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ORIGINAL ARTICLE

Simple, Rapid and Sensitive Determination of Epinephrine and Norepinephrine in Urine and Plasma by Non-competitive Enzyme Immunoassay, compared with HPLC Method

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SUMMARY

Background: **Measurement of the concentration of the free catecholamines epinephrine (adrenalin) and norepinephrine (noradrenalin) in humans is used for the diagnosis of pheochromocytoma and related diseases.**
Methods: **A non-competitive enzyme-linked immunosorbent assay (CatCombi ELISA) kit for the measurement of epinephrine and norepinephrine concentrations in plasma and urine was developed and validated. The assay procedure consists of sample extraction, chemical and enzymatic derivatization and immunological reaction steps. A sample volume of 10 µL urine or 300 µL EDTA plasma is required for duplicate determinations of both catecholamines. For method comparison we used a reversed phase HPLC by Chromsystems after extraction by aluminium oxide with electrochemical detection for the determination of epinephrine and norepinephrine.**
Results: **The CatCombi ELISA is accurate, sensitive, specific, and precise. Linear regression analysis of epinephrine and norepinephrine concentrations measured with the ELISA and with HPLC yielded highly significant correlations.**
Conclusions: **The CatCombi ELISA kit as an alternative to HPLC methods is very useful for clinical applications as well as for basic research where a simple, rapid, accurate and reproducible assay for epinephrine and norepinephrine determinations is required. (Clin. Lab. 202;48:61-71)**

KEY WORDS

enzyme-linked immunosorbent assay, catecholamines, epinephrine, adrenalin, norepinephrine, noradrenalin, pheochromocytoma, method comparison.

Abbreviations

BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
HPLC	high performance liquid chromatography
Thimerosal	sodium ethylmercurithiosalicylate
Tween	polyoxyethylenesorbitan
VMA	vanillic mandelic acid

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INTRODUCTION

The catecholamines epinephrine (adrenalin), norepinephrine (noradrenalin) and dopamine are synthesized in the adrenal medulla, the sympathetic nervous system and the brain. As they influence virtually all tissues and are involved with other hormonal and neuronal systems in the regulation of a wide variety of physiological processes, they are subjects of great scientific interest.
The concentrations of catecholamines and their metabolites are elevated in urine and plasma in several diseases and are of clinical interest, especially in the diagnosis of pheochromocytoma, neuroblastoma and ganglioneuroma [1].
Pheochromocytoma is a life-threatening tumor of the sympathetic nervous system and occurs mostly in the adrenal medulla. Correct diagnosis of the condition can result in the successful removal of the tumor in 90 percent of cases. However, if it is not correctly diagnosed, it is invariably fatal. Even today misdiagnosis is fre-

quent, so that approximately one in three pheochromocytomas is only identified post mortem [2-4].
Pheochromocytoma can produce an excess of adrenalin, noradrenalin, dopamine, metanephrines and vanillylmandelic acid (VMA). The detection of these substances forms the basis of pheochromocytoma diagnosis [5-6]. Currently there is no consensus as to which test is the most suitable for the biochemical identification of pheochromocytoma or what type of sample should be used.
In recent retrospective multicenter studies the usefulness of the different analytes has been investigated. Amongst laboratory assays the measurement of urinary VMA was the most frequently used screening method. However, this was also the method with the lowest diagnostic sensitivity [7-9] and should be considered as obsolete, to be replaced by more specific tests.
Several different analytical techniques, such as spectrophotometry and fluorometry, have been used in the past for the determination of catecholamines. Towards the end of the 1970s the introduction of HPLC with electrochemical detection for the measurement of urinary catecholamines was considered to be a major advance [10]. Several modifications to improve the sensitivity of the method in order to obtain reliable results for plasma catecholamines have been subsequently described [11]. However, all these methods are very time consuming and cumbersome so that their use is limited to highly specialized laboratories. Furthermore, the HPLC methods apply completely different extraction procedures for urinary and for plasma catecholamines prior to column separation.
The HPLC methods used differ between laboratories and some methods are prone to interference, often by antihypertensive agents (e.g. angiotensin-converting enzyme inhibitors, beta-blockers, calcium channel blockers and diuretics), which patients with suspected pheochromocytoma often receive [12-13].
The measurement of urinary catecholamines may not be appropriate for patients with advanced renal disease. In such cases plasma catecholamines ought to be measured, as these results are helpful in diagnosing pheochromocytoma in hemodialyzed patients [14]. The clinical value of plasma catecholamine measurement was also confirmed during hypertensive paroxysm [8,15] and the clonidine suppression test [16].
In this paper we present a commercially available, non-competitive enzyme immunoassay, which is suitable for both routine and research determinations of epinephrine and norepinephrine in urine and plasma samples (Cat-Combi ELISA). Sample preparation includes a common extraction procedure of urine or plasma so that they may be analysed simultaneously using the same standard curve. Results are available within six hours. The ELISA is based on the detection of metanephrine and normetanephrine and requires 300 µL plasma.

MATERIALS AND METHODS

Chemicals

All fine chemicals and biochemicals were purchased from Sigma (Munich, Germany) or Merck (Darmstadt, Germany), unless otherwise stated. All reagents used for the ELISA kit are provided by the manufacturer (IBL GmbH; Hamburg, Germany). The anti-N-acylmetanephrine and anti-N-acylnormetanephrine antisera used for the kit were produced by immunizing rabbits with bovine serum albumin conjugates using a conventional immunization technique, as previously described [17]. Catechol-o-methyltransferase (COMT) (E.C. No. 2.1.1.6.) was prepared from pig liver, as reported by Tilgmann and Kalkkinen [27].
N-succinimidyl-N-[6-(biotinoylamino)hexanoyl]-6-aminohexanoic acid (Biotin-XX-NHS) was synthesized by the manufacturer *(IBL GmbH; Hamburg, Germany).*

Samples

Urine specimens (24h) were collected and acidified with 10 mL of 6 mol/L HCl. Aliquots were stored at –20°C. Blood was collected in polyethylene tubes containing EDTA as anti-coagulant. The EDTA plasma was separated by centrifugation (3000g at 4°C for 20 min) and then stored aliquoted at –80°C until use. Urine and EDTA plasma samples for validation studies were provided by the University Hospital Hamburg-Eppendorf, the University Hospital Charité-Berlin and the blood-bank Eilbek (Germany). Additional samples were obtained from the UK National External Quality Assessment for urinary catecholamines and metabolites (UK NEQAS; Birmingham, United Kingdom). Urine and plasma control samples (Lyphochek®: Quantitative Urine Control Normal and Abnormal, Endocrine Control Levels 1 and 2) were obtained from BIO-RAD (Munich, Germany).

HPLC

Epinephrine and norepinephrine in plasma were extracted by aluminium oxide and separated by reversed phase HPLC using electrochemical detection. We used HPLC-columns from Chromsystems (Munich, Germany) and an electrochemical detector from BIO-RAD (Munich, Germany). The separation was controlled and corrected with the internal standard 3,4-dihydroxybenzylamine. The detection limit was found to be 10 pg/mL for both epinephrine and norepinephrine. The reference values were determined as 10-196 pg/mL (55-1070 pmol/L) for epinephrine and 78- 522 pg/mL (460 – 3080 pmol/L) for norepinephrine (n=140, adults).

Statistical Methods

All data were processed using Microsoft Excel of Office 95. Statistical calculations were performed according to Davies [18]. The slope of the calibration curves was cal-

culated according to Nix [19]. Calibration curves of the optical density plotted against the log of the Calibrator concentration were fitted using the spline-function program in the Melisa-Software (IBL, Hamburg, Germany). The catecholamine concentrations in each sample were determined from these calibration curves.

Description of the ELISA Method

Sample Pretreatment

We developed an epinephrine and norepinephrine immunoassay that included certain premeasurement steps, a cis-diole specific extraction (boronate extraction device, Patent no. 198 18 485.9 applied for by Jürgen Westermann, IBL Hamburg, Germany) and chemical derivatization.

The major premeasurement steps are (1) boronate extraction to remove interfering compounds and endogenous metaneprine and normetanephrine, (2) chemical conversion of the catechols to the XX-biotin-derivates with an activated ester and, after a washing step (3), elution of the derived catechols from the solid phase.

Samples (10 µL urine or 300 µL plasma) and standards (10 µL) were added to borate-coated wells of a macrotiter plate. The urine samples and standards were diluted by the addition of 300 µL distilled water. A volume of 1 mL extraction buffer (0.1 mol/L Tris-HCl buffer, 0.7 mol/L NaCl, 0.1 mmol/L EDTA, 0.3 mmol/L $Na_2S_2O_5$, pH 9.3) was then added to each well of the plate. After incubation (30 min at room temperature with shaking), washing (2 mL double distilled water per well) and the addition of 150 µL extraction buffer, catecholamines were acylated by the addition of 50 µL of 9 mmol/L N-succinimidyl-N-[6-(biotinoylamino)hexanoyl]-6-amino-hexanoic acid (Biotin-XX-NHS) in ethanol/ N,N-dimethylformamide (v/v=50/50) to each well and incubation (20 min. at room temperature with shaking). After the wells had been washed (see above) the extracted and acylated catecholamines were eluted from the macrotiter plate by shaking for 30 min. with 250 µL of release buffer per well.

Immunoassay

The assay procedure of the newly developed CatCombi ELISA kit follows the basic principle of a sandwich assay which utilizes all reagents in excess. The former competitive ELISA for epinephrine and norepinephrine was optimized to increase sensitivity for measurements in plasma. The epinephrine and norepinephrine concentrations in plasma of healthy subjects are approximately one-hundredth of those in urine.

After elution from the gel, there is an enzymatic methylation of the acylated catecholamines into N-acylmetanephrine and N-acylnormetanephrine. In the ELISA the derivatized catecholamine is sandwiched between a specific antibody (anti-N-acylmetanephrine or anti-N-acylnormetanephrine) immobilized on the surface of a microtiter plate and an alkaline phosphatase (AP) labelled antibody against biotin of the derivatized catecholamine molecules.

A solution of pig liver catechol-O-methyltransferase, S-adenosylmethionine (coenzyme) in 1 mol/L Tris-HCl buffer, 0.7 mol/L glycine, pH 9.1 was added into the wells of microtiter strips (50 µL for the determination of epinephrine and 25 µL for the determination of norepinephrine) coated with specific antibodies. The acylated catecholamines of the samples and standards were transferred from the macrotiter plate into the appropriate wells of the microtiter strips (50 µL for epinephrine and 10 µL for norepinephrine) for incubation (2h at room temperature with shaking). The wells were washed three times with wash buffer (0.02 mol/L Tris-HCl buffer, 0.1 mol/L NaCl, 5 mmol/L KCl, 0.2% Tween 80, pH 7.3) and 100 µL of a solution of anti-biotin antibodies conjugated with alkaline phosphatase (Roche, Mannheim, Germany) in 0.02 mol/L Tris-HCl buffer, 0.1 mol/L NaCl, 5 mmol/L KCl, 0.01% BSA, pH 9.1 were added afterwards. After 90 min. incubation (at room temperature with shaking) the wells were washed (see above) and subsequently 100 µL of substrate solution (amplification reagents 1, 2, and 3 in the ratio 1 to 1 to 1; UNITIKA, Osaka, Japan) were added and incubated (15 min. at room temperature with shaking). The substrate reaction was stopped by adding 50 µL of 1 mol/L H_3PO_4 to each well. The microtiter strips were shaken briefly, and the optical density at 490 nm (reference wave length 600-650 nm) was measured within 1 h using a microtiter plate reader (MR5000; DYNATECH, Denkendorf, Germany).

Signal Generation and Amplification

The sandwich complex fixed on the wells of the microtiter strips is visualized by use of a signal amplification system, which uses nicotinamide adenine dinucleotide phosphate (NADPH) as the substrate for alkaline phosphatase. For the CatCombi ELISA the signal intensity is proportional to the catecholamine concentration in the sample and is measured at 490 nm. The measured optical densities of the standards are used to generate a calibration curve against which the unknown samples are calculated.

The amplification system operates as follows: NADPH is converted by alkaline phosphatase (AP; anti-biotin-AP) to nicotinamide adenine dinucleotide (NADH). This dephosphorylation activates an alcohol dehydrogenase-diaphorase redox cycle. The oxidation of NADH to NAD^+ catalyzed by diaphorase is accompanied by a reduction of the colorless salt p-iodonitrotetrazolium (INT) to the highly colored compound formazan. Alcohol dehydrogenase oxidizes ethanol to acetaldehyde, thereby enabling the simultaneous reduction of the formed NAD^+ back to NADH. NADH can again be used in the diaphorase catalyzed reaction.The redox cycle is therefore repeated. The net result is an accumulation of red formazan by the cycling of NADH and NAD^+ in the presence of the enzymes diaphorase and

Table 1: Specificity

Compound	Epinephrine ELISA % Cross-Reactivity	Norepinephrine ELISA % Cross-Reactivity
Epinephrine	100	0.46
Norepinephrine	0.13	100
Metanephrine	2.00	0.05
Normetanephrine	<0.01	2.00
Dopamine	<0.01	<0.01
3-Methoxytyramine	<0.01	<0.01
3,4-Dihydroxyphenylalanine	<0.01	<0.01
Tyrosine	<0.01	<0.01
3-Methoxytyrosine	<0.01	<0.01
Vanillylmandelic acid	<0.01	<0.01
Homovanillic acid	<0.01	<0.01
3-Methox-4-hydroxyyphenylethylene glycol	<0.01	<0.01
3,4-Dihydroxycinnamic acid	<0.01	<0.01
4-Hydroxy-3-methoxycinnamic acid	<0.01	<0.01
4-Hydroxy-3-methoxyphenylpyruvate acid	<0.01	<0.01

Table 2: Precision

Sample	Epinephrine			Norepinephrine		
	Mean, µg/L	SD, µg/L	% CV	Mean, µg/L	SD, µg/L	% CV
Intra-assay (n = 20)						
Urine 1	4.8	0.4	8.3	13.5	1.1	8.1
Urine 2	8.5	0.2	2.4	28.6	1.4	4.9
Urine 3	27.3	1.3	4.8	45.4	4.1	9.0
Urine 4	31.9	1.2	3.8	114.7	7.7	6.7
Urine 5	76.8	6.2	8.1	195.6	21.9	11.2
Intra-assay (n = 20)						
Plasma 1	0.159	0.007	4.4	0.305	0.020	6.5
Plasma 2	0.263	0.018	6.8	0.707	0.071	10.0
Plasma 3	0.405	0.033	8.1	1.432	0.126	8.8
Plasma 4	1.042	0.076	7.3	2.490	0.119	4.8
Plasma 5	1.466	0.122	8.3	3.077	0.291	9.4
Inter assay (n = 20)						
Urine 1	3.5	0.3	8.6	10.3	1.2	11.6
Urine 2	7.8	0.8	10.2	30.3	2.6	8.6
Urine 3	26.6	3.6	13.5	44.8	4.9	10.9
Urine 4	34.1	3.8	11.1	99.7	10.8	10.8
Urine 5	91.9	12.3	13.4	188.8	27.7	14.7
Inter assay (n = 20)						
Plasma 1	0.104	0.015	14.4	0.264	0.043	16.3
Plasma 2	0.190	0.028	14.7	0.527	0.087	16.5
Plasma 3	0.294	0.035	11.9	0.831	0.100	12.0
Plasma 4	0.922	0.105	11.4	2.387	0.345	14.4
Plasma 5	1.543	0.185	12.0	3.892	0.599	15.4

Table 3: Recovery

Sample	Epinephrine					Norepinephrine				
	Endogenous µg/L	Added µg/L	Observed µg/L	Expected µg/L	Recovery %	Endogenous µg/L	Added µg/L	Observed µg/L	Expected µg/L	Recovery %
Urine 1	1.8	1.5	3.0	3.3	91	9.5	5.0	13.6	14.5	94
	1.8	5.0	6.0	6.8	88	9.5	15.0	21.4	24.5	87
	1.8	15.0	13.3	16.8	79	9.5	50.0	54.3	59.5	91
	1.8	50.0	48.3	51.8	93	9.5	150.0	139.7	159.5	88
Urine 2	2.2	1.5	3.6	3.7	97	15.4	5.0	18.5	20.4	91
	2.2	5.0	6.2	7.2	86	15.4	15.0	27.8	30.4	91
	2.2	15.0	14.2	17.2	83	15.4	50.0	62.3	65.4	95
	2.2	50.0	45.1	55.2	82	15.4	150.0	154.2	165.4	93
Urine 3	5.4	1.5	6.4	6.9	93	20.2	5.0	25.1	25.2	100
	5.4	5.0	9.9	10.4	95	20.2	15.0	35.0	35.2	100
	5.4	15.0	20.5	20.4	100	20.2	50.0	67.2	70.2	96
	5.4	50.0	58.7	55.4	106	20.2	150.0	155.2	170.2	91
Plasma 1	0.089	0.035	0.134	0.124	108	0.277	0.100	0.369	0.377	98
	0.089	0.116	0.177	0.205	86	0.277	0.300	0.437	0.577	76
	0.089	0.350	0.353	0.439	80	0.277	1.000	1.187	1.277	93
	0.089	1.162	1.212	1.251	97	0.277	3.000	3.235	3.277	99
Plasma 2	0.173	0.035	0.203	0.208	98	0.604	0.100	0.612	0.704	87
	0.173	0.116	0.278	0.289	96	0.604	0.300	0.786	0.904	87
	0.173	0.350	0.476	0.523	91	0.604	1.000	1.442	1.604	90
	0.173	1.162	1.253	1.335	94	0.604	3.000	3.783	3.604	105
Plasma 3	0.369	0.035	0.398	0.404	99	0.995	0.100	1.007	1.095	92
	0.369	0.116	0.475	0.485	98	0.995	0.300	1.46	1.295	113
	0.369	0.350	0.713	0.719	99	0.995	1.000	2.052	1.995	103
	0.369	1.162	1.272	1.531	83	0.995	3.000	4.933	3.995	123

alcohol dehydrogenase, as well as in an excess of INT and ethanol. After the substrate reaction is stopped, the optical density is measured. The developed color intensity is proportional to the catecholamine concentration in the sample.

RESULTS

The analytical performance of the CatCombi ELISA kit was assessed by evaluating its specificity, analytical and functional sensitivity, precision (intra-assay, inter assay), accuracy (recovery, linearity) and comparability with different HPLC methods.

Specificity

The specificity of the rabbit anti-N-acylmetanephrine and anti-N-acylnormetanephrine antisera was evaluated by determining cross-reactivities at 50% displacement of various compounds, listed in Table 1, that are structurally related to the intended analytes, and may therefore potentially interfere with the assays. Only the 3-O-methylated catecholamine metabolites showed an interference of 2% due to an incomplete removal of these metabolites during the extraction procedure and separation steps of the immunoassay methods. All other compounds tested showed a cross-reaction below 0.5%. In all cases the interference by these compounds in the ELISA is insignificant.

Hemoglobin, bilirubin and triglycerides were evaluated for possible interference in plasma catecholamine determination with the CatCombi ELISA. Hemoglobin was tested at concentrations from 0.5 to 4.0 mg/mL, bilirubin from 0.05 to 0.8 mg/mL and triglycerides from 0.3 to 5.0 mg/mL. Each of these compounds was added to aliquots of two different plasma samples at 10% of the total sample volume. Controls were prepared similarly by adding phosphate buffered saline only. Spiked samples and controls were assayed and the catecholamine contents evaluated (data not shown). No signifycant interference was seen by addition of hemoglobin, bilirubin or triglycerides. The mean calculated recover-

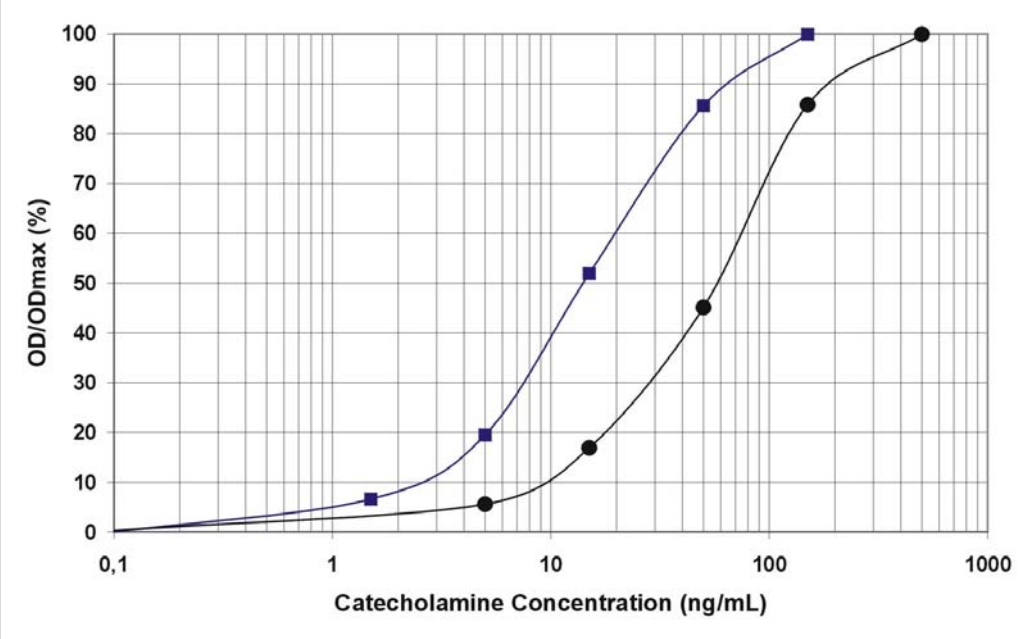

Figure 1: Standard curves (■ epinephrine ● norepinephrine) of the non-competitive CatCombi ELISA.

ies of epinephrine and norepinephrine from hemoglobin, bilirubin and triglycerides spiked plasma were 95.6 ± 7.3 %, 94.5 ± 6.3 % and 99.2 ± 6.7 %, respectively.

Analytical sensitivity and calibration

The analytical sensitivity for epinephrine as well as norepinephrine determinations was calculated from the mean absorbance values plus 2 times the standard deviation SD of the zero calibrator of 20 replicate analyses. The values were determined to be 0.3 µg/L (1.6 nmol/L) for epinephrine and 0.59 µg/L (3.5 nmol/L) for norepinephrine. Typical calibration curves are shown in Figure 1. The concentrations for the urinary catecholamine samples can be read directly from the calibration curve. The concentrations for plasma samples obtained from the calibration curve have to be multiplied by the factor 0.033 to correct for the difference in volumes used for the plasma samples (300 µL) and for the calibrators (10 µL) in pg/mL.

Precision

The intra-assay and inter assay precision data were determined by repeated measurements of urine and plasma samples. The results obtained are shown in Table 2. As can be seen the precision data obtained for intra-assay are in general below 10 % and for inter assay below 15 %.

Accuracy

The analytical recovery of catecholamines in the CatCombi ELISA was estimated at four different concentrations in three urine and three plasma samples (Table 3). Increasing amounts of epinephrine and norepinephrine were added to urine and plasma samples with various initial catecholamine concentrations. Each sample (non-spiked and spiked) was assayed in duplicate in one run. Epinephrine and norepinephrine concentrations were measured and the recovery percentage was calculated. The mean recovery of all six samples was 93% (SD 8%, range 79-108%) for epinephrine and 95% (SD 9%, range 76-123%) for norepinephrine.

As a further demonstration of assay accuracy, linearity studies were performed. Six individual urine and plasma samples with different catecholamine concentrations were serially diluted with 0.1 mol/L HCl (urine samples) or distilled water (plasma samples). The epinephrine and norepinephrine concentrations determined are shown in Table 4. Four dilutions were performed for each sample. Each dilution was measured in duplicate in one assay run. The ratio between concentration and dilution of samples did not significantly deviate from linearity across the concentration range studied.

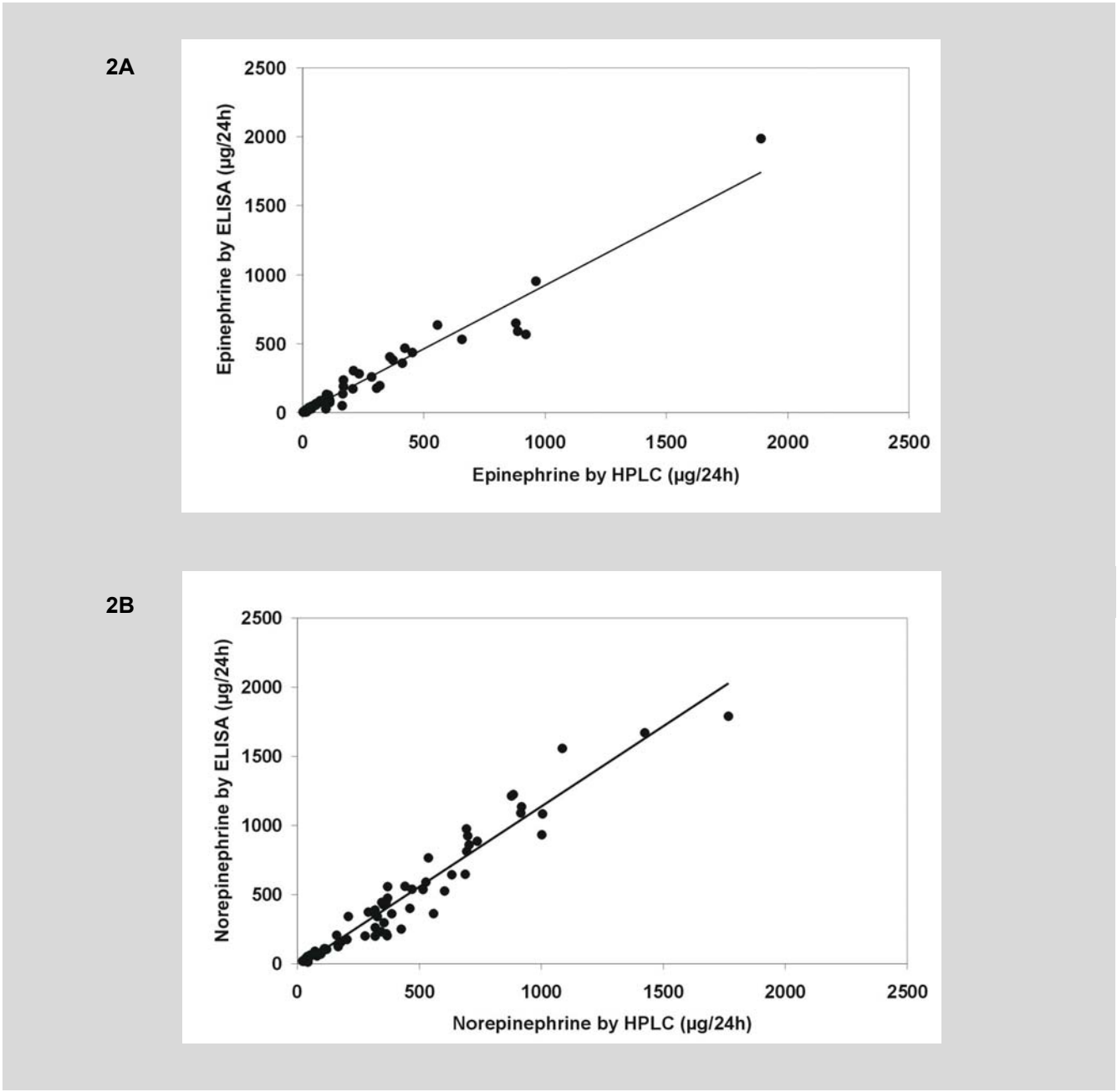

Figure 2: Method comparison urine (UKNEQAS).
Urine samples (n = 85) were assessed by HPLC and the CatCombi ELISA for epinephrine (2A) and norepinephrine (2B) concentrations and analyzed by linear regression analysis. The resulting regression line for epinephrine is: ELISA = 0.92 (HPLC) + 0.33, r = 0.97, and for norepinephrine is: ELISA ? 1.16 (HPLC) – 23.10, r = 0.97, repsectively.

Comparison of the CatCombi ELISA with HPLC

HPLC values were available for 85 urine samples from the UK National External Quality Assessment Schemes (Birmingham, UK). The epinephrine as well as norepinephrine content of these samples was assessed by the CatCombi ELISA. The mean concentration values of all HPLC methods (=reference, x) and the concentrations obtained by the CatCombi ELISA (y) were used for linear regression analyses (Figure 2.A, 2.B). We found regression coefficients of 0.97 for both analytes. The slopes were 0.92 and 1.16 for epinephrine and norepinephrine in urine, respectively. Within the normal range we found for epinephrine ELISA=0.947 (HPLC) + 0.57, r=0.93 (n=31), and for norepinephrine ELISA=0.895 (HPLC) + 1.58, r=0.94 (n=35).

In total 87 plasma samples were measured with HPLC in the Hospital Dresden-Friedrichstadt (Germany, see

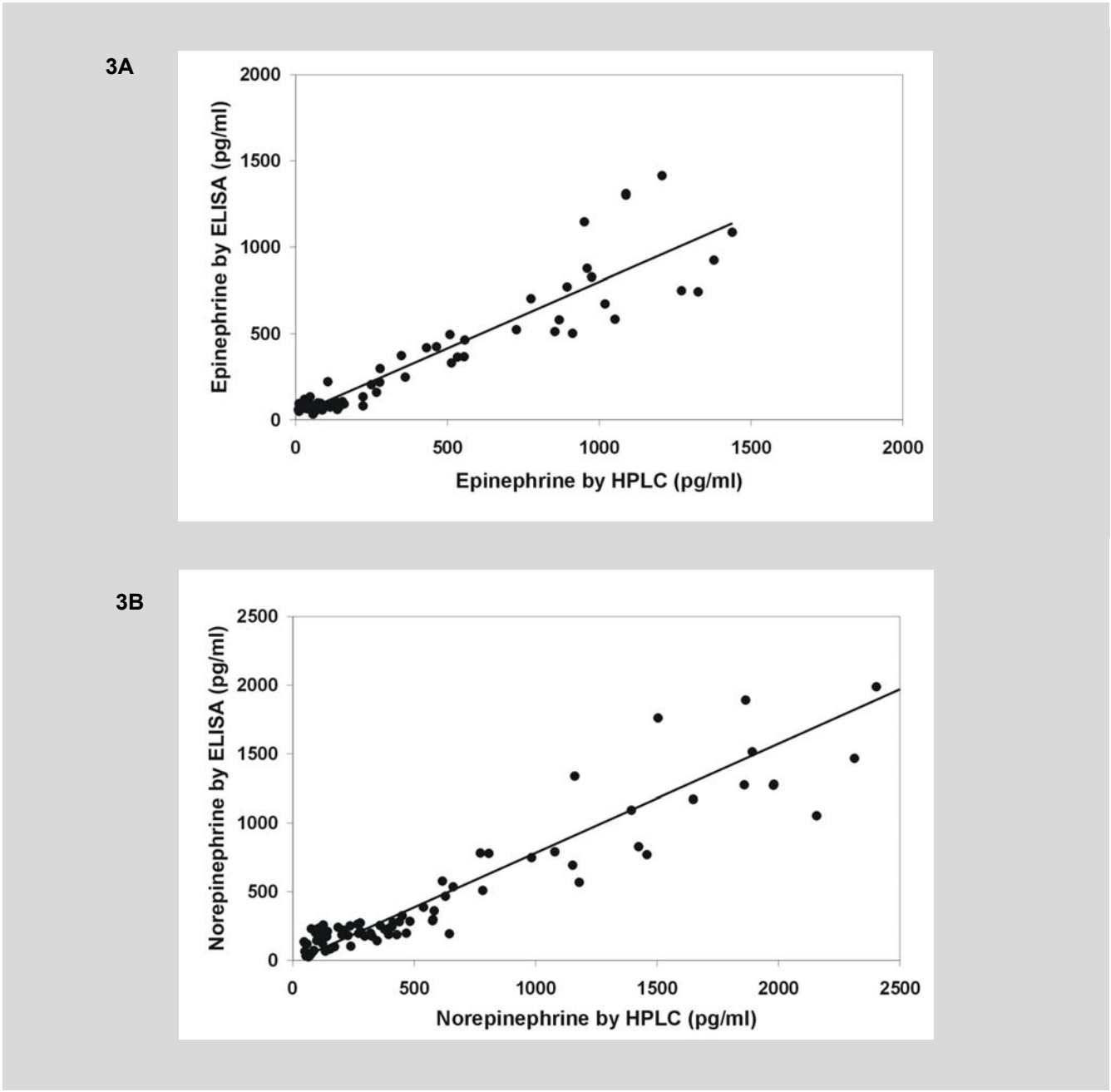

Figure 3: Method comparison plasma
Plasma samples (n = 87) were assessed by HPLC and the CatCombi ELISA for epinephrine (3A) and norepinephrine (3B) concentrations and analyzed by linear regression analysis. The resulting regression line for epinephrine is: ELISA = 0.771 (HPLC) + 29.3, r = 0.93, and for norepinephrine is: ELISA = 0,792 (HPLC) – 8.98, r = 0.95, respectively.

Methods) and with ELISA. The results are visualized in the linear regression curves in Figure 3.A and 3.B with the regression line for epinephrine ELISA=0.771 (HPLC) + 29.3, and for norepinephrine ELISA=0.792 (HPLC) – 8.98. In contrast to the results in urine we found slopes of 0.771 and 0.792 for epinephrine and norepinephrine in plasma, respectively. The regression coefficient for epinephrine was found to be 0.93 and 0.95 for norepinephrine in plasma. Within the normal range we found for epinephrine ELISA=0.528 (HPLC) + 42.2, r=0.73 (n=47), and for norepinephrine ELISA=0.475 (HPLC) + 84.8, r=0.77 (n=60).
Comparisons were performed according to Bablok et al. [20], and by using a method (difference plot) of Pollock et al. [21].

Table 4: Linearity

Sample	Dilution factor	Epinephrine			Norepinephrine		
		Observed µg/l	Expected µg/l	Recovery %	Observed µg/l	Expected µg/l	Recovery %
Urine 1	1:2	68.7	63.0	109	114.0	127.5	89
	1:4	28.3	31.5	90	60.9	63.8	95
	1:8	12.4	15.7	79	28.5	31.9	89
	1:16	6.3	7.8	81	13.7	15.9	86
Urine 2	1:2	42.2	43.3	97	101.0	108.0	94
	1:4	18.8	21.6	87	63.1	54.0	117
	1:8	8.8	10.8	81	27.9	27.0	103
	1:16	4.6	5.4	85	14.4	13.5	107
Urine 3	1:2	30.3	28.7	105	89.3	91.5	98
	1:4	14.7	14.4	102	48.9	45.8	107
	1:8	6.7	7.2	93	22.5	22.9	98
	1:16	3.6	3.6	100	11.5	11.4	101
Plasma 1	1:2	1.216	1.526	80	2.860	2.751	104
	1:4	0.531	0.763	70	0.979	1.375	71
	1:8	0.288	0.382	75	0.761	0.688	111
	1:16	0.192	0.191	101	0.401	0.344	117
Plasma 2	1:2	0.906	1.085	84	2.720	2.958	92
	1:4	0.431	0.542	80	1.235	1.479	84
	1:8	0.250	0.271	92	0.653	0.739	88
	1:16	0.174	0.136	128	0.373	0.370	101
Plasma 3	1:2	1.000	1.037	96	2.782	3.668	76
	1:4	0.516	0.519	99	1.305	1.834	71
	1:8	0.285	0.259	110	0.716	0.917	78
	1:16	0.156	0.130	120	0.401	0.458	88

Shelf Live Evaluation

Accelerated stability testing and real-time stability testing of the kit reagents, standards and controls were performed for determining expiration date, as reported in [22].

The stability of the CatCombi ELISA kit was investigated by real-time stability studies (data not shown). The kit was found to be stable for at least 6 months when stored at 2 to 8 °C.

DISCUSSION

The development of a simple and sensitive ELISA for the determination of epinephrine and norepinephrine in urine and plasma has proven more complicated than for other biogenic amines. The extraction procedure with immobilized boronate gels that we used is technically simple. The CatCombi ELISA is an improvement of our former competitive ELISAs and relies on the use of N-acyl-metanephrine and n-acylnormetanephrine antisera in which epinephrine and norepinephrine are detected by enzymatic conversion to metanephrine and normetanephrine and chemical derivatization to the corresponding N-acyl-derivatives.

At the present time, HPLC with various derivatization and detection methods is generally used for the measurement of catecholamines in urine and plasma.

The HPLC methods include extraction by aluminium oxide, reversed-phase separation, and electrochemical detection or fluorescence detection of the trihydroxyindole derivatives [23].

Comparison of ELISA with HPLC

The correlation coefficients and the regression lines show acceptable agreement between the two methods. The slopes for epinephrine and norepinephrine in plas-

ma have a trend toward slightly lower values for epinephrine and norepinephrine obtained by ELISA in comparison to HPLC. The comparison data obtained for urine samples show a better agreement than for plasma. This might be due to the different matrices and the higher concentrations of catecholamines in urine.
The agreement between the CatCombi ELISA values and HPLC suggests that the influence of interfering substances in most samples studied is minimal.
The detection of interference of drugs in immunoassays is more difficult than in HPLC because no visual hardcopy of the measured end product is produced. Wassell et al. (24) studied the influence of angiotensin-converting enzyme inhibitors, ß-blockers, calcium channel blockers, diuretics, statins, alpha-methyldopa and alpha-receptor antagonists in immunoassay and HPLC. They found that none of the drugs examined showed significant interferences in the immunoassay, but interferences were found in the HPLC system. They argue that this seems to be the result of the use of highly specific antibodies in the immunoassay (see table 1 for specificity).
In contrast to HPLC, this new ELISA is less time-consuming for large sample series and considerably more economical in its use of plasma sample volume, which is particularly advantageous where limited amounts of plasma are available.
The ELISA technique for catecholamines is easier to use than HPLC methods and is more accessible to laboratories. The analytical procedure of ELISA is relatively simple and requires lower equipment costs with a microtiter plate reader in comparison to the HPLC system.
Compared to HPLC, immunoassays are advantageous for analyzing numerous samples simultaneously, e.g. for screening purposes.

In conclusion, we have presented a new method that can measure epinephrine and norepinephrine accurately, which avoids the use of radioactive material.
In summary, the novel ELISA kit described here is useful for clinical applications as well as for basic research, where a simple, rapid, accurate and reproducible assay for the determination in urine or plasma is required.

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