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Novel ELISAs for Screening of the Biogenic Amines GABA, Glycine, β-Phenylethylamine, Agmatine, and Taurine Using One Derivatization Procedure of Whole Urine Samples

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The inhibitory neurotransmitters GABA, glycine and agmatine and neuromodulators β-phenylethylamine (β-PEA) and taurine are important biogenic amines of the sympathetic and parasympathetic nervous systems in the body. Abnormalities in the metabolism of these biomarkers have been implicated in a vast number of neurological diseases.

Novel competitive immunoassays, using one unique whole urine derivatization procedure applicable for all five biomarkers, have been developed. The determination of these biomarkers was highly reproducible: the coefficient of variance of inter- and intra-assay variation is between 3.9% and 9.8% for all assays. The assays show a good linearity in urine samples within the range of 100−400 mg Cr/dL and specificity when urine samples are spiked with biogenic amines. The recoveries are between 76 and 154%. The correlation between HPLC and ELISA for glycine and taurine (n = 10) showed regression coefficients of 0.97 and 0.98, respectively.

An in vivo study on the urinary clearance of β-PEA, agmatine and taurine after oral intake by healthy individuals demonstrated the specificity and clinical significance of these new immunoassays. The immunoassays are useful for clinical and basic research where a fast and accurate assay for the screening of biogenic amines in urine is required, without preclearance of the sample.

Both neurotransmitters and neuromodulators are the chemical messengers of the central nervous system (CNS) and are synthesized, metabolized, stored, and released from neurons. Via neurons, through an electrochemical process known as neurotransmission, the CNS communicates with all organ systems of the body. The highly specialized brain capillary endothelial cells of the blood brain barrier (BBB) regulate the passage of most neurotransmitters, neuromodulators and their precursors in and out of the CNS. The CNS also employs a mechanism to reuse neurotransmitters.

For the neurotransmitters GABA, agmatine, and glycine, transport from the brain to the blood has been shown. The transport of GABA occurs on the abluminal membrane transporters[1] and via still unidentified luminal transporters.[2] Agmatine crosses the BBB, as has been demonstrated by increased concentration of agmatine in cerebral spinal fluid (CSF) following intravenous injection.[3,4] Glycine also crosses the BBB, but not by a carrier- mediated process.[5] The two neuromodulators described in this paper, taurine and β-phenylethylamine (β-PEA), a lipid-soluble neuromodulator, freely cross the BBB.[6−9] Neurotransmitters cross cerebral endothelial membranes and support the existence of intact neurotransmitters and neuromodulators in plasma/serum and in urine after blood has filtered by the kidneys.[10] The value of testing urinary neurotransmitters or biogenic amines in general, as a noninvasive way to asses a person's health, has a long medical history and is becoming an increasingly popular method of assessing clinical conditions.[11−17] The role of norepinephrine and its metabolites as biomarkers of an adrenal tumor known as pheochromocytoma was the first result of clinical application of neurotransmitter testing in urine.[18]

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(1) Takanaga, H.; Ohtsuki, S.; Hosoya, K.; Terasaki, T. *J. Cereb. Blood Flow Metab.* **2001**, *21*, 1232–1239.
(2) Kakee, A.; Takanaga, H.; Terasaki, T.; Naito, M.; Tsuruo, T.; Sugiyama, Y *J. Neurochem.* **2001**, *79*, 110–118.
(3) Roberts, J. C.; Grocholski, B. M.; Kitto, K. F.; Fairbanks, C. A. *J. Pharmacol. Exp. Ther.* **2005**, *314*, 1226–1233.
(4) Piletz, J. E.; May, P. J.; Wang, G.; Zhu, H. *Ann. N. Y. Acad. Sci.* **2003**, *1009*, 64–74.
(5) Pollay, M. *J. Neurobiol.* **1976**, *7*, 123–128.
(6) Szabo, A.; Billett, E.; Turner, J. *Br. J. Sports Med.* **2001**, *35*, 342–343.
(7) Urquhart, N.; Perry, T. L.; Hansen, S.; Kennedy, J. *J. Neurochem.* **1974**, *22*, 871–872.
(8) Tsuji, A.; Tamai, I. *Adv. Exp. Med. Biol.* **1996**, *403*, 385–391.
(9) Salimäki, J.; Scriba, G.; Piepponen, T. P.; Rautolahti, N.; Ahtee, L. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2003**, *368*, 134–141.
(10) Moleman, P.; Tulen, J. H.; Blankestijn, P. J.; Man in 't Veld, A. J.; Boomsma, F. *Arch. Gen. Psychiatry* **1992**, *49*, 568–572.
(11) Wang, T.; Muthuswamy, *J. Anal. Chem.* **2008**, *80*, 8576–8582.
(12) Ailts, J.; Ailts, D.; Bull, M. *White paper NeuroScience Inc.* **2007**, 1–6.
(13) Jung, M. C.; Shi, G. Y.; Borland, L.; Michael, A. C.; Weber, S. G. *Anal. Chem.* **2006**, *78*, 1755–1760.
(14) Delahanty, D. L.; Nugent, N. R.; Christopher, N. C.; Walsh, M. *Psychoneuroendocrinology* **2005**, *30*, 121–128.
(15) Hughes, J. W.; Watkins, L.; Blumenthal, J. A.; Kuhn, C.; Sherwood, A. *J. Psychosom. Res.* **2004**, *57*, 353–358.
(16) Duncan, M. W.; Compton, P.; Lazarus, L.; Smythe, G. A. *N. Engl. J. Med.* **1988**, *319*, 136–142.
(17) Barthelemy, C.; Bruneau, N.; Cottet-Eymard, J.M. ; Domenech-Jouve, J.; Garreau, B.; Lelord, G.; Muh, J. P.; Peyrin, L. *J. Autism Dev. Disord.* **1988**, *18*, 583–591.
(18) Engel, A.; Euler von, U. S. *Lancet* **1950**, *2*, 387.

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Neurotransmitter testing in urine is also relevant to monitor changes in neurotransmitter balance and secretion as a result of therapeutic or pharmacokinetic studies.[3,19−23] In addition, neurotransmitter testing can be used to follow the turnover time and clearance of neurotransmitters, modulators and metabolic precursors in supplements and pharmaceuticals aimed to correct neurotransmitter imbalances, for example, 5-hydroxytryptophan for serotonin.[24,25] The five biomarkers described in this paper have been implicated in a wide array of neurological disorders including anxiety, neuropsychiatric diseases; pediatric neurotransmission diseases, and depression.[6,20,23,26−29] Four of the five biogenic amines are derived from decarboxylation of amino acids: GABA from glutamic acid, agmatine from L-arginine, taurine from cysteine sulfonic acid, and β-PEA from L-phenylalanine. Glycine is the only exception deriving from transamination of glyoxalate.[30]

The biogenic amines taurine, GABA and glycine described in this paper have been studied before either in histochemical studies to localize the neurotransmitters in brain tissue using specific antibodies[31−34] or by HPLC and gas chromatography to quantify biogenic amines in plasma and urine.[3,4,20,23,35,36] A review of the literature did not reveal any studies on screening of the biogenic amines with immunoassays as we described here in this paper.

The preparation of the antibodies for the GABA and glycine assays has been previously described.[37] The highly specific antibodies developed for urinary testing of β-PEA and agmatine will be described here for the first time. An antibody for taurine was also developed but has been previously described.[32,33] Whole urine samples, normalized by creatinine levels, are prepared by one simple derivatization procedure and each sample can be applied in multifold in five different immunoassays. The derivatization procedure of urine samples consists of cross-linking biogenic amine to itself or with other amine containing molecules in urine via glutaric aldehyde. This derivatization step of the urine sample with glutaric aldehyde mimics the preparation of the immunogens used to generate the highly specific antibodies against conjugated biogenic amines.[37]

We describe here, highly reproducible (CV < 9.8%) competitive immunoassays for (semi)quantitative urinary analysis of GABA, glycine, agmatine, β-PEA and taurine. An in vivo study to measure turnover rate and clearance of β-PEA, agmatine and taurine in urine samples, after oral intake by healthy individuals, demonstrated the specificity and clinical significance of these immunoassays for urinary testing of biogenic amines.

EXPERIMENTAL SECTION

Chemicals. All chemicals were purchased from Sigma Aldrich (Saint Louis, MO) Avidine conjugated with alkaline phosphatase. Keyhole limpet hemocyanin (KLH), ImmunoPure Immobilized Protein A, AminoLink coupling resin and Sulfo-NHS-LC-Biotin were purchased from Pierce (Rockford, IL). StabilGuard and StabilZyme Select were purchased from SurModics (Eden Prairie, MN).

Preparation of Immunogens. Agmatine sulfate salt was coupled to the carrier protein (CP) keyhole limpet hemocyanin (KLH); taurine and β-PEA-HCl were coupled to CP: bovine sera albumin (BSA) using the one-step linking through glutaric aldehyde essentially as has been described.[37]

Preparation of Conjugated Biogenic Amines for Coating, Standard and Control Conjugates for Cross-Reactivity Studies. All biogenic amines used in this study, including conjugated control amino acids and related biogenic amines for the competitive immunoassays, were made under the same conditions as the agmatine, taurine, glycine, GABA, and β-PEA protein conjugates. The glutaric aldehyde conjugates were made essentially according the procedure as described for immunogens, but with only BSA or thyroglobuline (TG) as the CP. After the end of the cross-linking incubation time, unsaturated aldehyde groups were quenched with excess Tris (hydroxymethyl)aminomethane (Tris-HCl pH 7.5) at a final concentration of 10 mM and Schiff's bonds were reduced with 30 mM $NaCNBH_3$ (5 M stock solution in 1 M NaOH from Sigma-Aldrich).

Polyclonal Antibodies. Immunizations were conducted at Maine Biotechnology Service (Portland, ME), using New Zealand White rabbits as described before.[37]

Antibody Isolation. The GABA and glycine antibody used in this paper were affinity purified IgG as previously described.[37] The β-PEA antibody, showing a high specific titer, was purified as total IgG by means of an immobilized Protein-A-column purification according to standard protocols. The agmatine antibody was affinity purified using agmatine sulfate salt coupled via an aldehyde linkage to AminoLink Coupling Resin from Pierce (Rockford IL). To improve the sensitivity of the agmatine assay the antibody was biotinylated using Sulfo-NHS-LC-Biotin purchased from Pierce (Rockford Il) and used in combination with avidine conjugated to alkaline phosphatase. The taurine antibody was purified from cross-reacting CP-antibodies using AminoLink coupling resin coupled with BSA pretreated with glutaric aldehyde.[37] The specificity of the three new antibodies for β-PEA, agmatine and taurine see Table 1, was assessed by a competitive ELISA (c-ELISA) as described before.

Competitive ELISA. A c-ELISA was created for each of the five biogenic amines. The procedure described here briefly is for

(19) Chen, Y.; Guo, C.; Lim, L.; Cheong, S.; Zhang, Q.; Tang, K.; Reboud, J. *Anal. Chem.* **2008**, *80*, 1133–1140, 2008.
(20) Kusaga, A.; Yamashita, Y.; Koeda, T.; Hiratani, M.; Kaneko, M.; Yamada, S.; Matsuishi, T. *Ann. Neurol.* **2002**, *52*, 372–374.
(21) Dutton, J.; Copeland, L. G.; Playfer, J. R.; Roberts, N. B. *Clin. Chem.* **1993**, *39*, 629–634.
(22) Zametkin, A. J.; Karoum, F.; Linnoila, M.; Rapoport, J. L.; Brown, G. L.; Chuang, L. W.; Wyatt, R. J. *Arch. Gen. Psychiatry* **1985**, *42*, 251–255.
(23) Bornstein, R. A.; Baker, G. B. *J. Neuropsychiatry Clin. Neurosci.* **1991**, *3*, 417–421.
(24) Turner, E. H.; Loftis, J. M.; Blackwell, A. D. *Pharmacol. Ther.* **2006**, *109*, 325–338.
(25) Khor, S. P.; Hsu, A. *Curr. Clin. Pharmacol.* **2007**, *2*, 234–243.
(26) Regunathan, S.; Dozier, D.; Takkalapalli, R.; Phillips, W. J. *J. Pain Palliative Care Pharmacother.* **2009**, *23*, 35–39.
(27) Reyes-García, M. G.; García-Tamayo, F. *J. Neuroimmunol.* **2009**, *216*, 20–31.
(28) Louzada, P. R.; Lima, A. C. P.; Mendonca-Silva, D. L.; Noël, F.; De Mello, F. G.; Ferreira, S. T. *FASEB J.* **2004**, *18*, 511–518.
(29) Wong, C. G.; Bottiglieri, T.; Snead, O. C. *Ann. Neurol.* **2003**, *54*, 3–12.
(30) McMurry, J.; Begley, T. *The Organic Chemistry of Biological Pathways*; Roberts and Company Publishers: Greenwood Village, CO, 2005.
(31) Seguala, P; Geffard, M; Buys, R. M.; Moal le, M. *Proc. Natl. Acad. Sci. U. S. A.* **1984**, *81*, 3888–3892.
(32) Campistron, G.; Geffard, M.; Buys, R. M. *J. Neurochem.* **1986**, *46*, 862–868.
(33) Ida, S.; Kuriyama, K.; Tomida, Y.; Kimura, H. *J. Neurosci. Res.* **1987**, *18*, 626–631.
(34) Meyer, K. H.; Behringer, D. M.; Veh, R. W. *J. Histochem. Cytochem.* **1991**, *39*, 749–760.
(35) Venta, R. *Clin. Chem.* **2001**, *47*, 575–583.
(36) Davis, B. A. *J. Chromatogr.* **1989**, *466*, 89–218.
(37) Huisman, H.; Wynveen, P.; Setter, P. *J. Neurochem.* **2010**, *112*, 829–841.

Table 1. Specificity of Anti-Agmatine, Anti-Taurine, and Anti-β-PEA Antibodies[a]

competitor	cross-reactivity ratio	antibody
BSA-agmatine	1:1	anti-agmatine
BSA-glutamate	>1:1000	
TG-L-lysine	>1:1000	
TG-L-arginine	1:641	
TG-cadavarine	>1:1000	
TG-β-phenylethylamine	1:1	anti-β-phenylethylamine
TG-β-alanine	>1:1000	(β-PEA)
TG-phenylalanine	>1:1000	
TG-tyramine	>1:1000	
TG-tyrosine	>1:1000	
BSA-taurine	1:1	anti-taurine
BSA-hypotaurine	1:92	
BSA-homocysteic acid	>1:1000	
BSA-cysteine	1:769	
BSA-taurocholic acid	>1:1000	

[a] Cross reactivity of agmatine, β-phenylethylamine, and taurine antibodies with related conjugated molecules. The specificity towards the conjugate was calculated by comparing the observed concentration to the actual concentration.[37] None of the antibodies show a significant response towards the free, non-cross-linked haptens ($n = 3$, %CV = 0.5−5.7%).

testing the specificity of the three new antibodies against taurine, agmatine, and β-PEA. Polystyrene 96 well flat-bottom immuno plates (Nalgene Nunc International, Rochester, NY) were coated overnight at 20 °C with BSA-taurine, TG-β-PEA, or BSA-agmatine prepared in coating buffer (50 mM sodium carbonate-bicarbonate, pH 9.2−9.6). Optimal coating antigen and antibody concentration were assessed by checkerboard titration. The wash protocols between each incubation, incubation times, blocking of the plates and buffers used throughout the c-ELISA have been described before.[37] Serial dilutions of conjugated β-PEA, taurine and competitors (75 μL/well) were incubated along with optimized concentration of the specific antibodies (75 μL/well) in PBS, 0.1% BSA, and 0.1% Tween-20) overnight at 32 °C with constant shaking. The agmatine assay was performed with a serial dilution of conjugated neurotransmitter and competitors (150 μL/well) and incubated with optimized biotinylated-affinity-purified IgG-agmatine (50 μL/well). The micro-titer plates for taurine and β-PEA were then incubated with 150 μL/well of secondary antibody, goat-antirabbit IgG-alkaline phosphatase, in PBS, 0.1% BSA, 0.5% normal goat serum, and 0.1% Tween-20 for 1 h with constant shaking at 20 °C at optimized concentrations. The agmatine plate was incubated with 200 μL/well optimized concentration of avidine conjugated with alkaline phosphatase in the same buffer as before. Finally, 150 μL/well of substrate was added for β-PEA and taurine assay or 200 μL/well for agmatine assay of alkaline phosphatase substrate buffer containing 2.5 mM p-nitrophenyl phosphate disodium hexahydrate in 1 M diethanolamine, pH 9.5. Absorbance at 405 nm was obtained using a Sunrise plate reader (Tecan, Männedorf, Switzerland) or SpectraMax M5/M5[e] from Molecular Devices Corp. (Sunnyvale CA). Antibody specificity was determined as described before using the c-ELISA and the results of the average of four independent analyses.[37] Cross-reactivity was expressed as the average of observed concentration to actual concentration in relation to the homologous conjugate,

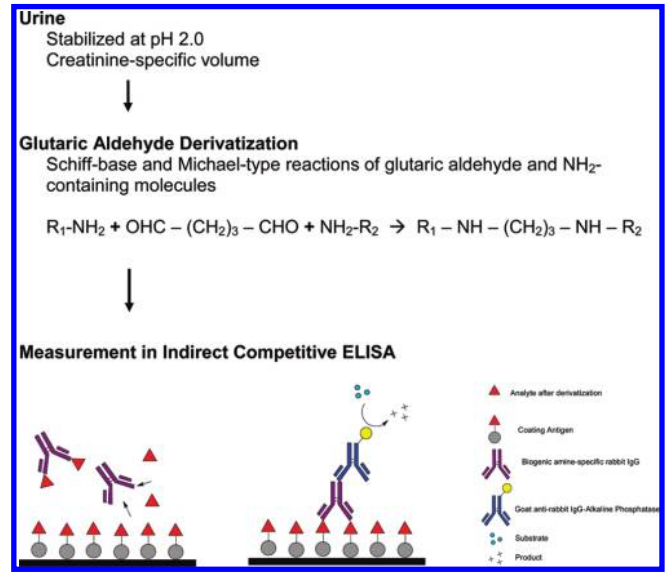

Figure 1. Schematic diagram of the method for measuring glutaric aldehyde conjugated biogenic amines in urine. A creatinine specific volume of urine is derivatized through glutaric aldehyde conjugations. These conjugates then compete with the coating antigen (biogenic amine coupled to a carrier protein) for specific antibiogenic amine rabbit IgG antibodies. Antibodies bound to the solid phase are measured with a secondary antibody (goat-antirabbit IgG conjugated to alkaline phosphatase) and subsequent substrate reaction. The specific antibody bound to the solid phase is inversely related to the amount of biogenic amine in the urine.

(e.g., A 1:200 ratio conveys that a 200 fold increase in concentration of the related heterologous conjugate is necessary to achieve the same response as the homologous conjugates).

Preparation of Standard Curves. For urinary screening of biogenic amines, expressed in arbitrary units, and for quantitative measurements of glycine and taurine, expressed in μmol/g Cr, standard curves are prepared of a serial dilution of a calculated amount of the free homologous biogenic amine in 100 μL of 50 mM HCl. After neutralization with 20 μL of 1 M $NaHCO_3$ containing 16 μg of BSA, in situ conjugation is performed with 40 μL glutaric aldehyde (267 mM). The reaction is stopped with the same Tris quenching buffer. This procedure for the preparation of standard curves in situ is therefore essentially the same as the derivatization of urine samples. The performance of the c-ELISA is as described before.

Derivatization of Urine Samples. Morning urine samples of approximately 5 mL were collected in plastic tubes containing 100 μL of 150 mM hydrochloric acid. Urine samples were used immediately after centrifugation for 6 min at 8000*g* or kept frozen at −20 °C. Urinary creatinine (Cr) was assayed using Roche COBAS INTEGRA Creatinine (Jaffé Gen.2) system. Only urine samples with creatinine values between 100 mg Cr/dL and 400 mg Cr/dL were assayed. A urine volume containing 100 μg Cr was taken (between 100 and 25 μL) and 100 mM HCl was added to adjust the volume to 100 μL, if appropriate. The urine samples were neutralized by adding 20 μL of neutralizing buffer containing 1 M $NaHCO_3$ pH 8.0 and 0.1% Tween-20 (final pH between 7.5 and 8.0). A stock solution of 267 mM glutaric aldehyde was made in sodium phosphate buffer 0.1 M pH 7.5 containing 150 mM sodium chloride (BupH Phosphate buffered saline packs

Table 2. Reproducibility of the c-ELISAs for GABA, Glycine, β-PEA, Agmatine, and Taurine as Expressed in Coefficient of Variance (%)[a]

assay	intra-assay (% CV)	inter-assay (% CV)
GABA	4.7	5.7
glycine	4.0	9.2
β-PEA	3.9	5.1
agmatine	6.5	9.8
taurine	4.9	5.7

[a] Urine samples ($n = 160$) were taken randomly from healthy and clinical urine samples with Creatinine values ranging between 100 and 400 mg/dL and derivatized in duplicate. Each set of the two derivatized samples was tested by two different lab-workers in triplicate on two different plates (24 analyses per sample).

purchased from Pierce (Rockford IL). Forty μL of glutaric aldehyde was added to the neutralized urine sample (final concentration glutaric aldehyde 66.7 mM) and mixed rapidly. The mixture was allowed to incubate for 60 min at 20 °C in the dark. Forty μL quenching buffer (Tris-HCl 666 mM; NaCl 344 mM; Tween-20 0.25%; BSA 0.5%; sodium azide 0.02%) was added to the mixture to quench excess of glutaric aldehyde and to prepare the sample for c-ELISA. The samples were allowed to incubate for 30 min at 20 °C in the dark and centrifuged for 5 min at 10 000*g*, before applying to the c-ELISA or stored frozen at −20 °C upon use. This basic protocol for derivatization of urine samples can be scaled up to allow multiple analyses of one urine sample.

In Figure 1 we present a schematic diagram of the test principle, including the derivatization of biogenic amines via cross-linking with glutaric aldehyde and the competitive-ELISA.

Urinary Clearance of Agmatine, Taurine, and β-PEA after Oral Intake. Three capsules each containing either 300 mg agmatine, 600 mg taurine, or 1200 mg β-PEA, all of pharmaceutical grade, were taken orally immediately after collecting the first urine sample. The first urine sample served to establish the baseline values of agmatine, taurine, and β-PEA and the control biogenic amines GABA and glycine. Urine samples were collected every hour over the course of 5 h. Each set of urine samples (baseline and five consecutive samples) of the eight healthy participants were tested at the same time in the c-ELISAs after derivatization of the urine samples as described before. The relative concentration of each biogenic amine is expressed in (semi-) quantitative arbitrary units.

RESULTS

c-ELISA for Screening Biogenic Amines: Reproducibility. The reproducibility of the c-ELISAs for GABA, glycine, agmatine, β-PEA, and taurine was tested on 160 randomly taken urine samples. The panel of 160 samples was first derivatized in duplicate. Two different lab-workers tested each one set of the derivatized samples in the assays in triplicate and on two different plates. The reproducibility of each assay is expressed in intra- and interassay coefficient of variation (CV %) and is the results of 24 measurements of each sample belonging to the panel of 160 urine samples. The results are depicted in Table 2.

c-ELISA for Screening Biogenic Amines: Linearity. The linearity was investigated on urine samples representing a wide range of creatinine clearance in individuals (100−400 mg Cr/dL). Each urine sample of this panel was tested in duplicate and diluted 1.0, 1.7, 2.7, and 4.5-fold with 100 mM HCl before cross-linking with glutaric aldehyde (see Experimental Section). Each sample was analyzed in triplicate in the five biogenic amine c-ELISA. The dilution corrected analyte concentrations yielded between 80−125% recoveries between dilutions. The results are depicted in Table 3.

c-ELISA for Screening Biogenic Amines: Assay Specificity and Recovery. For the analysis of each assay eight urine samples were tested first on endogenous concentration of biogenic amines (nonspiked). A fixed amount of each of the biogenic amines was added to the urine samples and the specificity and recovery was

Table 3. Linearity: Proportionate Measurements of Differences in Concentration[a]

		GABA		β-PEA		taurine		glycine		agmatine	
creatinine range (mg/dL)	dilution factor	measured (AU)	dilution Adjusted	measured (AU)	dilution Adjusted	measured (AU)	dilution Adjusted	measured (AU)	dilution Adjusted	measured (AU)	dilution Adjusted
100−150	1.0	10.5	10.5	118.5	118.5	1246.4	1246.4	4215.7	4215.7	6.9	6.9
	1.7	5.2	8.6	82.4	136.0	750.2	1237.8	2232.3	3683.3	4.1	6.7
	2.7	3.3	9.0	56.7	154.3	443.1	1206.4	1586.7	4319.9	2.9	7.8
	4.5	1.8	8.3	39.6	178.1	300.7	1350.8	889.9	3997.4	1.7	7.9
150−200	1.0	7.5	7.5	29.0	29.0	1217.8	1217.8	846.6	846.6	7.7	7.7
	1.7	4.3	7.1	20.9	34.5	699.7	1154.6	619.8	1022.7	4.8	7.8
	2.7	2.6	7.1	15.0	40.8	454.8	1238.2	305.0	830.4	3.2	8.8
	4.5	1.5	6.6	9.2	41.5	257.7	1157.6	150.5	676.0	2.1	9.6
200−300	1.0	3.4	3.4	41.8	41.8	810.7	810.7	265.6	265.6	10.2	10.2
	1.7	2.0	3.3	29.2	48.1	445.1	734.4	200.0	330.0	5.0	8.3
	2.7	1.4	3.8	18.8	51.2	276.5	752.9	96.0	261.3	3.0	8.1
	4.5	1.0	4.4	12.2	55.0	194.4	873.3	65.0	292.0	2.1	9.2
300−400	1.0	2.3	2.3	39.5	39.5	237.0	237.0	247.6	247.6	4.3	4.3
	1.7	1.4	2.4	26.1	43.1	137.5	226.9	165.4	272.9	2.8	4.6
	2.7	0.9	2.4	18.5	50.4	104.3	284.1	120.3	327.5	1.7	4.7
	4.5	0.6	2.5	13.1	58.8	68.8	309.1	86.8	390.0	1.2	5.2

[a] The linearity of each c-ELISA was investigated using four different dilutions of urine samples within four different creatinine ranges. After adjustment for the dilution factors, all assays yield 80−125% recovery between dilutions.

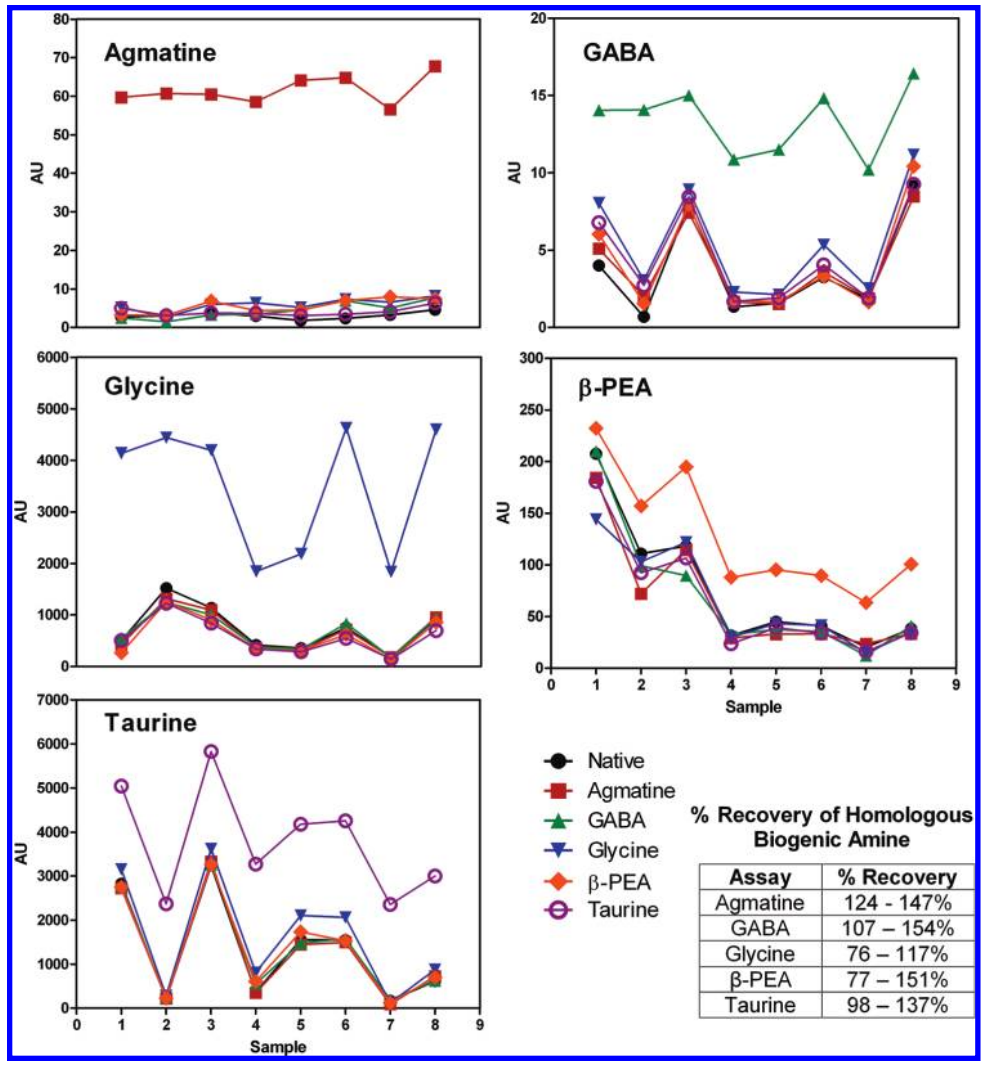

Figure 2. In situ specificity and recovery. Eight urine samples spiked with constant amounts of each biogenic amine were measured in each of the assays. The specificity toward the homologous biogenic amine is shown in the graphs. The table insert depicts the percentage recovery of the homologous biogenic amines.

determined in each c-ELISA as well as the nonspecific reaction for the spiked sample in the heterologous assays. Figure 2 displays the specificity and recovery of each assay (76−154%) and the low to absent cross-reactivity for the heterologous biogenic amines as depicted in the profiles of the endogenous amounts of biogenic amines.

Comparison of c-ELISA for Glycine and Taurine with HPLC. For a validation study 10 urine samples were split and analyzed by the c-ELISA and HPLC. HPLC analysis of glycine and taurine in 10 urine samples was performed by ARUP Laboratories Inc. (Salt lake City, UT) (Test: Amino Acids Quantitative Urine: 0080044). The glycine and taurine content expressed in μmol/g

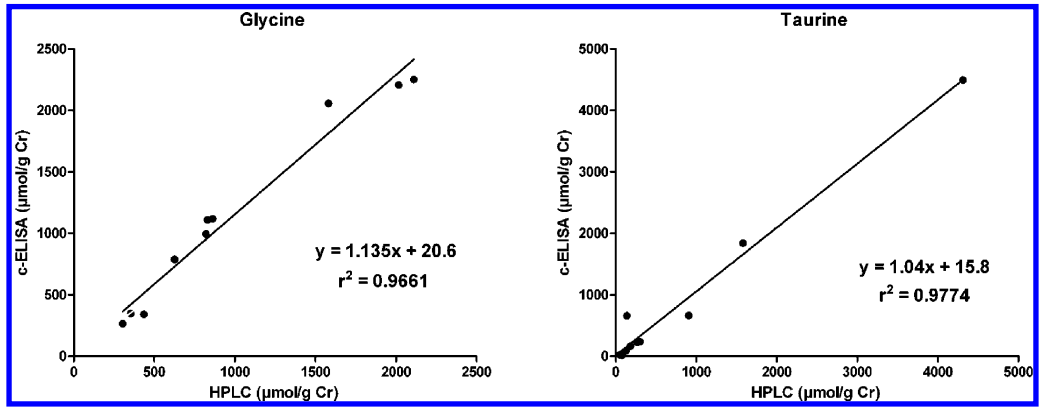

Figure 3. c-ELISA-HPLC Correlation. Urine samples (n = 10) were analyzed under the c-ELISA conditions as well as by an independent HPLC urinary amino acid analysis.

Table 4. Biological Relationship of the Biogenic Amines: Agmatine, GABA, β-PEA, Taurine, and Glycine as Measured in Urine Samples and Expressed in Pearson's Correlation[a]

	agmatine	GABA	β-PEA	taurine	glycine
agmatine		14.8%	34.9%	14.5%	14.6%
GABA			14.0%	23.1%	33.2%
β-PEA				10.8%	8.8%
taurine					15.1%

[a] Biological relationship was investigated by analyzing 200 urine samples using the five specific competitive ELISAs. The degree of biological relationship between the five biogenic amines is expressed in a range of 100% (perfect biological relationship) to 0% (no biological relationship).

Cr were assessed by the immuno assays as described in this paper using the in situ prepared calibration curve. The mean concentrations values of all HPLC measurements and the concentration obtained by our assays were used for linear regression analyses (see Figure 3). We found regression coefficients of 0.97 for glycine and 0.98 for taurine.

In Vivo Significance of c-ELISAs for Screening Biogenic Amines-1. We determine the relative concentration of each biogenic amine and used the Pearson's Correlation to identify pairs of biogenic amines which could be biologically related. Two hundred urine samples were tested in duplicate, and Table 4 shows the degree of biological relationship between each of combination of two biogenic amines ($n = 10$ combinations).

In Vivo Significance of c-ELISAs for Screening Biogenic Amines-2. In Figure 4 we show the (semi)quantitative concentration of the five biogenic amines in urine samples after intake of the biogenic amines agmatine, taurine, and β-PEA. The appearance and clearance over time of these compounds were monitored in urine samples by each corresponding c-ELISA. The study was conducted under the guidelines for the protection of human subjects. Each volunteer gave informed consent. Variable turnover/clearance rates were observed between the eight volunteers as well as between the biogenic amines. Both the control biogenic amines GABA and glycine displayed only the endogenous variable amount of these biogenic amines in each of the participants.

DISCUSSION

Derivatization of Urine Samples for Screening of Agmatine, β-PEA, Taurine, GABA and Glycine. The rationale for the derivatization of urine samples with glutaric aldehyde is to mimic the same chemical reaction we used for preparing the immunogens to raise specific antibodies. We anticipated that biogenic amines can be conjugated either to themselves or conjugate with different molecules in urine carrying an ionizable amine group to act as "carrier protein". In both cases, the biogenic amines are presented via Schiff bond coupling to the bivalent glutaric aldehyde and, as such, are recognizable by the antibodies. The addition of exogenous albumin to act as the carrier protein did not show to have any beneficial effect (data not shown). The reproducibility of the derivatization step of urine depends on two important factors: the pH of the urine samples after neutralization with 1 M $NaHCO_3$ and the final concentration of glutaric aldehyde. A pH shift influences the cross-linking reaction for biogenic amines with ionizable moieties or different pK_a values[38] (data not shown). Both the neutralization buffer and the phosphate buffered cross-linking reaction appeared to be sufficient to maintain the pH between 7.5 and 8.0. We have established that reduction of the Schiff's bond with $NaCNBH_3$ was not necessary, apparently urine contains sufficient concentrations of reducing compounds, for example, cysteine and reduced glutathione.

As pointed out in the Experimental Section, we used urine samples with creatinine levels in the range of 100−400 mg Cr/dL^{-1}. As accepted in clinical chemistry, creatinine values act as in internal standard for the glomular filtration rate (GFR) of blood in the kidneys and the secretion into urine. As a consequence, highly concentrated urine (>100 mg Cr/dL^{-1}) has to be diluted to be normalized at 100 μg Cr/analysis. It is well accepted that creatinine is the best marker to normalize urine samples with different creatinine values for comparative studies.[39,40] However, we have observed that the linearity of creatinine as a marker for GFR becomes less appropriate for urine samples with creatinine values lower than 100 mg Cr/dL^{-1}. This observation is currently under study.

It is well-known that concentrations of biogenic amines and their metabolites in urine could differ in concentrations.[35,36] As a consequence, the derivatization of urine samples with one concentration of glutaric aldehyde (66.7 mM final) will conjugate a proportional amount of each biogenic amine. We prepared calibration curves based on fixed amounts of the biogenic amines and the concentrations calculated from the calibration curve will provide μmol biogenic amines/g Creatinine. However, only in two cases, glycine and taurine, the amounts of these two biogenic amines were in concordance with published amounts of glycine and taurine in urine. This was further substantiated in a comparative study with HPLC (see Figure 3). The amounts of the other three biogenic amines were not in concordance with published data as a consequence of one derivatization procedure for all five assays, which was apparently not optimal to quantify the other three amines. We do not want to suggest that we quantify the absolute amounts of biogenic amines, (except for glycine and taurine), and therefore prefer to express their amounts in a (semi)quantitative way by arbitrary units, which will suffice for screenings assays. (See also the Conclusions section).

The Reliability of the Immunoassays for Screening Agmatine, β-PEA, Taurine, GABA, and Glycine. Immunoassays are inherently less precise than chromatographic assays due to the greater interassay variation imprecision.[41,42] Recent publications have recommended that the 20% acceptance limit be made less restrictive for immunoassays.[42] The intra-assay and inter assay precision data for our five immunoassays were determined by multifold measurements ($n = 24$) of each of the 160 urine samples. As can be seen from Table 2, the combined precision data (CV 3.9−9.8%) is well within the accepted limit of immunoassay

(38) Migneault, I; Dartiguenave, C; Bertrand, J; Waldron, K. *BioTechniques* **2004**, *37*, 790–802.
(39) Ropero-Miller, J. D.; Paget-Wilkes, H.; Doering, P. L.; Goldberger, B. A. *Clin. Chem.* **2000**, *46*, 295–297.
(40) Goldberger, B. A.; Loewenthal, B.; Darwin, W. D.; Cone, E. J. *Clin. Chem.* **1995**, *41*, 116–117.
(41) Braggio, S.; Barnaby, R. J.; Grossi, P.; Cugola, M. *J. Pharm. Biomed. Anal.* **1996**, *14*, 375–388.
(42) Findlay, J. W. A.; Smith, W. C.; Lee, J. W.; Nordblom, G. D.; Das, I.; DeSilva, B. S.; Khan, M. N.; Bowsher, R. R. *J. Pharm. Biomed. Anal.* **2000**, *21*, 1249–1273.

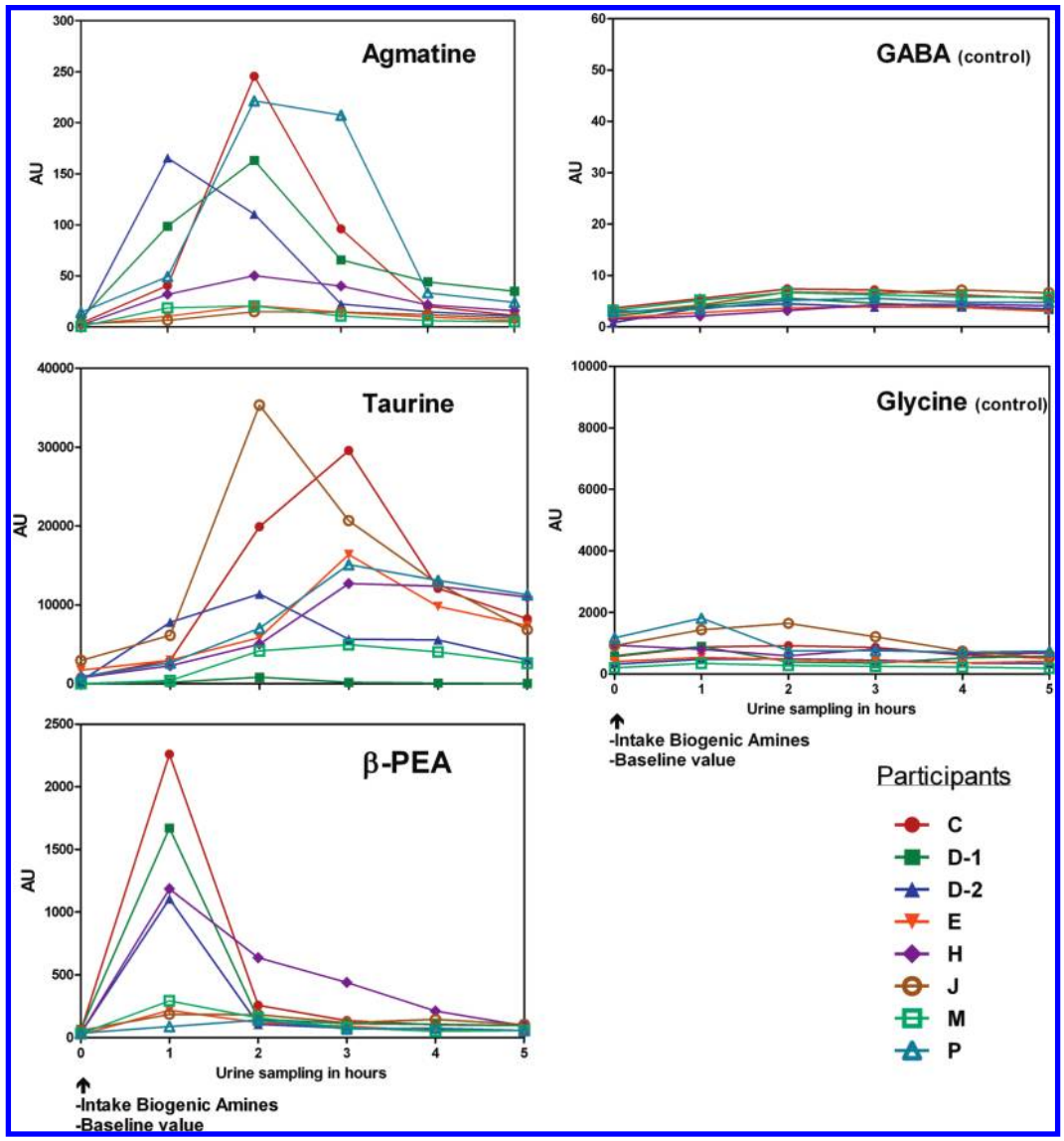

Figure 4. Clearance and turnover rate of three biogenic amines agmatine, taurine, and β-PEA as detected in urine after oral intake of 300 mg agmatine, 600 mg taurine, and 1200 mg β-PEA at the same time. Urine samples of each healthy participant (n-8) were collected prior to intake and over a 5 h period. Glycine and GABA were used as control biogenic amines. (AU = arbitrary units)

reliability.[42] The linearity of the urinary c-ELISA analysis of the five biogenic amines was estimated in four groups of urine representing four ranges of creatinine concentrations. Each dilution was measured in triplicate, and as can be seen in Table 3, the ratio between concentration and dilution of samples did not significantly deviate from the linearity across the concentration studied. The difference between the multiple dilutions is in agreement with validation criteria of immunoassays.[42] A majority of assays for the determination of small molecules in biological fluids needs a preclearance and/or a specific isolation of the compounds of interest before derivatization.[37,43−45] This could have consequences for the efficiency of recovery of the analyte, but has the advantage that it reduces the matrix effect of biological fluids. The derivatization in our immunoassays is performed on whole urine and we have investigated the effect of the urine matrix on the specificity and recovery of each immunoassay. Eight urine samples were spiked with a constant amount of biogenic amines and tested in each of the assay for specificity and recovery. As can be seen in Figure 2, each assay has its own endogenous profile of biogenic amines (nonspiked) which remain unaffected with the spiked heterologous biogenic amines (see Figure 2). The recovery of biogenic amines in the homologous assay was between 76 and 154%.

Comparison c-ELISA and HPLC for Glycine and Taurine. We describe in this paper the (semi)quantitative measurements of five biogenic amines based on one derivatization procedure. Since the concentration of both taurine and glycine as calculated from a standard curve prepared in situ, were in concordance with what has been published before[35,36] we performed a comparative study between our assay and HPLC. The correlation coefficients for both taurine and glycine analyses show excellent agreement between the two methods ($r^2 > 0.96$). The agreement between the two assays also indicates the absence of interfering

(43) Vuorensola, K.; Sirén, H.; Karjalainen, U. *J. Chromatogr., B* **2003**, *788*, 277–289.
(44) Westermann, J.; Huble, W.; Kaiser, N.; Salewski, L. *Clin. Lab.* **2002**, *48*, 61–71.
(45) Westerink, B. H. C.; Bosker, F. J.; O'Hanlon, J. F. *Clin. Chem.* **1982**, *28*, 1745–1748.

substances (matrix effect) of whole urine on the assay performance after derivatization, and the high specificity of the antibodies.

In Vivo Significance of Immunoassays for Agmatine, β-PEA, Taurine, GABA, and Glycine. Since the five assays can be considered as specific we used the immunoassays to investigate to which degree the secreted amounts of biogenic amines are correlated biologically (Pearson's correlation). The results are summarized in Table 3 and show that we could identify pairs of biogenic amines with a biological relationship of >30%, while other pairs of biogenic amines displayed a low relationship (<10%). Glycine and GABA shows a correlation of 33.2%, although speculative, this correlation could be attributed to the fact that both neurotransmitters are considered inhibitory neurotransmitters and could have similar physiological or cellular activities in vivo.[46,47]

The in vivo significance of our assays was further validated in a study of urinary clearance of β-PEA, agmatine, and taurine after oral intake by eight healthy individuals. The results as presented in Figure 4 can be interpreted as follows: despite the secretion of large amounts of β-PEA, agmatine and taurine in most of the participants, baseline values for the control biogenic amines GABA and glycine remain normal, indicating the absence of nonspecific matrix effect on the immunoassays. The profile for clearance of β-PEA in a sharp early peak differs significantly from agmatine and taurine, which were secreted into urine in a slower fashion. This also indicates the independency of each assay. It was our aim to demonstrate the clinical significance and specificity of urinary measurement of biogenic amines after intake of a mixture of β-PEA, agmatine, and taurine. Any clinical or biological implication of the observed clearance profiles is speculative and is beyond the scope of this study.

(46) Muller, E.; Le-Corronc, H.; Legendre, P. *Front Mol. Neurosci.* **2008**, *1*, 3.
(47) Bischofberger, J. P.; Sandkühler, J. *Science* **1998**, *281*, 419–424.
(48) Lechin, F.; Dijs van der, B.; Lechin, M. E. *Psychother. Psychosom.* **1996**, *65*, 293–318.
(49) Salomé, N.; Viltart, O.; Lesage, J.; Landgraf, R.; Vieau, D.; Laborie, C. *Psychoneuroendrocrinology* **2006**, *6*, 724–735.
(50) Cook, I. A. *Primary Psychiatry* **2008**, *15*, 54–59.
(51) Chau, C. H.; Rixie, O; McLeod, H; Figg, W. D. *Clin. Cancer Res.* **2008**, *14*, 5967–5976.

CONCLUSIONS

At present, it is generally accepted that testing for biogenic amines can result in relevant information on neurological processes, or pharmacological response to a therapeutic intervention.[12,24,48−50] We describe here five different immunoassays for a reproducible screening and (semi)quantitative measurements of β-PEA, agmatine, glycine, GABA and taurine in derivatized urine samples. We have used one simple derivatization procedure of urine that allows us to reproducibly analyze each urine sample in multiple assay formats. These new immunoassays can perform urinary screening of five different biogenic amines accurately, without preclearance of the urine samples. As a consequence for using a fixed concentration of glutaric aldehyde in the derivatization step, biogenic amines are measured in a (semi)quantitative way for GABA, agmatine, and β-PEA, expressed in arbitrary units and in a quantitative way for glycine and taurine. Quantitative assays for all biogenic amines would require optimal derivatization for each of the biogenic amine of interest. However, since biogenic amines in plasma and urine are not immediate indicators for central nervous system activity, but a reflection of activity of both the sympathetic and parasympathetic nervous system, we consider biogenic amines as indirect or surrogate biomarkers for various clinical and neurological conditions.[51] The screening assays described here, based on one derivatization procedure of whole urine, are useful for clinical application as well as for basic research where a fast, simple, and accurate assay for the screening of biogenic amines in urine is required.

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