Development of a voltammetric technique for monitoring brain dopamine metabolism: compensation for interference caused by DOPAC electrogenerated during homovanillic acid detection

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The established stability of carbon-paste electrodes (CPEs) in brain extracellular fluid was exploited to develop a voltammetric technique to monitor the dopamine metabolite, homovanillic acid (HVA), at 10 s intervals. At the scan rates needed for this time resolution, 3,4-dihydroxyphenylacetic acid (DOPAC), electrogenerated as a result of HVA oxidation, was observed in the cyclic staircase voltammograms, and this interfered with the straightforward reliable quantification of HVA. However, correction of the HVA signal, recorded in mixtures, with currents from the DOPAC and ascorbate regions of the voltammogram allowed the reproducible construction of well behaved HVA calibration plots. These showed good linearity, LOD values, selectivity and stability during six days of continuous CPE exposure to a lipid medium, which served as an *in-vitro* model of CPE implantation in brain tissue for future applications.

Introduction

Dopamine (DA) is a catecholamine neurotransmitter in a number of brain regions, and is important in the expression of a wide range of behaviours, including motor control, cognitive functions and reinforcement emotions.[1–4] Indeed, many of the common drugs of abuse have specific actions on brain DA reward systems, and this mechanism may be involved in their addictive properties.[5] The ability to monitor a highly temporally resolved index of DA activity in discrete brain areas over extended periods, during well defined behaviours and in response to pharmacological challenges, would provide an important key to understanding more fully the role this molecule plays in brain function.

A growing number of *in-vivo* monitoring (IVM) methodologies are being developed, including sampling,[6,7] spectroscopic[8] and electrochemical,[9–11] to study neurochemical phenomena in the intact brain. One subset of these techniques focuses on the *in-situ* detection of substances in brain extracellular fluid (ECF), using *in-vivo* voltammetry (IVV) with implanted amperometric electrodes. An important aspect of developing an IVV technique is the choice of target analyte whose concentration can be used as a reliable index of neurotransmitter release. The most obvious candidate for monitoring DA release is the concentration of DA itself in ECF, resulting from its overflow from the synapse,[10] and a number of successful IVV techniques have been devised to monitor DA in neurochemical applications.[10–16] One problem with the direct detection of DA is its very low baseline concentration in the ECF (<50 nM[17–20]), due to efficient DA re-uptake mechanisms. Thus, the established IVV technique for monitoring neurotransmitter DA in the ECF with sub-second time resolution, using fast cyclic voltammetry and carbon fibre electrodes, is limited to a time range of minutes, because the small DA signal is overwhelmed by metabolite contamination (mainly 3,4-dihydroxyphenylacetic acid, DOPAC) after this time.[21] DOPAC itself is considered to be more closely coupled to DA synthesis than release,[22–26] and the very high affinity of the DA re-uptake carrier for the metabolite 3-methoxytyramine[27] precludes the detection of this potentially useful DA marker in the ECF.[28]

Detection of another DA metabolite, homovanillic acid (HVA), using implanted carbon-paste electrodes (CPEs), has been used in previous studies to investigate DA function.[26,29–34] The advantages and limitations of this approach have been reviewed recently, together with a summary of DA metabolic pathways.[29] The main attraction of the HVA signal recorded with CPEs is its stability over many weeks of monitoring.[35] However, an important drawback with previous IVM studies involving HVA, including those using microdialysis,[36] is the low sampling frequency, which was of the order of 10–30 min intervals. In the present work, where the emphasis was on the development of a technique suitable for long-term monitoring of DA function at high time resolution (seconds), the CPE HVA signal was the index of choice. The aim was not to compete with approaches that exploit the detection of DA directly, but rather to provide a complementary tool for studying DA function over different time scales.

Here we report the novel observation that DOPAC is electrogenerated from HVA during cyclic voltammetry, and that this leads to interference in HVA quantification. Correction methods to compensate for DOPAC and ascorbate interference in HVA calibrations are also demonstrated as a prerequisite to future *in-vivo* characterisation. Many reviews on the complex principles of IVV (some of which are referred to above) have been published, and a representative selection is cited here.[30,37–49]

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Experimental

Carbon paste was prepared by thoroughly mixing 2.83 g of carbon powder (UCP-1-M, Ultra Carbon Corporation, Bay City, MI) and 1.0 mL silicone oil (Aldrich, Cat. No. 17 563-3). The CPEs were prepared using approximately 4 cm lengths of Teflon-coated Ag wire (Advent Research Materials, Eynsham, England; 370 μm diameter wire). The Teflon insulation was slid along the wire to create an approximately 1 mm-deep cavity. The cavity was tightly packed with the paste mixture, using a bare Ag wire as plunger, and the disk surface smoothed by rubbing gently on a clean card.

Experiments were carried out in a phosphate buffer saline (PBS) solution, pH 7.4, containing NaCl (BDH, Poole, Dorset, UK, AnalaR grade, 0.15 M), NaH_2PO_4 (BDH, AnalaR grade, 0.04 M) and NaOH (Merck, Darmstadt, Germany, analytical-reagent grade, 0.04 M). The lipid phosphatidylethanolamine (PEA, sheep brain cephalin, type II-S, Sigma), homovanillic acid (HVA, Sigma), 3,4-dihydroxyphenylacetic acid (DOPAC, Sigma) and L-ascorbic acid (AA, Aldrich) were used as supplied. 100 mM stock solutions of AA, HVA and DOPAC were prepared using 0.01 M HCl. A ~10% PEA suspension was made by vibrating 0.1 g PEA in 1 mL PBS.

The majority of the working electrodes were treated with the PEA medium for 24 h (CPE_{PEA}) before being rinsed and transferred to the electrochemical cell containing 20 mL PBS at room temperature, together with a saturated calomel reference electrode (SCE) and a stainless steel needle auxiliary electrode. The voltammetric experiments were controlled using a low-noise potentiostat (Biostat IV, ACM Instruments, Cumbria, UK) linked to a PC through a Biodata Microlink interface system.

Cyclic staircase voltammograms (CVs) for AA, DOPAC and HVA in PBS were recorded between −300 and 700 mV *vs.* SCE at 1 or 0.2 V/s, using a step size of 10 or 5 mV, unless stated otherwise. This is a fair approximation to cyclic voltammetry;[50,51] however, because no rigorous kinetic parameters were determined in this study, the similarity is fortuitous. Scans were recorded initially in background electrolyte (PBS) under continuous mild (1 Hz) stirring conditions to allow the electrodes to condition and equilibrate; aliquots of stock HVA, DOPAC and AA were then added while recording continued under the same conditions, which allowed efficient mixing. The concentration ranges used were appropriate to physio-pharmacological conditions *in vivo*: tens of micromolar for the metabolites,[17,52] and hundreds of micromolar for AA.[53,54] Averaged background scans, recorded in PBS and comprising mainly of capacitance currents, were subtracted from voltammograms of analyte in some analyses to highlight the Faradaic response. This practice is useful when dealing with electrodes with relatively high double-layer capacitance, such as surfactant-treated CPEs,[55,56] and/or voltammetric techniques employing high scan rates.[57,58]

Data are reported as mean ± SEM, with n = number of electrodes. The significance of differences observed was estimated using Student's two-tailed t-tests. Paired tests were used for comparing signals recorded with the same electrode (*e.g.*, before and after a treatment); unpaired tests were used for comparing responses obtained with different electrodes.

Results and discussion

The presence of AA in relatively high concentrations in brain ECF (~0.5 mM),[53] coupled with its low electrode potential,[59,60] means that IVV techniques designed to monitor other neurochemical analytes, such as HVA, must be able to separate the AA and target signals. The long-term stability of CPEs in brain tissue (up to several weeks of continuous recording)[29,35] results from the ability of brain lipids to protect this electrode surface from protein fouling,[61] and a number of studies have established that exposing CPEs to lipids such as PEA *in vitro*, producing CPE_{PEA}, serves as a good model of the behaviour of CPEs in brain tissue.[56,61–65] In addition, lipids (and other surfactant media)[66,67] can remove surface pasting oil from CPEs, enhancing electron transfer kinetics and improving signal resolution.[64] The voltammetric behaviour of AA at very slow sweep rates at carbon electrodes has been described extensively,[56,63,68–73] as has that of HVA under similar conditions,[29,30,74,75] and the ability of CPE_{PEA} to resolve AA and HVA signals recorded at 10 min intervals *in vivo* at scan rates as low as 5 mV/s is clear.[29,33,76,77] However, because our aim was to develop a technique to monitor HVA with a time resolution of the order of seconds, the cyclic voltammetric response of AA and HVA at CPE_{PEA} for a scan rate of 1 V/s was determined, initially in separate solutions (Fig. 1).

AA and HVA studied separately

Fig. 1 shows sections of the voltammetric responses for HVA and AA, recorded separately with a CPE_{PEA} in PBS at 1 V/s. The oxidation of AA at many electrode materials is irreversible due to following chemical reactions,[78,79] while that for HVA is irreversible due to the breaking of the O–C methoxy bond (see Fig. 2). For clarity, therefore, only the forward (anodic) sweep of the CV is shown in Fig. 1. When voltammetric waves are broad or sigmoidal, such as those in Fig. 1, the potential of maximum

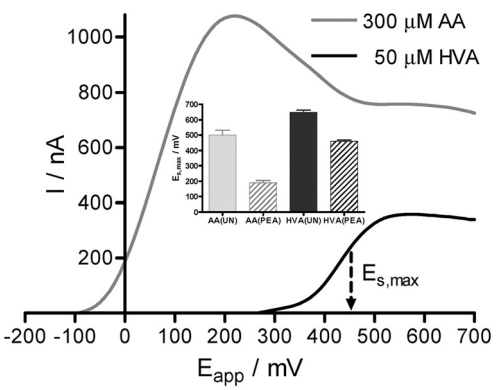

Fig. 1 Sections of cyclic staircase voltammograms at 1 V/s for AA and HVA in separate experiments, using a CPE_{PEA} in phosphate buffered saline (PBS, pH 7.4), illustrating that the intrinsic separation (~270 mV) between the rising kinetic waves for these two signals is maintained even at relatively high scan rates. Background scans have been subtracted in both cases to eliminate capacitance currents, emphasising the Faradaic component of the response. A scan-1 section was used for HVA to avoid DOPAC generation (see Fig. 2 and Fig. 3). Inset: potential of maximum slope ($E_{s,max}$) for AA and HVA at both untreated (UN, $n = 10$) and lipid-treated (PEA, $n = 19$) CPEs.

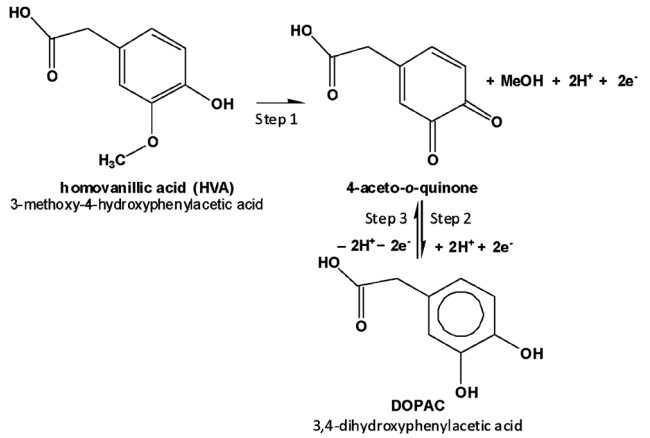

Fig. 2 Electrochemical generation of a reversible redox couple (DOPAC/4-acetoquinone) from the irreversible oxidation of HVA (step 1), which also requires the addition of a H_2O molecule (not shown). The reversible couple involves the addition of $2H^+$ and $2e^-$ for the formation of DOPAC (step 2) and their removal upon oxidation (step 3). See Fig. 3 and text for electrochemical evidence for this scheme.

slope ($E_{s,max}$) is superior to the peak potential (E_p) for quantifying the wave position on the voltage axis. Shifts in $E_{s,max}$ ($\delta E_{s,max}$) can therefore be used as an index of *changes* in the heterogeneous electron transfer coefficient, $k^{o\prime}$, for a given redox couple on different surfaces determined under the same conditions (pH, temperature, *etc.*).[61,80]

The $E_{s,max}$ values (Fig. 1, inset) for AA and HVA at CPE_{PEA} showed good wave separation ($\Delta E_{s,max} = 270 \pm 18$ mV, $n = 19$) at 1 V/s, which compares favourably with peak separation observed at 5 mV/s *in vivo* (~350 mV),[81] considering the 200-fold difference in scan rates and the irreversible nature of the electro-oxidations involved. This $\Delta E_{s,max}$ value was statistically better than that observed at fresh untreated CPEs (149 ± 35 mV, $n = 10$, $p < 0.002$; see Fig. 1, inset), mainly because the effect of lipid

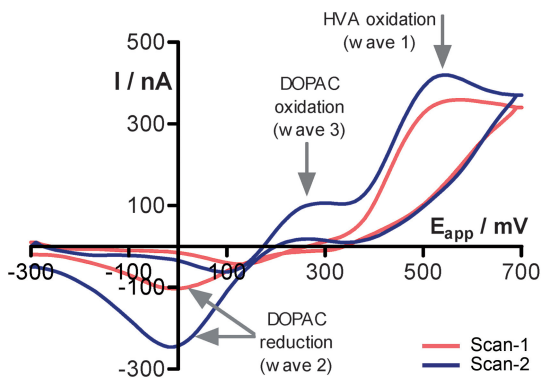

Fig. 3 Cyclic staircase voltammograms at 1 V/s for 50 μM HVA in PBS, using a CPE_{PEA}, illustrating the electrochemical generation of DOPAC (see Fig. 2). Scan-1 shows the irreversible electro-oxidation of HVA on the forward sweep, and a small reduction peak for DOPAC on the backward scan. Scan-2 shows the oxidation of DOPAC (newly formed from HVA oxidation at the electrode surface) and a larger reduction peak for DOPAC on the backward scan due to accumulation of electro-generated products around the CPE_{PEA} disk surface. Background scans recorded in PBS have been subtracted to eliminate capacitance currents.

on decreasing $E_{s,max}$ (increasing $k^{o\prime}$) for AA ($\delta E_{s,max} = -311 \pm 36$ mV, $n = 10$) was greater than the corresponding effect on HVA (-190 ± 17 mV, $n = 10$; $p < 0.008$). These initial results indicated that CPE_{PEA} had the ability to resolve AA and HVA signals recorded at 1 V/s, although this remained to be verified for mixtures (see below). Before progressing, however, a novel complication with the electrochemistry of HVA was observed which could seriously undermine its quantification, *i.e.*, the electrosynthesis of 3,4-dihydroxyphenylacetic acid (DOPAC) from HVA on the electrode surface (see Fig. 2 and Fig. 3).

HVA oxidation on lipid-treated CPEs

Fig. 3 shows CV scans-1 and -2 at 1 V/s for 50 μM HVA in PBS, using a CPE_{PEA} and subtraction of averaged background electrolyte scans to eliminate capacitance currents. The forward (anodic) sweep of scan-1 generated a typical electro-oxidation response (Fig. 3, wave 1), with an E_p value of ~550 mV *vs.* SCE. The backward (cathodic) sweep of scan-1, however, generated a reduction peak (−20 mV, wave 2), unexpected for an irreversible process (Fig. 2, step 1). A new peak appeared on the forward sweep of scan-2 (wave 3 at ~270 mV) followed by an augmented HVA wave and cathodic peak (Fig. 3). The current intensity continued to increase on subsequent scans for the same 50 μM concentration of HVA, indicating adsorption of one or more electroactive species.

The reaction scheme in Fig. 2 is consistent with the voltammetric data shown in Fig. 3. More explicitly, on the first scan, only HVA is present in the cell and this is electro-oxidised *via* step 1 (Fig. 2) to produce wave 1 (Fig. 3). The product of step 1 is the oxidised form of DOPAC, and thus on the backward scan this is reduced to DOPAC (step 2) to generate wave 2. The DOPAC thus formed is oxidised on subsequent scans to reform the aceto-*o*-quinone and more of this quinone is generated from additional oxidation of HVA at higher applied potentials. The suggestion that waves 2 and 3 correspond to electrogenerated DOPAC is in line with literature data showing that DOPAC and other catechols oxidise at potentials between the E_p for AA and HVA at CPE_{PEA}.[74,82] To test the DOPAC hypothesis further, aliquots of either 4-methylcatechol, 3,4-dihydroxyphenylethylamine (dopamine) or DOPAC were injected into the electrochemical cell during CV recording of HVA. Only added DOPAC produced the same redox signature as waves 2 and 3 in Fig. 3. Taken together, these data indicate that DOPAC is produced from HVA electro-oxidation. It remained to be determined, however, what impact this would have on the ability of CPE_{PEA} to monitor HVA unambiguously (see below).

HVA calibrations with lipid-treated CPEs

To achieve maximum temporal resolution for the detection of HVA, CV was carried out 1 V/s in continuous scanning mode, which gave a scan every 2 s. Fig. 4 shows the time course of the current at 700 mV in response to injections of aliquots of HVA into the electrochemical cell. The potential was chosen more anodic than the E_p value to maximise separation from DOPAC at lower potentials (see Fig. 3), but not too high to drive the oxidation of amino acids, such as tyrosine, in later *in-vivo* applications. The current at this potential for CVs recorded in

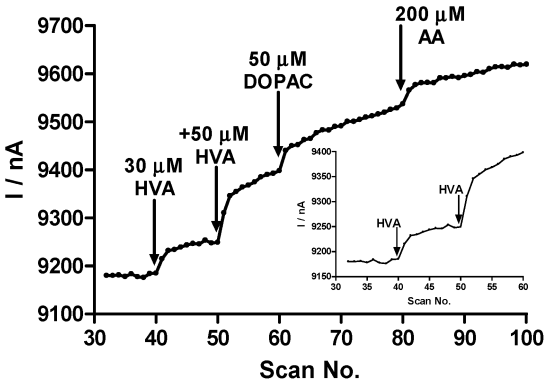

Fig. 4 Calibration time course of the current at the plateau potential for HVA (700 mV; see Fig. 3) recorded at 2 s intervals using CV at 1 V/s with a CPE_{PEA} in PBS, illustrating the problem of signal drift and interference by DOPAC and AA (the *Inset* highlights the two HVA injections). Background currents have not been subtracted in this analysis to model future monitoring conditions *in vivo* where these values would not normally be available.

background electrolyte only (PBS) was stable and consisted mainly of capacitance effects (Fig. 4). Injection of 30 μM HVA caused a step increase in current which continued to drift higher; a following 50 μM HVA injection repeated this pattern (Fig. 4, inset).

The irreversibility of HVA oxidation (Fig. 2) would not allow a build-up of adsorbed HVA on the surface, because all such molecules would be converted to DOPAC electrochemically by the end of each scan. Thus, HVA was not directly responsible for the problem of upward drift in current in Fig. 4. In contrast, the reversibility of DOPAC electro-oxidation (Fig. 2) suggests that adsorbed DOPAC molecules could remain bound on the surface in either oxidation state and, since each scan produced additional DOPAC, the number of such molecules would increase scan by scan. Indeed, the adsorption of catechols on carbon electrodes has been reported widely.[83–88] Using different potentials to quantify the HVA signal produced no major improvement in either the current drift caused by DOPAC adsorption or interference caused by DOPAC and AA injected into the cell (see Fig. 4). The next step was to ascertain whether a correction for interference by DOPAC and AA could be applied, using currents from the voltammogram corresponding to these compounds.

Interference correction of HVA calibrations

Using the CV characteristics of AA (Fig. 1) and DOPAC (Fig. 3) at CPE_{PEA}, a variety of corrections of the form $I_{HVA} = I_{700} - I_{COR}$ were plotted, where the correction potential varied from 100 to 450 mV. No single value of I_{COR} was found that could correct for both the HVA signal drift and the response to added DOPAC and AA shown in Fig. 4. Because we were attempting to correct for two interference species, currents of the form $I_{HVA} = \frac{1}{2}(2I_{700} - I_{DOPAC} - I_{AA})$ were also explored, with little success. (The factor of 2 in the I_{700} term was necessary to balance the large charging currents of the lipid-modified CPEs.[56,63]) Some combinations did remove the drifting, but AA injections produced a step response, or *vice versa*. Only rarely did a set of corrections lead to the elimination of both problems simultaneously, and this was deemed to be inadequate as a reliable technique to monitor HVA.

To overcome this failure to separate reproducibly the three signals recorded at 1 V/s, the electrochemical resolution of AA, DOPAC and HVA was increased by reducing the scan rate to 0.2 V/s. Although this lowered the time resolution to one scan every 10 s, it was still vastly superior to literature values of several minutes.[29] Following calibrations with HVA and the interference species, the correction potentials were determined by the iterative method used above for the 1 V/s data. Firstly, I_{HVA} was approximated by I_{700}, and plotted *versus* scan number, giving a graph similar to Fig. 4. Next, I_{HVA} was adjusted with a single correction ($I_{700} - I_{DOPAC}$) in such a way as to compensate approximately for signal drifting. Then I_{700} was adjusted with a second single correction ($I_{700} - I_{AA}$) to compensate approximately for responses to injected AA. Finally, these two corrections were averaged and values of the potentials for I_{DOPAC} and I_{AA} fine-tuned to minimise both problems. The final optimum correction currents were typically obtained in the region of 100 and 250 mV, and so can reasonably be labelled I_{AA} and I_{DOPAC}, respectively, although the ultimate justification lay phenomenologically in the generation of a near-ideal calibration plot for HVA (Fig. 5).

Fig. 5 shows that this two-point iterative correction was successful at the 0.2 V/s scan rate, for both individual CPE_{PEA} sensors (top) and the averaged normalised response over a small population of electrodes ($n = 5$, bottom). Starting the scans in PBS only, corrected I_{HVA} values showed some residual background currents, which stabilised by scan 10 (see Fig. 5).

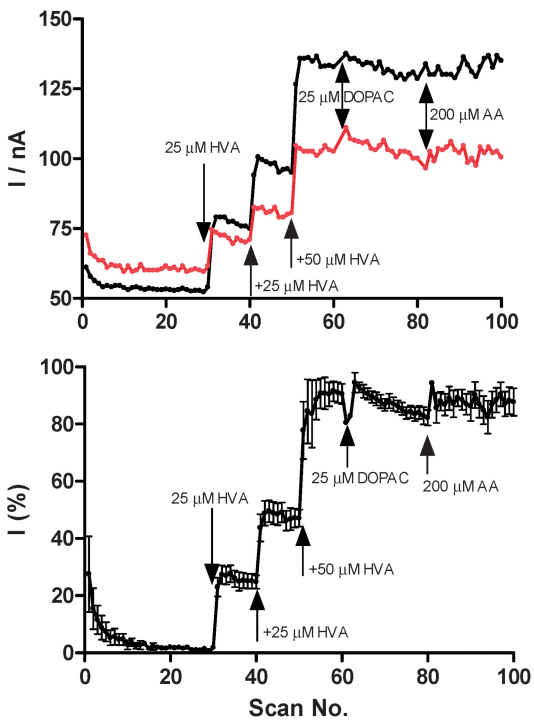

Fig. 5 Calibrations for HVA, using fine-tuned correction currents from the AA and DOPAC regions of the voltammogram, recorded at 0.2 V/s at 10 s intervals. Examples for two individual CPE_{PEA} sensors (top) and averaged normalised values (bottom, $n = 5$).

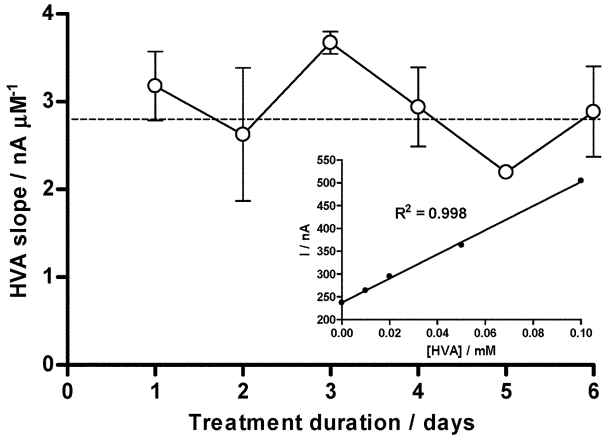

Fig. 6 Stability of calibration slope values for the corrected HVA response over six consecutive days. The CPEs ($n = 4$) were exposed to PEA on each day to model continuous implantation in brain tissue. The average sensitivity was 2.9 ± 0.5 nA/μM with no significant difference observed over the 6-day period. Inset: example of a 5-point calibration (0, 10, 20, 50 and 100 μM HVA) illustrating good linearity ($R^2 = 0.998$) over this physiologically and pharmacologically relevant concentration range.

Successive injections of HVA produced rapid, step responses with no drifting. Subsequent additions of DOPAC (25 μM) and AA (200 μM) did not affect the corrected signal significantly.

Linearity and stability of HVA responses

The baseline concentration of HVA in the ECF of rodent brain regions receiving a strong dopaminergic input, such as striatum and accumbens, is of the order of 10 μM.[17,89] Using the criterion of three standard deviations of the background current, the estimated LOD of CPE_{PEA} electrodes was 3 μM (see Fig. 5), indicating that both physiological and pharmacological changes in ECF HVA levels could be detected with this significantly improved technique.[29]

The linearity of the HVA response, determined using 5-point calibrations (see Fig. 6, inset), was excellent, with $R^2 = 0.998$. The stability of the response was investigated by treating the same set of CPEs ($n = 4$) with the lipid suspension for six consecutive days, and calibrating daily with HVA. There was no significant change in HVA sensitivity under these conditions which served as a model for CPEs chronically implanted in brain tissue (Fig. 6). The exact value of the calibrated HVA sensitivity *in vitro* is not as important as the selectivity and stability of the technique because factors such as tissue tortuosity *in vivo* prevent accurate conversion of calibration data into *in-vivo* analyte concentrations.[90,91]

Conclusions

The novel complication of adsorption of DOPAC, electrosynthesised during the voltammetric detection of HVA on lipid-treated CPEs, precluded reliable monitoring of HVA at 2 s intervals, using CV at a scan rate of 1 V/s. Decreasing the scan rate to 0.2 V/s, in combination with two carefully chosen correction currents, did allow the construction of a useful HVA signal at 10 s intervals. This represents a significant improvement on existing temporal resolution values for HVA monitoring, using either *in-vivo* voltammetric or microdialysis techniques, which is of the order of several minutes.

Following specialised training, certification and licensing of the project, the next step in the development of this technique will involve implantation of the sensors in specific brain regions. These electrodes can then be characterised *in vivo*, using the microinfusion of compounds directly into the tissue, as well as pharmacological challenges known to influence AA and DA metabolism. These studies will be required to ascertain whether the sensitivity, selectivity and stability of the HVA signal constructed here will translate to reliable *in-vivo* monitoring.

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