercept and from 1.00 for slope could be observed, both for M and NM (data not shown).

Table 2. Cross-reactivity experienced in the M and NM ELISA kits for substances with related structures.

Compound	Cross-reactivity, %	
	M	NM
Metanephrine	100	0.6
Normetanephrine	0.3	100
Adrenaline	<0.5	<0.02
Noradrenaline	<0.01	<0.02
3-Methoxytyramine	<0.01	0.4
Dopamine	<0.01	<0.02
L-DOPA	<0.01	<0.02
4-hydroxy-3-methoxyphenylglycol	0.01	0.15
VMA	<0.01	<0.02
Homovanillic acid	<0.01	<0.05
4-hydroxy-3-methoxyphenylpyruvic acid	<0.01	<0.05
4-hydroxy-3-methoxylactic acid	<0.01	<0.02
3-methoxytyrosine	<0.01	<0.02
L-Tyrosine	<0.01	<0.02
Tryptophan	<0.01	<0.02
Caffeic acid	<0.01	<0.02
Ferulic acid	<0.01	<0.01

Discussion

In surveying the data obtained for the two described ELISA kits for urinary M and NM, the following conclusions can be drawn. Cross-reactivity by potentially interfering compounds in urine, such as unmetabolized catecholamines (adrenaline, noradrenaline, and dopamine) and acid catecholamine metabolites such as homovanillic acid and VMA, very likely is minimal to absent, taking into account the data shown in Table 2. Also, no significant interference between each of the two metanephrines (small cross-reactivity of NM in the M test kit and vice versa) has been observed. Reproducibility data, linearity data (Table 1), and recovery data taken together indicate that the precision of an obtained test result for both urinary metanephrines is in the order of 10–20%, which, although less than experienced for chromatographic techniques, is in principle sufficient to discriminate between normal and pathological states. ELISA data demonstrate a

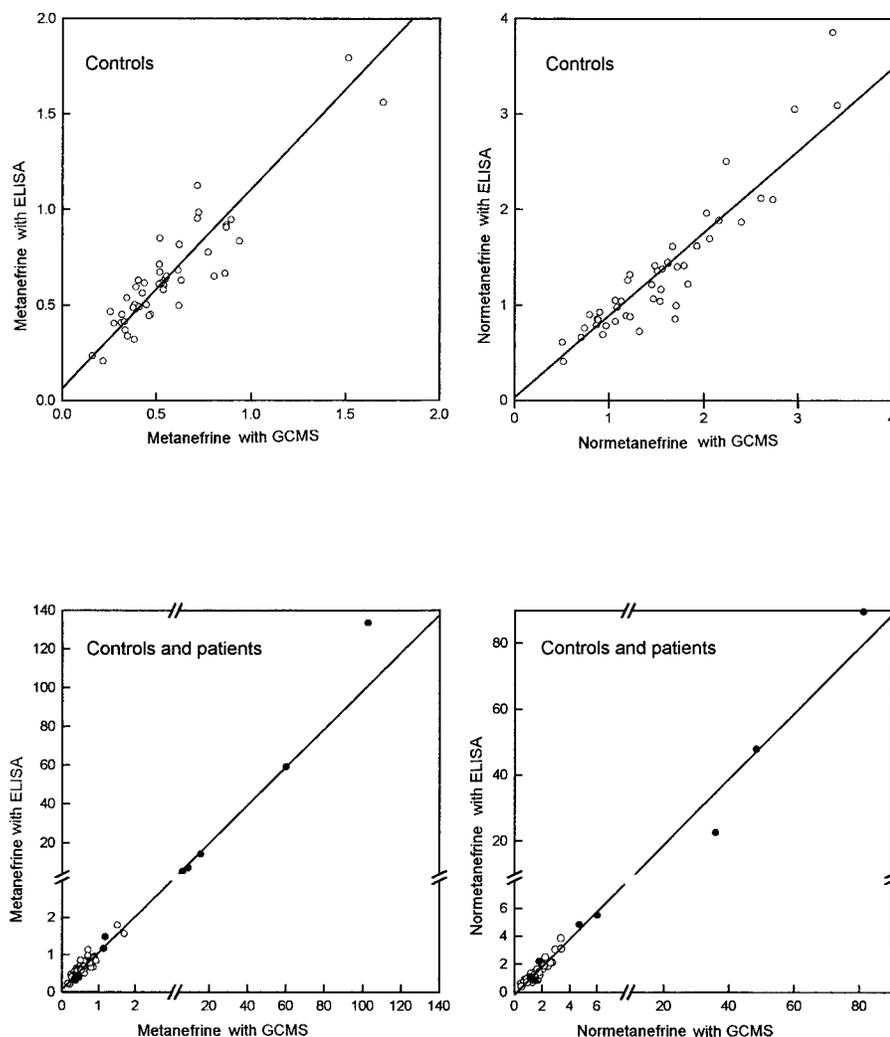


Fig. 3. Comparisons between the ELISA methods and GC-MS method for urinary M and N, both for patients and healthy controls.

Concentrations of M and NM are plotted in $\mu\text{mol/L}$. The upper panels represent the results for the controls ($n = 47$), the lower panels those of controls + patients ($n = 56$) (one of the ELISA results for M in the patient group and for NM in the control group, because of experimental circumstances, could not be obtained and had to be skipped, together with the matching GC-MS results, resulting in one data point less in both the NM as well as the M data in the combined group); on the x-axes GC-MS data are plotted, on the y-axes ELISA data. ●, patient data; ○, control data.

good accordance with GC-MS data, for concentrations both in the normal and pathological ranges.

To establish whether or not a determined concentration of urinary M and (or) NM indicates the presence of pheochromocytoma, the amount of metanephrines present in a 24-h urine collection or expressed as $\mu\text{mol/mol}$ creatinine (in that case no 24-h collection is necessary) must be calculated and compared with the respective reference values. As to which M and NM concentrations (in $\mu\text{mol/24 h}$ or in $\mu\text{mol/mol}$ creatinine) should be consid-

ered abnormal and thus may be indicative of pheochromocytoma, no general agreement exists, as they depend more or less on the analytical methods used and the choice of the reference group. In Table 4 some upper limits of reference values for M and NM expressed in $\mu\text{mol/24 h}$ are given, derived from the cited review papers, the GC-MS method used by us, and the values reported for the present ELISA kits.

Taking into account that the GC-MS and the ELISA method were concordant in identifying the pathological

Table 3. Calculated (regression) parameters with Passing-Bablok statistics in the method comparison for urinary NM and M (in $\mu\text{mol/L}$) as determined by ELISA (y-values) and GC-MS (x-values) in the two series of measurements.

Analyte	Series	n ^a	Slope	Intercept	r	S _{y/x}
NM	controls	46	0.86	0.038	0.928	0.27
M	controls	47	1.04	0.065	0.911	0.13
NM	patients	10	1.00	-0.043	0.984	5.60
M	patients	9	0.98	0.011	0.992	12.12
NM	patients + controls	56	0.98	-0.152	0.988	2.17
M	patients + controls	56	0.98	0.083	0.993	4.37

^a In the control group one ELISA result for NM and in the patient group one ELISA result for M could not be obtained because of experimental circumstances.

Table 4. Reported reference values (upper limits) in $\mu\text{mol}/24\text{ h}$ for M and NM.

Source	M	NM
Graham et al. [10] (HPLC)	2.0	5.1
Gerlo and Sevens [14] (HPLC) males	1.8	4.4
females	1.2	3.3
Rosano et al. [3] (GC)	1.31	1.86
(HPLC)	1.51	1.94
Kema et al. [17] (GC-MS)	1.3	3.4
Present ELISA methods	1.4	1.75

urinary samples (see Fig. 3, lower panels), we are justified in stating that the ELISA test kits can be successfully applied in the detection of pheochromocytoma patients. The analytical procedures are relatively simple and require only low-cost instrumentation (microtiter plate washer and reader) generally available in the clinical laboratory. Therefore the present ELISA methods for determining urinary metanephrines can be considered acceptable alternatives for chromatographic methods, allowing any clinical laboratory to extend with ease their arsenal of laboratory tests with a procedure for detection of pheochromocytoma.

For those laboratories that would like to determine plasma metanephrines or urinary *free* (unconjugated) metanephrines, the described test kits do not possess the required sensitivity, since concentrations $<1\text{ nmol/L}$ must be measured with sufficient accuracy, which is below the detection limits of these kits. As argued above, there is no valid reason to prefer such determinations over those of urinary *total* metanephrines and, therefore, clinically there is no need for the availability of such sensitive M or NM test kits.

One may speculate that in the near future, apart from catecholamines, other diagnostically important biogenic amines, e.g., serotonin, histamine, and their metabolites in body fluids, will be determined likewise by simple immunoassay, becoming the method of choice for the analysis of these molecules. However, one should realize that, although easier to perform, with immunoassays each analyte (e.g., M and NM) must be determined with separate kits, which might lead to higher costs and in some cases might consume more analytical time in comparison with chromatographic methods, in which several analytes can be quantified together within one assay run (e.g., M and NM).

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