analytical chemistry

Article

pubs.acs.org/ac

Development and Characterization of an Implantable Biosensor for Telemetric Monitoring of Ethanol in the Brain of Freely Moving Rats

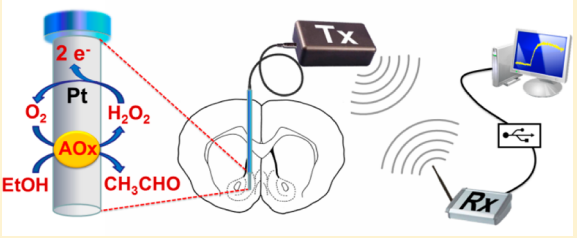
Gaia Rocchitta,[†] Ottavio Secchi,[†] Maria Domenica Alvau,[†] Rossana Migheli,[†] Giammario Calia,[†] Gianfranco Bazzu,[†] Donatella Farina,[†] Maria Speranza Desole,[†] Robert D. O'Neill,[‡] and Pier Andrea Serra*[,†]

[†]Department of Clinical and Experimental Medicine, Medical School, University of Sassari, Viale S. Pietro 43/b, 07100 Sassari, Italy

[‡]UCD School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland

Supporting Information

ABSTRACT: Ethanol is one of the most widespread psychotropic agents in western society. While its psychoactive effects are mainly associated with GABAergic and glutamatergic systems, the positive reinforcing properties of ethanol are related to activation of mesolimbic dopaminergic pathways resulting in a release of dopamine in the *nucleus accumbens*. Given these neurobiological implications, the detection of ethanol in brain extracellular fluid (ECF) is of great importance. In this study, we describe the development and characterization of an implantable biosensor for the amperometric detection of brain ethanol in real time. Ten different designs were characterized in vitro in terms of Michaelis–Menten kinetics (V_{MAX} and K_M), sensitivity (linear region slope, limit of detection (LOD), and limit of quantification (LOQ)), and electroactive interference blocking. The same parameters were monitored in selected designs up to 28 days after fabrication in order to quantify their stability. Finally, the best performing biosensor design was selected for implantation in the *nucleus accumbens* and coupled with a previously developed telemetric device for the real-time monitoring of ethanol in freely moving, untethered rats. Ethanol was then administered systemically to animals, either alone or in combination with ranitidine (an alcohol dehydrogenase inhibitor) while the biosensor signal was continuously recorded. The implanted biosensor, integrated in the low-cost telemetry system, was demonstrated to be a reliable device for the short-time monitoring of exogenous ethanol in brain ECF and represents a new generation of analytical tools for studying ethanol toxicokinetics and the effect of drugs on brain ethanol levels.



Alcoholic drinks are usually considered a behavioral stimulant; on the contrary, however, ethanol is primarily a CNS depressant.[1] The psychoactive effects of ethanol may result from enhancement of the effects of GABA, the major inhibitory neurotransmitter present in the brain.[1] The consumption of ethanol is also associated with the activation of the mesolimbic dopaminergic "reward" system and the consequent increase in extracellular dopamine levels in the *nucleus accumbens*,[2] a mechanism which plays a pivotal role in the development of alcohol abuse and addiction.[3−5] Immediately following its administration, ethanol starts to be metabolized first to acetaldehyde, subsequently converted to acetic acid, mainly by alcohol dehydrogenase and aldehyde dehydrogenase enzymes. Cytochrome P450 (CYP2E1) and catalase can also convert ethanol to acetaldehyde, reducing its effects in the CNS. Because of the significance of its effects and toxicokinetics, the detection of ethanol in the brain is of particular interest, especially its concentration dynamics. One of the most common techniques used to obtain this kind of information is brain microdialysis, by monitoring the composition of the interstitial fluid through a dialysis probe inserted into brain tissues.[6] Indeed, in vivo microdialysis has been applied to investigate the relationship between ethanol-associated behavior and its underlying neurochemistry.[7] Microdialysis is very powerful because it allows the simultaneous sampling and unambiguous assignment of a wide range of chemical species in the extracellular space of the tissue. Furthermore, local perfusion of ethanol through the microdialysis probe into a defined brain region has also been used to investigate the potential involvement of local mechanisms.[7] Although microdialysis possesses many advantages, it also has significant drawbacks. For example, temporal resolution is clearly a major limiting factor: several minutes are usually needed to collect a sufficient volume in order to analyze the sample with a chosen technique. In addition, microdialysis is very commonly done under nonequilibrium conditions, and dialysate concentrations are only a fraction of real concentrations in the medium surrounding the microdialysis probe.[8]

The study of brain ethanol concentration dynamics in real time, therefore, requires a different approach. In this regard, amperometric biosensors are proving useful as they allow detection of a range of nonelectroactive analytes by virtue of the presence of a biological element (usually an enzyme) that

Received: May 16, 2012
Accepted: July 23, 2012
Published: July 23, 2012

   dx.doi.org/10.1021/ac301253h | *Anal. Chem.* 2012, 84, 7072−7079

Table 1. In Vitro Characterization of Ten Different Biosensor Designs at Day 1 (n = 4 for each group) in Terms of the Apparent Michaelis–Menten Kinetic Parameters (V_{MAX} and K_M, eq 4) and AA Interference Parameters (1 mM Current and ΔI for the Transition from 0.5 to 1 mM AA) for Each Design[a]

	in vitro electrochemical characterization at day 1				
	Michaelis–Menten kinetics			AA interference	
biosensor design	V_{MAX} (nA)	K_M (mM)	R^2	AA 1 mM (nA)	AA ΔI (nA)
1: Pt_c/AOx_{10}/PPD	3.0 ± 0.8	82 ± 10	0.862	1.90 ± 0.21	0.57 ± 0.18
2: Pt_c/PPD/$[AOx]_{10}$/PU(1%)	3.8 ± 0.3	73 ± 12	0.983	0.99 ± 0.02	0.19 ± 0.01
3: Pt_c/PPD/$[PEI(1\%)/AOx]_5$/PU(1%)	13.0 ± 0.9	23 ± 4	0.968	1.21 ± 0.11	0.23 ± 0.13
4: Pt_c/PPD/$[PEI(1\%)/AOx]_{10}$/PU(1%)	35.7 ± 0.7	27 ± 1	0.997	2.01 ± 0.25	0.49 ± 0.21
5: Pt_c/PPD/$[PEI(1\%)/AOx]_{15}$/PU(1%)	42.9 ± 3.4	36 ± 6	0.938	2.55 ± 0.15	0.84 ± 0.15
6: Pt_c/PPD/$[PEI(1\%) + AOx]_{10}$/PU(1%)	6.6 ± 0.2	58 ± 13	0.933	1.37 ± 0.11	0.72 ± 0.13
7: Pt_c/PPD/$[Glyc(1\%)/AOx]_{10}$/PU(1%)	2.7 ± 0.3	76 ± 12	0.969	1.70 ± 0.10	0.63 ± 0.08
8: Pt_c/PPD/$[\{PEI(1\%) + Glyc(1\%)\}/AOx]_{10}$/PU(1%)	116 ± 6	41 ± 5	0.978	1.72 ± 0.36	0.55 ± 0.12
9: Pt_c/PPD/$[PEI(1\%)/AOx]_{10}$/BSA(10%)/GA(1%)	56 ± 2	36 ± 2	0.986	0.80 ± 0.08	0.17 ± 0.03
10: Pt_c/PPD/$[\{PEI(1\%) + Glyc(1\%)\}/AOx]_{10}$/BSA(10%)/GA(1%)	52 ± 2	28 ± 3	0.978	0.93 ± 0.06	0.19 ± 0.04

[a]Abbreviations as in Figure 1. In the notation used here, for example, PEI/AOx represents a dip in the PEI solution and a subsequent dip in the AOx solution, whereas PEI + AOx denotes a dip in the pre-mixed PEI + AOx solution.

can interact specifically with a target, producing an electroactive byproduct.[9−13] In this study, different designs of alcohol biosensors were developed and characterized, where the biological element was alcohol oxidase (AOx; EC 1.1.3.13), a flavoprotein with eight subunits, each containing a flavin adenine dinucleotide (FAD) cofactor molecule that plays a pivotal role in the enzyme activity (reaction 1). AOx is capable of catalyzing the oxidation of primary, aliphatic short-chain alcohols (such as ethanol or methanol) to the respective aldehydes (reactions 1 and 2).

$$RCH_2OH + AOx/FAD \rightarrow RCHO + AOx/FADH_2 \quad (1)$$

$$AOx/FADH_2 + O_2 \rightarrow AOx/FAD + H_2O_2 \quad (2)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

The hydrogen peroxide produced by reaction 2 can be amperometrically detected at, for example, a Pt surface by applying a high fixed anodic overpotential (reaction 3).

As discussed previously,[14] the form of the Michaelis–Menten enzyme kinetics equation, even for a two-substrate system (reactions 1 and 2), can be expressed for the alcohol EtOH as eq 4,

$$v = \frac{V_{MAX}}{1 + \frac{K_M(EtOH)}{[EtOH]}} \quad (4)$$

which represents the rate of formation of the product and, therefore, of hydrogen peroxide, a fraction of which is detected at the electrode surface.[15] Thus, eq 4 is usually expressed in terms of current or current density, for enzymes immobilized on amperometric biosensors, and V_{MAX} is the maximum biosensor signal recorded when the enzyme is saturated with substrate.[14] Therefore, different values of V_{MAX} determined under the same conditions can reflect differences in the activity of enzyme on the surface. On the other hand, K_M is the apparent Michaelis constant and is determined by factors such as the binding of substrate to the enzyme. Phenomenologically, K_M is the concentration of substrate that gives half the V_{MAX} response (see eq 4). The apparent Michaelis constant is also important for determining the linear region slope (LRS), and LRS can be approximated by V_{MAX}/K_M.[14]

Several papers in the literature have studied ethanol pharmacokinetics and concentrations in the brain after systemic injection and showed that ethanol in the CNS could reach concentrations of about 30 mM.[7,16−18] In all the above-cited papers, neither "real-time" techniques nor implanted biosensors were used. Thus, the aim of the present study was to develop and characterize an implantable biosensor capable of detecting ethanol in a linear concentration range up to 40 mM under normoxic conditions; a full analysis of the oxygen dependence of AOx-based biosensors will be reported separately.

EXPERIMENTAL SECTION

Chemicals. All chemicals were purchased from Sigma-Aldrich (Milano, Italy). PBS (pH 7.4) was prepared by dissolving NaCl (8.9 g), NaOH (1.76 g), and NaH_2PO_4 (6.89 g) in 1 L of bidistilled water. The stock solution of alcohol oxidase from *Hansenula polymorpha* (AOx, EC 1.1.3.13) was prepared as 200 U mL^{-1} in PBS. Ethanol solutions (1 M and 10 mM) were obtained from absolute ethanol by dilution in bidistilled water. Ascorbic acid solution (AA, 100 mM) was prepared by dissolving L-ascorbic acid in 0.01 M HCl. Polyethyleneimine (PEI) solution 1% w/v was obtained by diluting the stock solution (50% w/v) in bidistilled water. Glycerol (glyc, 1% w/v), glutaraldehyde (GA, 1% w/v), and bovine serum albumin (BSA, 10% w/v) solutions were prepared in bidistilled water. *ortho*-Phenylenediamine monomer solution (oPD, 300 mM) was prepared in deoxygenated PBS. Special caution is needed when phenylenediamine and glutaraldehyde are used; please refer to their respective material safety data literature. Polyurethane solution (PU, 1% w/v) was obtained dissolving PU beads in tetrahydrofuran (THF). Ranitidine hydrochloride solution was prepared in saline (0.9% w/v) immediately before in vivo administration. Teflon-coated platinum (90% Pt, 10% Ir; ⌀ = 125 μm) and silver wires (⌀ = 250 μm) were purchased from Advent Research Materials (Eynsham, England).

Biosensor Fabrication and Characterization. Ten alcohol biosensor designs were developed, all based on the same cylindrical geometry (1 mm length and 125 μm diameter) obtained by exposing the bare metal by cutting away the Teflon insulation. Different strategies were implemented in order to differentiate biosensor design-modifying components and

dx.doi.org/10.1021/ac301253h | Anal. Chem. 2012, 84, 7072−7079

Table 2. In Vitro Ethanol Sensitivity of Ten Different Biosensor Designs at Day 1 (n = 4 for Each Group) in Terms of the Linear Region Slope (LRS) and LOD and LOQ Calculated as 3.3σ/LRS and 10σ/LRS, Respectively (Eqs 5 and 6)[a]

	in vitro electrochemical characterization at day 1				
	linear regression			limits of detection (LOD) and quantification (LOQ)	
biosensor design	concentration limit for LRS (mM)	LRS (nA mM^{-1})	R^2	LOD ± SEM (mmol L^{-1})	LOQ ± SEM (mmol L^{-1})
1: $Pt_c/AOx_{10}/PPD$	20	0.05 ± 0.05	0.879	2.7 ± 1.1	8.1 ± 3.3
2: $Pt_c/PPD/[AOx]_{10}/PU(1\%)$	40	0.032 ± 0.001	0.994	3.0 ± 1.4	9.0 ± 4.1
3: $Pt_c/PPD/[PEI(1\%)/AOx]_5/PU(1\%)$	10	0.370 ± 0.005	0.998	0.56 ± 0.04	1.7 ± 0.1
4: $Pt_c/PPD/[PEI(1\%)/AOx]_{10}/PU(1\%)$	40	0.63 ± 0.03	0.966	0.32 ± 0.10	1.0 ± 0.3
5: $Pt_c/PPD/[PEI(1\%)/AOx]_{15}/PU(1\%)$	40	0.81 ± 0.02	0.982	0.18 ± 0.06	0.6 ± 0.2
6: $Pt_c/PPD/[PEI(1\%) + AOx]_{10}/PU(1\%)$	40	0.114 ± 0.001	0.994	1.1 ± 0.2	3.3 ± 0.5
7: $Pt_c/PPD/[Glyc(1\%)/AOx]_{10}/PU(1\%)$	20	0.023 ± 0.002	0.907	2.8 ± 0.3	8.4 ± 0.7
8: $Pt_c/PPD/[\{PEI(1\%) + Glyc(1\%)\}/AOx]_{10}/PU(1\%)$	40	1.73 ± 0.08	0.996	0.09 ± 0.03	0.27 ± 0.09
9: $Pt_c/PPD/[PEI(1\%)/AOx]_{10}/BSA(10\%)/GA(1\%)$	40	0.78 ± 0.04	0.973	0.57 ± 0.14	1.7 ± 0.4
10: $Pt_c/PPD/[\{PEI(1\%) + Glyc(1\%)\}/AOx]_{10}/BSA(10\%)/GA(1\%)$	50	0.76 ± 0.04	0.967	0.35 ± 0.23	1.0 ± 0.7

[a]Abbreviations and notation as in Figure 1 and Table 1.

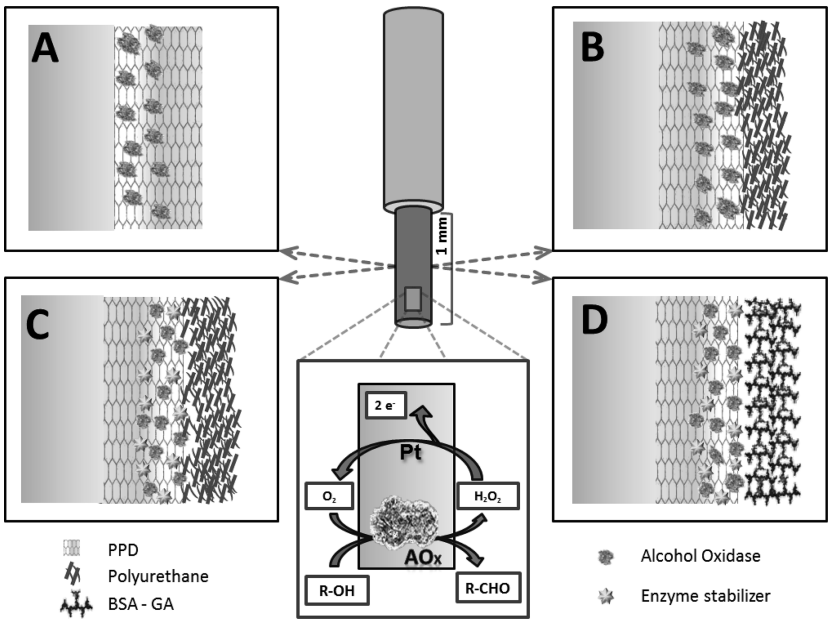


Figure 1. Schematic representation of the four main designs of implantable alcohol biosensors developed and characterized in this study. A: Pt_c/AOx/PPD; B: Pt_c/PPD/AOx/PU; C: Pt_c/PPD/$[PEI/AOx]_x$/PU, Pt_c/PPD/$[Glyc/AOx]_x$/PU, and Pt_c/PPD/$[\{PEI + Glyc\}/AOx]_x$/PU; D: Pt_c/PPD/$[PEI/AOx]_x$/BSA/GA. Pt_c: 1 mm long, 250 μm diameter Pt cylinder; AOx: alcohol oxidase; PPD: poly-*ortho*-phenylenediamine; PU: polyurethane; Glyc: glycerol; PEI: polyethyleneimine; BSA: bovine serum albumin; GA: glutaraldehyde. Enzyme stabilizer: PEI or/and Glyc. The subscript "x" represents the number of dip−evaporation deposition steps.

procedures. All designs had in common the same strategy for blocking AA interference: the electro-deposition of a poly-*ortho*-phenylenediamine (PPD) nanometer-thick membrane.[19] The PPD electrosynthesis was performed as follows: stock solutions of oPD monomer (300 mM) were freshly prepared immediately before electropolymerization, carried out amperometrically at +0.7 V versus Ag/AgCl for 15 min. The deposition of the PPD occurred at different stages of biosensor manufacture for various biosensor designs. The different designs are shown in Tables 1 and 2, and the main features in four groups are as follows: (1) The AOx was deposited on the metal surface by dip−evaporation from the enzyme solution. Afterward, the PPD layer was deposited as described above (Figure 1A). (2) The PPD layer was deposited on bare metal, and then, the enzyme was loaded by dip−evaporation. In order to immobilize the enzyme, the biosensor was quickly dipped in a 1% PU solution (Figure 1B). (3) The PPD electrosynthesis was first performed on bare Pt, and then, the AOx was loaded in conjunction with an enzyme stabilizer (PEI, glycerol, or both). Finally, the biosensor was dipped in a 1% PU solution (Figure 1C). (4) The PPD polymer was electrodeposited on bare metal, and then, the AOx was loaded in conjunction with an enzyme stabilizer. The final layer was deposited by dipping biosensors in a BSA (10%) and GA (1%) solution to promote cross-linking and immobilization of the enzyme (Figure 1D).

All biosensors were characterized in vitro for ethanol response and AA interference blocking. AA was chosen as the archetypal interference species for biosensor function because of its relatively high levels in brain extracellular fluid (ECF)

dx.doi.org/10.1021/ac301253h | *Anal. Chem.* 2012, 84, 7072−7079

compared with other electroactive molecules.[20] The electrochemical studies were performed in a cell consisting of four biosensors as working electrodes (WE), an Ag/AgCl (NaCl, 3 M) electrode as reference electrode (RE), and a large surface-area Pt wire as auxiliary electrode (AE). Both electropolymerization and calibrations were performed by applying a constant potential of +0.7 V versus Ag/AgCl, using the four-channel equipment (eDAQ QuadStat, e-Corder 410, eDAQ, Australia). In vitro response to ethanol was assessed by means of a full calibration (0−120 mM; see Figure 2A), performed

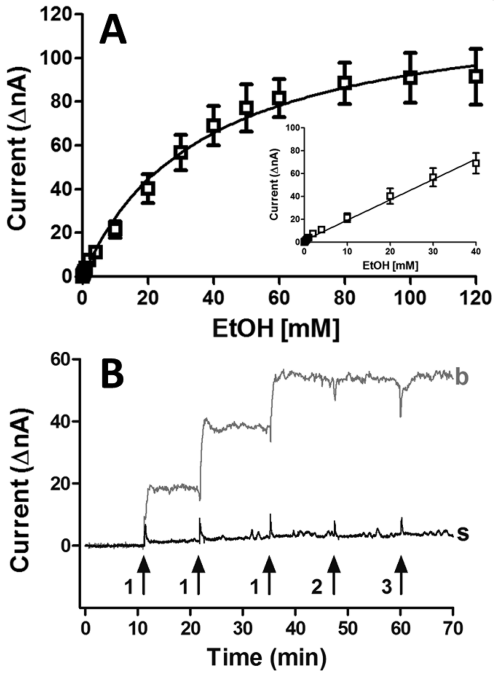


Figure 2. In vitro calibration (panel A) of implanted ethanol biosensors (Pt_c/PPD/[{PEI + Glyc}/AOx]$_{10}$/PU, design 8) performed immediately before neurosurgery (day 0). The resulting calibration curve showed reasonable Michaelis−Menten kinetics (R^2 = 0.965) with a V_{MAX} of 126 ± 10 nA and a K_M of 38 ± 7 (n = 4). The response to pharmacologically relevant concentrations of ethanol (0−40 mM; panel A, inset) revealed good linearity (R^2 = 0.992) with a slope of 1.78 ± 0.44 nA mM^{-1}. In a separate series of experiments (panel B), the potential interference of ranitidine was studied in vitro on a biosensor (Pt_c/PPD/[{PEI + Glyc}/AOx]$_{10}$/PU, b) and on an enzyme-free sentinel microsensor (Pt_c/PPD/[PEI + Glyc]$_{10}$/PU, s) to demonstrate that the drug did not interfere with AOx activity or had electroactive properties at the concentrations used. For this purpose, three successive injections of ethanol (10, 20, and 30 mM, arrows 1) were made in PBS succeeded by two injections of ranitidine hydrochloride (1 and 10 μM, arrows 2 and 3, respectively).

with 16 successive injections of known volumes of ethanol stock solutions (10 mM and 1 M) in 20 mL of fresh PBS at room temperature (23 ± 2 °C) and fitted to eq 4, using nonlinear regression. Injections of 0.5 mM and 1 mM of AA were made in fresh PBS, in order to assess the blocking ability of the biosensor against the main interference species present in rat brain.[21]

All biosensors were calibrated immediately after their construction (day 0) and after 24 h (day 1). Among all studied designs (at day 1), the most promising were selected on the basis of the respective values of parameters such as K_M, V_{MAX}, linear region slope, AA blocking, limit of detection (LOD), and limit of quantification (LOQ), as shown in Tables 1 and 2. Four selected biosensor designs were chosen on the basis of their better ethanol sensitivity and AA shielding capability for a stability study, carried out by means of full calibrations of ethanol and AA from day 2 up to day 28 from manufacture (Figures 2-S and 3-S of Supporting Information). On the basis of these in vitro characterizations, the most promising biosensor was implanted for in vivo experiments.

Telemetry System. The telemetry system used in this study was based on previous designs.[21,23] A detailed description of the biotelemetric device is provided in the Supporting Information. During in vivo experiments, sensor data were acquired second-by-second, averaged, and transmitted to the personal computer every 15 s.

Animals. Male Sprague−Dawley rats (Charles Rivers, Milan, Italy), weighing 280−330 g, were used for in vivo experiments. The rats were maintained under standard animal care conditions (12 h/12 h light/dark cycle, light on at 07.00 h; room temperature 21 °C), with food and water provided ad libitum. Prior to the start of any experimental procedure, the health of the animals was assessed according to published guidelines.[24] All procedures were specifically licensed under the European Community directive 86/609 included in Decreto No. 116/1992 of the Italian Ministry of Public Health.

Stereotaxic Surgery and in Vivo Experimental Procedures. The detailed surgical procedure was described in previous studies.[21,22] The alcohol biosensor was implanted under chloral hydrate (400 mg kg^{-1} i.p.) anesthesia in the right *nucleus accumbens* (shell) using the following coordinates from the atlas of Paxinos and Watson:[25] A/P +1.7 from the bregma, −0.9 M/L, and −7.6 D/V from the dura. After surgery, the animals were housed in large plastic bowls in a temperature- and light-controlled environment with free access to food and water.

The in vivo experiments started 24 h after surgery (day 1) with the animal in its own bowl: this arrangement, which allowed the rat free movement, reduced handling and other forms of stress.[21,22] The alcohol biosensor was polarized at +0.7 V, and the telemetric signal was recorded until a stable baseline was obtained. In a control group of animals (n = 3), intragastric (i.g.) ethanol (1 g kg^{-1}) was administered 20 min after the intraperitoneal (i.p.) injection of saline (1.5 mL). Using a similar protocol, a selective inhibitor of alcohol dehydrogenase, ranitidine (30 mg kg^{-1} dissolved in 1.5 mL of saline), was administered i.p.[26] in a second group of rats (n = 3). The biosensor current was monitored for 2 h after ethanol administration.

Histology. Following the experiments, rats were sacrificed with an overdose of chloral hydrate (800 mg kg^{-1} i.p.). The location of biosensors was confirmed by postmortem histology. Brains were fixed in formal saline, and 50 mm coronal sections were made with a cryostat. The slices were stained with cresyl violet and examined under a microscope.

Statistical Analysis. Currents were expressed in nanoampere (nA) and given as baseline-subtracted values ± standard error of the mean (nA or ΔnA ± SEM). The AA ΔI value represents the difference between the current resulting from the injection of 1 mM and 0.5 mM of AA in the electrochemical cell, as discussed previously.[20] The limit of detection (LOD, eq 5) and limit of quantification (LOQ, eq 6) were determined using a statistical method based on the standard deviation (σ) of the response and the linear region slope (LRS) of the calibration curve.[27]

dx.doi.org/10.1021/ac301253h | *Anal. Chem.* 2012, 84, 7072−7079

$$\mathrm{LOD} = 3.3\sigma/\mathrm{LRS} \quad (5)$$

$$\mathrm{LOQ} = 10\sigma/\mathrm{LRS} \quad (6)$$

In vivo statistical significance between groups was evaluated by averaging signals at different times and calculating unpaired *t*-tests, as illustrated in Figure 3.

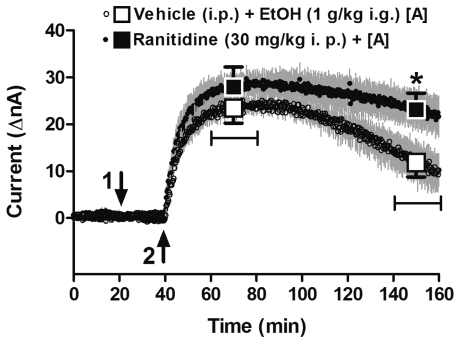


Figure 3. In vivo response of ethanol biosensors implanted in the right *nucleus accumbens* after systemic administration of ethanol (1 g kg^{-1} i. g., arrow 2). The selected biosensor (Pt_c/PPD/[{PEI + Glyc}/AOx]$_{10}$/PU, design 8) was prepared, calibrated in vitro, and implanted immediately after calibration (day 0). The in vivo experiments were performed 24 h after neurosurgery (day 1) on untethered, freely moving animals (n = 3 per group). Each curve represents the mean ± SEM of baseline-subtracted biosensor currents (ΔnA, n = 3). In one group of rats, an alcohol dehydrogenase inhibitor (ranitidine, 30 mg kg^{-1} i.p., arrow 1) was administered 20 min before ethanol (●). In the control group (○), the vehicle (saline solution, arrow 1) was administered instead of ranitidine. Statistical differences were evaluated by averaging currents at different times (60−80 min and 140−180 min, n = 80) and calculating unpaired *t*-tests between groups (* = p < 0.05 vs ranitidine group at 140−180 min).

RESULTS

In Vitro Responses to Ethanol and AA for Different Biosensor Designs. Since day 1 would be the day of the in vivo monitoring of the implanted biosensors (see in vivo experimental procedures), we decided to compare the responses to ethanol and AA for different biosensor designs (n = 4 for each group) at this time. These results are summarized in Tables 1 and 2. The basic design (Figure 1A; Table 1 and 2, design 1), which demonstrates enzyme loading on bare metal followed by electro-deposition of the PPD layer, gave a low response in terms of V_{MAX} (2.95 ± 0.75 nA, R^2= 0.962) and slope (0.05 ± 0.05 nA mM^{-1}, R^2= 0.979) and high K_M (82 ± 10 mM); the response to 1 mM AA was 1.90 ± 0.21 nA. The disposition of PU as a "containment net" over the PPD layer (Figure 1B; Tables 1 and 2, design 2) did not give a significant increase in V_{MAX} (3.84 ± 0.30 nA, R^2= 0.993), slope (0.032 ± 0.035 nA mM^{-1}, R^2= 0.994) or any change in K_M (73 ± 12 mM). This design, however, showed one of the best shielding powers against 1 mM AA (0.99 ± 0.02 nA, with a ΔI of 0.19 ± 0.01 nA). The addition to the design of the enzyme stabilizer PEI (Figure 1C; Tables 1 and 2, design 3) caused a significant increase in V_{MAX}, K_M, and slope relative to the basic design. The response to 1 mM AA (and ΔI) was similar to design 2. Increasing the number of PEI/AOx dips to 10 (design 4) and 15 (design 5) resulted in a proportional increase in V_{MAX}, K_M, and slope but also in AA oxidation. Mixing PEI and AOx together (design 6) induced a partial precipitation of the enzyme resulting in no benefits for biosensor performance. The substitution of PEI with another enzyme stabilizer (glycerol, design 7) caused a decrease in terms of V_{MAX} (2.65 ± 0.25 nA, R^2= 0.969) and slope (0.023 ± 0.002 nA mM^{-1}, R^2= 0.907) while causing an increase in K_M (76 ± 12 mM). AA blocking properties were unaffected. An interesting response was obtained when PEI and glycerol were mixed together (design 8). The presence of the mixture caused a significant increase in V_{MAX} (115 ± 6 nA, R^2= 0.978), slope (1.73 ± 0.08 nA mM^{-1}, R^2= 0.996), and K_M (41 ± 5 mM) compared to PEI alone. The response to AA was similar to other designs. The substitution of PU with BSA cross-linked with GA as the "containment net" (Figure 1D; Tables 1 and 2, designs 9 and 10) gave results comparable with design 5 in terms of K_M and slope, while producing a slightly higher V_{MAX} and a lower response to AA interference.

Limits of Detection (LOD) and Quantification (LOQ) and Response Time. The LOD and LOQ values (eqs 5 and 6) were determined at day 1 and are shown in Table 2. The basic design (design 1) had a LOD of 2.7 ± 1.1 mM and a LOQ of 8.1 ± 3.3 mM; design 2 showed similar values. The introduction of PEI (designs 3−5) dramatically reduced the LOD and LOQ down to 182 ± 62 μM and 551 ± 186 μM, respectively (design 5). The increase in ethanol sensitivity was directly proportional to the number of PEI/AOx dips (5−15), resulting in a reduction of the LOD value of −38 ± 5 μM per dip (R^2 = 0.980, n = 4) and an increase in the LRS (+44 ± 5 pA mM^{-1} per dip; R^2 = 0.989, n = 4). Mixing PEI and AOx (design 6) and the substitution of PEI with glycerol (design 7) caused an increase in LOD and LOQ when compared with design 4. In contrast, mixing PEI with glycerol (design 8) resulted in the lowest observed LOD and LOQ values (88 ± 28 μM and 265 ± 86 μM, respectively). The substitution of PU with BSA cross-linked with GA (designs 9 and 10) caused a doubling of LOD and LOQ values compared with the corresponding PU design (designs 4 and 8, respectively).

Response times were recorded in constantly stirred solution, using a data acquisition rate of >100 Hz. A $t_{90\%}$ parameter was defined as the time taken for the analyte response to reach 90% of its maximum value from the start of the current upswing, similar to a definition used previously.[28] Design 8 was used here because of its use in in vivo studies. The response time for the addition of ethanol aliquots was fast ($t_{90\%}$ = 1.6 ± 0.7 s, n = 20; 4 biosensors × 5 determinations; mean ± SD) and suitable for real-time neurochemical monitoring.

Stability of Biosensor Michaelis−Menten Kinetics, Sensitivity, and Selectivity. In order to ascertain the stability of biosensors over time, a study on aging was conducted on selected designs that displayed the best V_{MAX}, K_M, slope, and AA shielding properties at day 1 (Tables 1 and 2). Michaelis−Menten kinetic parameters (V_{MAX} and K_M), sensitivity (LRS), and 1 mM AA current were monitored in vitro from day 0 (when biosensors were made, calibrated, and eventually implanted for in vivo experiments) up to day 28 (Figure 2-S of Supporting Information). When not undergoing calibration, the biosensors were stored in air at 4 °C in a fridge (following rinsing with deionized water) during the entire period of the study. All the selected designs showed a global, rather homogeneous, decay in terms of V_{MAX} and slope. However, only design 8 (Pt_c/PPD/[{PEI(1%) + Glyc(1%)}/AOx]$_{10}$/PU(1%)) showed values statistically superior to other designs, remaining so over a period of one week and then aligned itself with other designs over the following three weeks. K_M values also showed a rather homogeneous behavior for all designs,

dx.doi.org/10.1021/ac301253h | Anal. Chem. 2012, 84, 7072−7079

increasing up to day 2 and remaining quite stable up to day 28. In contrast, the studied designs showed a different trend with respect to the AA shielding power. In particular, BSA/GA-based designs (9 and 10) revealed the worst behavior, while AA interference rejection remained stable during the first week for the PU-based designs (5 and 8). The biosensors ability to prevent AA interference deteriorated over the next three weeks, making them unsuitable for long-term implantation.

As discussed in the introduction, several papers showed that ethanol in the CNS could reach a concentration of about 30 mM after i.p. injection.[16−18] On the basis of this observation, we exposed the selected designs (5, 8, 9, and 10) to 30 mM of ethanol and, in a separate series of experiments, studied the AA ΔI currents for a period of 28 days (Figure 3-S of Supporting Information). Design 8 also showed the best performance in this regard. The initial stability of this design (up to day 7) confirmed its selection for in vivo implantation.

In Vivo Response of the Biosensor to Systemic Administration of Ethanol and Effect of Ranitidine. The biosensors selected for implantation (Pt_c/PPD/[{PEI(1%) + Glyc(1%)}/AOx]$_{10}$/PU(1%), design 8) in the rat *nucleus accumbens* were made and calibrated immediately before neurosurgery (day 0). The results of these calibrations are shown in Figure 2A and explained in the corresponding legend. Biosensors were also exposed to AA (0.5 mM and 1 mM) to confirm their AA blocking properties (data not shown). Because a selective inhibitor of alcohol dehydrogenase, ranitidine, was administered i.p. in some in vivo studies, its potential interference on the biosensor was studied in vitro before implantation. As illustrated in Figure 2B, ranitidine did not interfere with AOx activity or exhibited electroactive properties (at +0.7 V vs Ag/AgCl). The in vivo experiments started 24 h after surgery (day 1) by polarizing the alcohol biosensor at +0.7 V and recording the telemetric signal until a stable baseline was reached (by 40−50 min). In a control group of animals (n = 3), ethanol (1 g kg^{-1} i.g.; Figure 3, arrow 2) was administered 20 min after the i.p. injection of saline (1.5 mL; Figure 3, arrow 1) and resulted in an increase of the biosensor current that reached its maximum amplitude (23.4 ± 3.2 nA) 20−40 min after ethanol administration (i.e., at 60−80 min). The biosensor current, recorded for 160 min, then decreased to 11.8 ± 3.1 nA. In a second group of rats (n = 3), ranitidine (30 mg kg^{-1} dissolved in 1.5 mL of saline), was administered i.p. 20 min before the i.g. administration of ethanol. In this case, an increase in the biosensor current was also observed (27.9 ± 4.3 nA at 60−80 min) while the subsequent decrease was less pronounced (23.2 ± 3.5 nA at 140−160 min) and statistically different from the control group (p < 0.05). Following the experiments, rats were sacrificed and postmortem histology was performed demonstrating that all biosensors were located in the shell of the *nucleus accumbens* (data not shown).

DISCUSSION AND CONCLUSIONS

The aim of the present study was to develop and characterize an amperometric biosensor capable of monitoring ethanol concentration changes in brain ECF. Several alcohol biosensor designs were developed and characterized in terms of V_{MAX}, K_M, LRS, and AA shielding power in vitro. These parameters are extremely important in order to evaluate responses to ethanol and the main interference species and for choosing the best implantable design. Changing the amount of loaded enzyme and modifying the surface with different kinds of "molecular net" to trap the enzyme and other components were strategies that aimed to maximize ethanol-related sensitivity parameters and minimize AA interference. V_{MAX} is a measure of the amount of active enzyme molecules on related biosensor surfaces, provided the sensitivity to hydrogen peroxide (reaction 3) is similar across the designs, as was the case here.[19,28] The presence of PEI led to an increase in V_{MAX} compared to the basic biosensor (design 1). On the other hand, the presence of glycerol gave greater stability to the biosensor, in terms of enzyme loading, over time. In view of the diffusible nature of glycerol, it is likely that its beneficial effects on biosensor performance are due to its influence during enzyme deposition, cross-linking, and drying stages of biosensor fabrication, rather than during biosensor operation per se. Both PEI and glycerol are known in the literature as enzyme stabilizers,[29,30] but in our study, PEI acted more as an "enzyme activity enhancer", as previously described,[31] and glycerol acted as an enzyme stabilizer: mixing together both PEI and glycerol, we obtained an addictive effect evidenced by the increased number of active enzyme molecules (demonstrated by the enhanced V_{MAX}) and by the maintenance of such high V_{MAX} values over time (glycerol effect). Moreover, the apparent K_M is the substrate concentration that gives half the V_{MAX} response and reflects both the affinity and access of the substrate to the enzyme.[32] In biosensor design, K_M has a dual importance: it determines the amplitude of linear region slope of the biosensor substrate (LRS = V_{MAX}/K_M), as well as the concentration range of the linear response ($\sim 1/2K_M$);[14,19] thus, the higher the K_M value, the smaller is the LRS but the wider is the linear response region. All the approaches used here aimed to increase as much as possible V_{MAX} and K_M values in order to achieve the highest ethanol LRS up to 30 mM, a value reached in the CNS after i.p. injection in rats.[17,18] The introduction of the enzyme enhancer PEI provided a sustained increase in LRS values. Even though LRS is generally valid only up to half the K_M value,[19,28] our implantable biosensors showed a good linearity up to 40 mM with R^2 = 0.996 (Table 2) because of slight deviations from Michaelis−Menten behavior (R^2 < 0.98, Table 1) in the form of a flattening of the calibration plot at low concentrations (see Figure 2A).

All biosensor designs showed a good AA shielding power with a range of values between 0.8 and 2.6 nA for 1 mM AA on day 1. Indeed, an important parameter that can be extracted from calibration data is the percentage interference by AA relative to the measured biosensor current for 30 mM ethanol. It is commonly known in the literature that ethanol administered i.p. can increase AA levels by about 200% in the striatum,[33−36] the most commonly targeted brain region in in vivo monitoring studies. Although the response of AA in some brain areas, such as the hippocampus, can be markedly different from that in the striatum,[37] AA baseline levels and responses in the striatum and nucleus accumbens are often quite similar.[38,39] From AA calibration, we evaluated the variations in the AA current when its concentration changed from 0.5 (striatal ECF baseline level) to 1 mM (expressed in nA as AA ΔI). On the basis of these values (Table 1), we examined the impact of AA ΔI on the current resulting from 30 mM ethanol (Figure 3-S of Supporting Information). For the implantable biosensor (design 8), the percentage of interference was less than 1% up to day 2 and about 2% up to day 7 but reached a value of 25% at day 14, increasing week by week in conjunction with the worsening of the polymer shielding performance. From these data, it is clear that the predictable variations in AA concentrations in vivo are unable to interfere significantly

with the currents produced by 30 mM ethanol during the first week, making the biosensor reliable for ethanol monitoring in vivo. Unfortunately, these results also showed that the most promising biosensor design for in vivo use (Pt_c/PPD/[{PEI(1%) + Glyc(1%)}/AOx]$_{10}$/PU(1%), design 8) cannot be implanted chronically for more than one week because of the decrease in its selectivity associated with the increase in AA interference. No significant interference signals were observed on exposing biosensors to other electroactive molecules (dopamine, 3,4-dihydroxyphenylacetic acid, and uric acid) present in the CNS extracellular fluids, even at pharmacologically relevant concentrations (data not shown), as reported previously for other PPD-based biosensor designs.[20]

As shown in the in vivo results section, the systemic administration of ethanol resulted in an increase of its current recorded by the biosensor implanted in the *nucleus accumbens*. The maximum concentration of ethanol in the extracellular compartment (from Figure 3, vehicle-treated group), estimated using the preimplantation calibration (Figure 2A), was equivalent to approximately 14 mM (60−80 min) and decreased down to ∼6 mM (140−160 min). These results, although lower than those obtained using in vivo microdialysis by Jamal and collaborators[16] (23 mM), are in agreement with data recorded by Yoshimoto et al. (18 mM).[17] In another microdialysis study, Yim and co-workers[40] found a maximum concentration of ethanol in the *nucleus accumbens* of about 6 mM after i.p. injection of 1 g kg^{-1} of ethanol. Using proton magnetic resonance spectroscopy, Adalsteinsson et al.[18] obtained similar results administering the same dose of ethanol i.g.; however, the maximum averaged ethanol concentration (∼30 mM) was reached in the CNS after its i.p. administration. These differences in attempts at absolute determination of in vivo concentrations may be related both to different experimental setups and analytical methods used for ethanol quantification and are not surprising in view of the assumptions made in these estimations.[41]

In this context, preimplantation sensitivity cannot be used as a straightforward measure of in vivo sensitivity. Instead, preimplantation sensitivity was used in this work mainly to rank biosensor performance in terms of likely beneficial attributes in later applications. However, postimplantation sensitivity is also not a reliable measure of in vivo sensitivity, especially for biosensor-type electrochemical devices. For example, PPD-based glucose biosensors showed excellent rejection of AA interference in vivo several days after implantation;[15] yet, postimplantation calibrations indicated that the polymer−enzyme permselective layer was damaged when removed from the tissue following chronic implantation. Although not perfect, therefore, the preimplantation substrate sensitivity is a useful measure of biosensor substrate sensitivity but gives only estimates of substrate concentration in the ECF. However, the detailed real-time response dynamics achievable with implanted biosensors, such as those shown in Figure 3, are of greater import than absolute levels at this stage of in vivo monitoring methodology development.

Ranitidine is a competitive histamine receptor (H_2) antagonist widely used in the treatment of peptic ulcer disease.[21] This drug is considered to be an inhibitor of cytochrome P450 (CYP2E1),[42] alcohol dehydrogenase, and aldehyde dehydrogenase[26,43] enzymes. Because of this multitarget pharmacological profile, ranitidine is able to increase the bioavailability of ethanol by modifying its pharmacokinetics. For example, blood alcohol levels have been shown to increase in drinkers following the administration of ranitidine.[44,45] Walt and co-workers showed that ranitidine does cross the blood brain barrier in small quantities (1−20 ng mL^{-1}),[46] and for this reason, we studied the effects of the drug on the implantable biosensor in vitro (Figure 2B) before its administration in vivo. The administration of ethanol to both ranitidine-pretreated and saline-control animals (n = 3) resulted in an increase of extracellular ethanol levels estimated at ∼16 mM (60−80 min; Figure 3). The subsequent decrease in biosensor signal, however, was significantly smaller in the ranitidine-pretreated group (∼14 mM at 140−160 min; $p < 0.05$; Figure 3), consistent with the inhibition of ethanol metabolism.

According to the results of this study, we can affirm that the developed ethanol biosensor is a reliable device for the short-time monitoring of exogenous ethanol in CNS extracellular fluids. The implantable biosensor, integrated into a low-cost telemetry system, represents a new generation of analytical tools for studying ethanol pharmacokinetics and the effect of drugs on ethanol levels in real time. Additional research is in progress to reduce the effects of aging on biosensor functionality and to extend its use to study methanol intoxication.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +39079228558. Fax: +39079228525. E-mail: paserra@uniss.it.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The research was supported by the University of Sassari (ex 60% fund).

■ REFERENCES

(1) Hardman, J. G.; Limbird, L. E. *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, 11th ed.; Mcgraw-Hill: New York, 2005.

(2) Theile, J. W.; Morikawa, H.; Gonzales, R. A.; Morrisett, R. A. *Neuroscience* **2011**, *172*, 94−103.

(3) Karahanian, E.; Quintanilla, M. E.; Tampier, L.; Rivera-Meza, M.; Bustamante, D.; Gonzalez-Lira, V.; Morales, P.; Herrera-Marschitz, M.; Israel, Y. *Alcohol.: Clin. Exp. Res.* **2011**, *35* (4), 606−612.

(4) Mostofa, J.; Kiyoshi, A.; Mitsuru, K.; Setsuko, A.; Takako, K.; Weijuan, W.; Iwao, I. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2003**, *798* (1), 155−158.

(5) Munson, P. L.; Mueller, R. A.; Breese, G. R. *Principles of Pharmacology. Basic concepts & clinical application*, 1st ed; Chapman & Hall Inc.: New York, USA, 1995.

(6) Nurmi, M.; Kiianma, K.; Sinclair, J. D. *Alcohol* **1994**, *11* (4), 315−321.

(7) Gonzales, R. A.; McNabb, J.; Yim, H. J.; Ripley, T.; Bungay, P. M. *Alcohol.: Clin. Exp. Res.* **1998**, *22* (4), 858−867.

(8) Brunner, M.; Langer, O. *AAPS J.* **2006**, *8* (2), Article 30.

(9) Khan, A.; Ab Ghani, S. *Biosens. Bioelectron.* **2012**, *31*, 433−438.

(10) Yarman, A.; Grobe, G.; Neumann, B.; Kinne, M.; Gajovic-Eichelmann, N.; Wollenberger, U.; Hofrichter, M.; Ullrich, R.; Scheibner, K.; Scheller, F. W. *Anal. Bioanal. Chem.* **2012**, *402*, 405−412.

(11) Kowalska, M.; Tian, F.; Smehilova, M.; Galuszka, P.; Frebort, I.; Napier, R.; Dale, N. *Anal. Chim. Acta* **2011**, *701*, 218−223.
(12) Vasylieva, N.; Barnych, B.; Meillerd, A.; Maucler, C.; Pollegioni, L.; Lin, J. S.; Barbier, D.; Marinesco, S. *Biosens. Bioelectron.* **2011**, *26*, 3993−4000.
(13) Njagi, J.; Chernov, M. M.; Leiter, J. C.; Andreescu, S. *Anal. Chem.* **2010**, *82*, 989−996.
(14) O'Neill, R. D.; Lowry, J. P.; Rocchitta, G.; McMahon, C. P.; Serra, P. A. *TrAC, Trends Anal. Chem.* **2008**, *27* (1), 78−88.
(15) Lowry, J. P.; McAteer, K.; El Atrash, S. S.; Duff, A.; O'Neill, R. D. *Anal. Chem.* **1994**, *66*, 1754−1761.
(16) Jamal, M.; Ameno, K.; Kumihashi, M.; Ameno, S.; Kubota, T.; Wang, W.; Ijiri, I. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2003**, *798* (1), 155−158.
(17) Yoshimoto, K.; Komura, S. *Alcohol Alcohol.* **1993**, *28* (2), 171−174.
(18) Adalsteinsson, E.; Sullivan, E. V.; Mayer, D.; Pfefferbaum, A. *Neuropsychopharmacology* **2006**, *31* (12), 2683−2691.
(19) Kirwan, S. M.; Rocchitta, G.; McMahon, C. P.; Craig, J. D.; Killoran, S. J.; O'Brien, K. B.; Serra, P. A.; Lowry, J. P.; O'Neill, R. D. *Sensors* **2007**, *7* (4), 420−437.
(20) Ryan, M. R.; Lowry, J. P.; O'Neill, R. D. *Analyst* **1997**, *112*, 1419−1424.
(21) Calia, G.; Rocchitta, G.; Migheli, R.; Puggioni, G.; Spissu, Y.; Bazzu, G.; Mazzarello, V.; Lowry, J. P.; O'Neill, R. D.; Desole, M. S.; Serra, P. A. *Sensors* **2009**, *9* (4), 2511−2523.
(22) Bazzu, G.; Puggioni, G. G.; Dedola, S.; Calia, G.; Rocchitta, G.; Migheli, R.; Desole, M. S.; Lowry, J. P.; O'Neill, R. D.; Serra, P. A. *Anal. Chem.* **2009**, *81* (10), 3911−3918.
(23) Barberis, A.; Bazzu, G.; Calia, G.; Puggioni, G. M.; Rocchitta, G. G.; Migheli, R.; Schirra, M.; Desole, M. S.; Serra, P. A. *Anal. Chem.* **2010**, *82* (12), 5134−5140.
(24) Wolfensohn, S.; Lloyd, M. *Handbook of Laboratory Animal Management and Welfare*, 3rd ed.; Blackwell Publishing: Oxford, U.K., 2003.
(25) Paxinos, G.; Watson, C. *Rat Brain in Stereotaxic Coordinates*, 6th ed.; Academic Press: San Diego, CA, 2007.
(26) El-Bakary, A. A.; El-Dakrory, S. A.; Attalla, S. M.; Hasanein, N. A.; Malek, H. A. *Hum. Exp. Toxicol.* **2010**, *29* (2), 93−101.
(27) ICH Q2 (R1). *Validation of analytical procedures: text and methodology*, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005, pp 11−12; www.ich.org.
(28) Rothwell, S. A.; Killoran, S. J.; O'Neill, R. D. *Sensors* **2010**, *10*, 6439−6462.
(29) Bolivar, J. M.; Rocha-Martin, J.; Mateo, C.; Cava, F.; Berenguer, J.; Fernandez-Lafuente, R.; Guisan, J. M. *Biomacromolecules* **2009**, *10*, 742−747.
(30) Qian, J. M.; Suo, A. L.; Yao, Y.; Jina, Z. H. *Clin. Biochem.* **2004**, *37* (2), 155−161.
(31) McMahon, C. P.; Rocchitta, G.; Serra, P. A.; Kirwan, S. M; Lowry, J. P.; O'Neill, R. D. *Analyst* **2006**, *131*, 68−72.
(32) Moran, L. A.; Perry, M.; Horton, R.; Scrimgeour, G.; Rawn, D. *Principles of Biochemistry*, 4th ed.; Prentice Hall: New Jersey, USA, 2005.
(33) Liu, J.; Wu, C. F.; Liu, W.; Zhang, H. L.; Li, C. L. *Addict. Biol.* **1999**, *4* (3), 273−281.
(34) Hou, Y.; Yang, J. Y.; Wu, C. F.; Huang, M. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **2005**, *29* (1), 83−89.
(35) Hou, Y.; Wu, C. F.; Yang, J. Y.; Gu, P. F.; Bi, X. L.; Dai, F. *Pharmazie* **2007**, *62* (2), 158−160.
(36) Dai, F.; Yang, J. Y.; Gu, P. F.; Hou, Y.; Wu, C. F. *Brain Res.* **2006**, *1125* (1), 163−170.
(37) Grunewald, R. A.; O'Neill, R. D.; Fillenz, M.; Albery, W. J. *Neurochem. Int.* **1983**, *5*, 773−778.
(38) Brose, N.; O'Neill, R. D.; Boutelle, M. G.; Fillenz, M. *Neuropharmacology* **1989**, *28*, 509−514.
(39) Fillenz, M.; O'Neill, R. D. *J. Physiol.* **1986**, *374*, 91−110.
(40) Yim, H. J.; Schallert, T.; Randall, P. K.; Gonzales, R. A. *Alcohol.: Clin. Exp. Res.* **1998**, *22* (2), 367−374.
(41) Chefer, V. I.; Zapata, A.; Shippenberg, T. S.; Bungay, P. M. *J. Neurosci. Methods* **2006**, *155*, 187−193.
(42) Brett, S. *Crit. Care* **2005**, *9* (1), 45−50.
(43) Yokoyama, H.; Matsumoto, M.; Shiraishi, H.; Miyagi, M.; Kato; Ishii, H. *Alcohol.: Clin. Exp. Res.* **2001**, *25* (6 Suppl), 24S−8S.
(44) DiPadova, C.; Roine, R.; Frezza, M.; Gentry, R. T.; Baraona, E.; Lieber, C. S. *JAMA* **1992**, *267* (1), 83−86.
(45) Arora, S.; Baraona, E.; Lieber, C. S. *Am. J. Gastroenterol.* **2000**, *95* (1), 208−213.
(46) Walt, R. P.; LaBrooy, S. J.; Avgerinos, A.; Oehr, T.; Riley, A.; Misiewicz, J. J. *Scand. J. Gastroenterol., Suppl.* **1981**, *69*, 19−25.