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DETERMINATION OF THE PRIMARY METABOLITE OF CENTRAL NERVOUS SYSTEM NOREPINEPHRINE, 3-METHOXY-4-HYDROXY-PHENETHYLENEGLYCOL, IN MOUSE BRAIN AND BRAIN PERFUSATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

JOHN F. TOWELL and V. GENE ERWIN*

School of Pharmacy, Campus Box 297, University of Colorado, Boulder, CO 80309 (U.S.A.)

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SUMMARY

Assays are described for the determination of picomole levels of 3-methoxy-4-hydroxy-phenethyleneglycol (MHPG) in mouse brain and in the perfusate of an intact mouse brain. High-performance liquid chromatography with electrochemical detection yielded a MHPG detection limit of 0.37 pmol. This technique offers a sensitive and inexpensive alternative to gas chromatography with mass spectrometry.

INTRODUCTION

Neuronal noradrenergic systems in brain have been implicated in a variety of behavioral and psychopharmacological phenomena [1—3]. It has been proposed that the rate of production of 3-methoxy-4-hydroxyphenethyleneglycol (MHPG), the principal metabolite of norepinephrine in mammalian brain, is an indicator of the rate of norepinephrine turnover [4, 5]. Therefore, new methods of MHPG isolation and detection are important in furthering our knowledge of the role of norepinephrine in normal and dysfunctional mammalian brain.

Among the methods available for MHPG detection, gas chromatography with electron-capture or mass spectrometric detection is certainly the most definitive technique currently in use [6, 7]. The disadvantages of these methods are that derivatization of MHPG is required to produce a volatile compound and that the mass spectrometric instrumentation is expensive. High-performance liquid chromatography with electrochemical detection (HPLC—EC) offers a relatively inexpensive, simple, and highly sensitive technique

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for the routine assay of MHPG. This paper describes the methodology we have developed for measuring MHPG in mouse brain and in artificial blood which had perfused an intact mouse brain [8—10].

EXPERIMENTAL

Reagents

DL-4-Hydroxy-3-methoxyphenylglycol, piperazine salt (No. 454205, Calbiochem-Behring Corp., Los Angeles, CA, U.S.A.; No. 14,879-2, Aldrich Chemical Co., Milwaukee, WI, U.S.A.); 3-hydroxybenzyl alcohol (No. H2,060-1, Aldrich Chemical Co.); hydroquinone (No. 374387, Matheson, Coleman and Bell, East Rutherford, NJ, U.S.A.); spectral grade ethyl acetate, redistilled (J.T. Baker, Phillipsburg, NJ, U.S.A.); HPLC mobile phase, 0.01 *M* potassium phosphate buffer (pH 7.0); mice, C3H/Ibg males (45—65 days old); artificial blood, washed bovine erythrocytes in an artificial serum [11].

MHPG purity and stability

The molar extinction coefficients of newly synthesized and dried MHPG (piperazine salt, $C_{22}H_{34}N_2O_8$, with the following elemental analysis: theoretical C = 58.1%, H = 7.54%, N = 6.17%; found C = 58.2%, H = 7.62%, N = 6.14%) are $\epsilon_{280} = 6.26 \cdot 10^3\ M^{-1}\ cm^{-1}$ and $\epsilon_{230} = 1.45 \cdot 10^4\ M^{-1}\ cm^{-1}$ in 100% ethanol [12]. MHPG ordered from Calbiochem-Behring Corp. and Aldrich Chemical Co. was assayed within a week after it had been received and stored at —20°C. The ϵ_{280} molar extinction coefficients were $5.75 \cdot 10^3\ M^{-1}\ cm^{-1}$ (Calbiochem-Behring) and $5.82 \cdot 10^3\ M^{-1}\ cm^{-1}$ (Aldrich) which indicated decreases of 8.2% and 7.0%, respectively, when compared to the theoretical ϵ_{280} noted above. These data suggested that the MHPG was slightly impure, which may have been due to possible hydration and/or contamination by products of chemical deterioration. The piperazine salt of MHPG is known to be hygroscopic and oxygen-labile [12], and thus aqueous solutions of the piperazine salt of MHPG, which are basic, should be buffered or neutralized to retard oxidation. Throughout most of the studies reported here, stock MHPG solutions were prepared weekly and stored at 5°C in 1 m*M* HCl. However, later on in our studies it was found that stock MHPG solutions can be stored for two months in 100% methanol at —20°C without significant deterioration. This was advantageous because it minimized the atmospheric exposure of the stock bottle of MHPG which accompanied weighing procedures.

Apparatus

Solvent metering pumps, Altex 110A (Altex Scientific); sample injection and window valves, Rheodyne 7010, RP-2 MPLC cartridge and holder, and RP-2 analytical column, 25 cm × 4.6 mm I.D., 10-μm LiChrosorb (Rheodyne, Berkeley, CA, U.S.A.); μBondapak C_{18} analytical column, 30 cm × 3.9 mm I.D., 10 μm (Waters Assoc., Milford, MA, U.S.A.); electrochemical detector (LC-4), CP-S carbon paste, and TL-4 cell (Bioanalytical Systems); recorder (Houston Instruments Omniscribe); sample filters (Millipore Corp. No. GSWP 01300; Schleicher and Schuell No. RC55; Bio-Rad Labs. No. 313-5009); filter holders (Millipore Corp., Swinnex No. SX00 01300; Bio-Rad Labs., Liqui-Holder, 13 mm, No. 342-0001).

Procedure

The chromatographic flow-rate was 1 ml/min and the detector potential was +0.8 V against a Ag|AgCl reference electrode. Because of extraneous components that eluted from the analytical column 60—75 min after the injection, we utilized the system shown in Fig. 1 to minimize the time between injections. The sample was injected onto an MPLC RP-2 column which had been precalibrated for the retention times of MHPG and the internal standard 3-hydroxybenzyl alcohol (3HBA). During that interval in which these compounds eluted from the pre-column, the window valve (Fig. 1) was opened, allowing them to flow on to the analytical column, after which the window valve was closed and the extraneous components were washed off the MPLC column into the waste container.

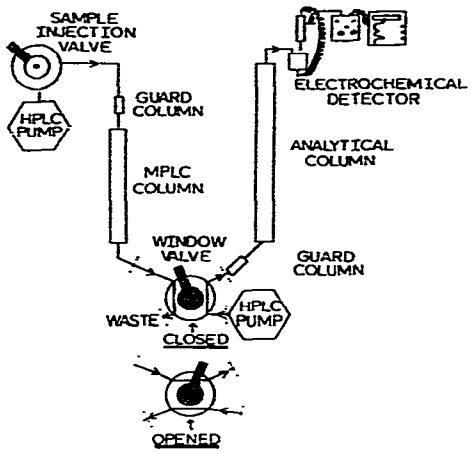


Fig. 1. Window-valve HPLC—EC system. Shown with valve in closed (upper) and opened (lower) positions. Equipment described in text.

Extraction of mouse brain tissue

C3H/Ibg male mice were sacrificed by decapitation; the heads were immediately frozen with liquid nitrogen. The frozen brains were removed, weighed, and pulverized with 1 ml of frozen 50 m*M* perchloric acid with a deeply cooled mortar and pestle. The mixture was then thawed on an ice bath after which 2 ml of cold 50 m*M* perchloric acid, along with the internal standard 3HBA (100 pmol) and MHPG (when appropriate), were added. The mixture was homogenized and then centrifuged for 15 min at 15,000 *g*. The supernatant was neutralized with 1 ml of 3 *M* tris(hydroxymethyl)aminomethane—HCl (Tris—Cl) buffer (pH 8.6) and extracted twice with 5 ml of ethyl acetate on an automatic shaker (5-min shaking intervals). The ethyl acetate extracts were maintained at 5°C until they were rotoevaporated to dryness, which was done as soon as possible following the extraction procedure. The remaining residues were dissolved in methanol, transferred to conical tubes, and evaporated under nitrogen. The warming baths for the evaporation steps were maintained below 40°C. The residues were then dissolved in 0.1 ml of the mobile phase, millipore-filtered, and injected onto the HPLC system.

Extraction of mouse brain perfusate

The perfusion fluid was a suspension of washed bovine erythrocytes in an artificial serum [11]. MHPG was extracted from the perfusate of a mouse brain by shaking the serum (10 ml), from which the erythrocytes had been separated by centrifugation, with ethyl acetate (10 ml) for three 10-min intervals. Again, the ethyl acetate extracts were maintained at 5°C until they were rotoevaporated to dryness. The residues were then dissolved in methanol, dried, dissolved in mobile phase, and filtered as described above in the mouse brain MHPG determination.

Chromatographic peaks obtained from extractions of brain perfusates were confirmed by gas chromatography—mass spectrometry (GC—MS). The peaks from twelve injections (approximately 72 pmol) were collected, pooled, and rotoevaporated to dryness. The residue was derivatized by adding bis(trimethylsilyl)trifluoroacetamide and heating at 60°C for 15 min. The GC column employed was 1.52 m × 2 mm I.D., packed with 3% SE-30 and operated at 150—200°C at 10°C/min. The mass spectrometer (Finnigan 3200 with a Model 6100 data system) was used to scan the mass range from *m/z* 50 to 500. The GC retention times and mass spectra obtained corresponded to those of authentic derivatized MHPG [13].

MHPG levels in the mouse brain were depleted by treatment with intraventricular injections of 6-hydroxydopamine [14, 15], which chemically lesioned the central noradrenergic systems. In other studies we have demonstrated that 99% of the brain MHPG was eliminated after this method of treatment [10].

RESULTS AND DISCUSSION

Chromatography

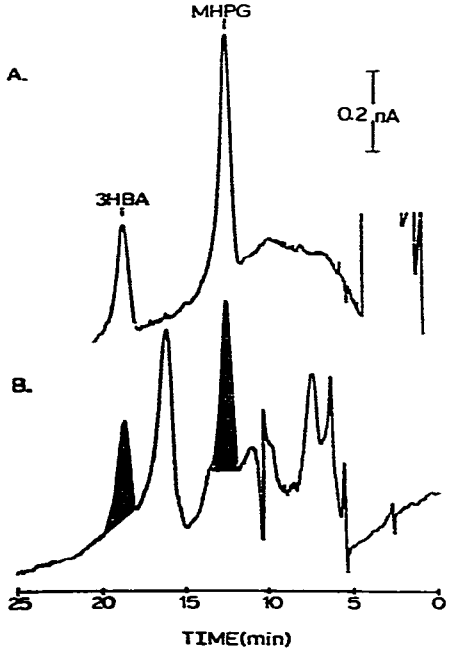
During the development of the MHPG assay it was found that filtration of the sample before injection on to the HPLC system introduced a component which eluted from the C-18 analytical column in a very wide band with a retention time of 60—75 min. This artifact complicated the analysis and prohibited further sample injections until the component had eluted from the column. This extraneous component was found in all the membrane filters cited in the experimental section. Through the use of the window-valve system (Fig. 1), injections on to the HPLC column were made at 20-min intervals and the subsequent detections of MHPG and 3HBA were accomplished without interference from extraneous components from the previous injection. Because the carbon-paste electrode of the electrochemical detector was spared the chemical perturbation that typically accompanies the injected solvent front in single-column systems, we found improved stability, sensitivity, and lifetime of the carbon-paste electrode. The detection limit (where the signal-to-noise ratio became less than 2.5) of MHPG in an aqueous (1 m*M* HCl) standard was 0.37 pmol.

Mouse brain MHPG determination

In Fig. 2 are shown HPLC—EC chromatograms of MHPG and 3HBA controls (Fig. 2A) and an extract of a mouse brain homogenate to which 100 pmol

of 3HBA have been added (Fig. 2B). The retention times for MHPG and 3HBA on the system shown in Fig. 1, when the RP-2 analytical column was employed, were 12.5 and 19.0 min, respectively.

The calibration curve for the determination of MHPG in homogenates of mouse brain tissue (Fig. 3) was generated by the following procedure. Brain homogenates from mice that had been treated with intraventricular injections of 6-hydroxydopamine were mixed with known amounts of MHPG (50—150 pmol) and 3HBA (100 pmol) and extracted as described above. The ratio of the MHPG and 3HBA detector responses (MHPG/3HBA) was plotted against the MHPG (pmol) that was added to the brain homogenates (open circles, Fig. 3). In the second type of experiment, 3HBA was added to the brain homogenates from normal mice which were then divided in half and a known amount of MHPG (25—100 pmol) was added to one half. Thus, the MHPG content of a half-brain homogenate contained either the endogenous MHPG level (approximately 60 pmol) or the endogenous level plus the amount of MHPG added. The endogenous level of each half brain was determined from the calibration curve generated from the 6-hydroxydopamine-treated animals. Then the numerical value of the endogenous level was combined with the amount of MHPG that had been added to the other half, and these data (closed



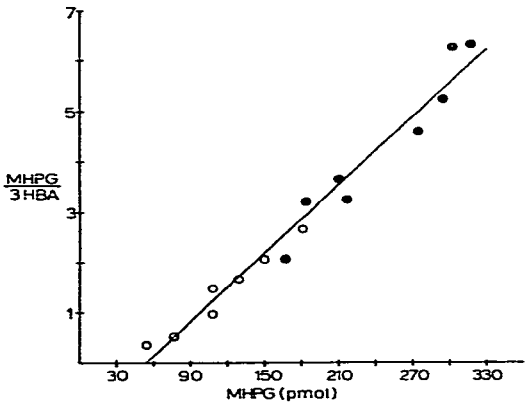


Fig. 2. HPLC—EC chromatograms. (A) MHPG (10 pmol) and 3HBA (5 pmol) standard solution. (B) Extract of brain homogenate. 100 pmol 3HBA added to homogenate before extraction. Flow-rate, 1 ml/min. Mobile phase, 10 m*M* potassium phosphate buffer (pH 7.0). Analytical column, Rheodyne RP-2.

Fig. 3. Calibration curve for determining MHPG in mouse brain homogenates. Ratio of detector responses for MHPG and internal standard 3HBA plotted against MHPG in 0.45 g of brain tissue. (○) 6-Hydroxydopamine-treated brains; (●) normal brains. 100 pmol 3HBA added to brain homogenate before extraction. Slope = $2.28 \cdot 10^{-2}$ $pmol^{-1}$; *y* intercept = —1.26.

circles, Fig. 3) were plotted with the data from the 6-hydroxydopamine-treated animals. The advantage of this method is the increased reliability in the measurement of brain MHPG levels that are lower than the endogenous level. Also, the inclusion of both methods ensured that the drug treatment did not affect the extraction efficiency.

The calibration curve shown in Fig. 3 has a slope of $2.28 \cdot 10^{-2}$ $pmol^{-1}$ and a y intercept of −1.26. The open and closed circles in Fig. 3 were generated from the extractions of 6-hydroxydopamine-treated and normal brains, as indicated. Because of the negative intercept in Fig. 3, the linear fit to the data is a good approximation only in the range of 54—317 pmol MHPG per 0.45 g of mouse brain.

The techniques described above were used to determine the MHPG levels in the brains of male C3H mice, the mean of which was 275 ± 12 pmol/g of brain (mean ± S.E.M., n = 9). This value is in agreement with data determined from other published methods using gas chromatography with electron-capture detection [16] and selected ion monitoring mass spectrometry [17]. The absolute recovery of MHPG, which was determined from that half of the mouse brain homogenate to which a known amount of MHPG had been added, was 23.8 ± 1.4% (mean ± S.E.M., n = 9). This was sufficient to produce amounts required for the HPLC—EC determination of MHPG levels in a whole mouse brain.

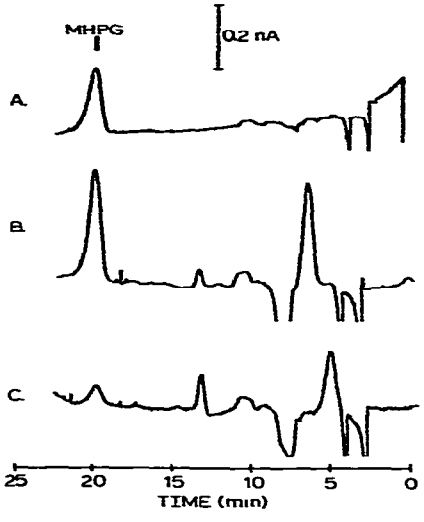
The mouse brain MHPG determination procedure described here can be used in conjunction with brain catecholamine determination. When the supernatant from a mouse brain homogenate is incubated with alumina in 3 *M* Tris—Cl buffer (pH 8.6) [18], the catecholamines are adsorbed by the alumina but the MHPG is not. Hence, the MHPG can be subsequently extracted from the Tris—Cl buffer [10] which is washed from the alumina. This is the reason for adding Tris—Cl buffer to the brain homogenate before the ethyl acetate extraction procedure as described in the Experimental section.

MHPG determination in mouse brain perfusate

The chromatogram of MHPG on the window-valve system with a C-18 analytical column is shown in Fig. 4A. Fig. 4C shows the chromatography of an extraction of perfusion fluid which had not passed through a mouse brain. The background levels of MHPG shown here were routinely subtracted from the levels determined in the perfusate of a mouse brain (Fig. 4B).

The C-18 analytical column was routinely used in the perfusate MHPG determinations because of occasional interferences by additional peaks (not shown in Fig. 4) which could not be resolved from the MHPG peak by the C-2 analytical column. However, the use of the C-18 column resulted in prohibitively long retention times for 3HBA which was used for the internal standard in the mouse brain MHPG determination. This is why the external standard hydroquinone was used in the brain perfusate MHPG determination. MHPG itself was not used as the external standard because of its instability and the relative ease of using another compound (such as hydroquinone) that does not require storage at low temperatures.

The MHPG calibration curve (Fig. 5) was normalized to 2.37 pmol of hydroquinone injected onto the column. The slope of the curve is $1.53 \cdot 10^{-1}$ $pmol^{-1}$



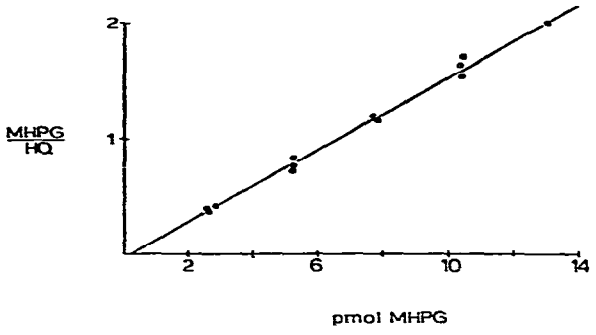


Fig. 4. HPLC—EC chromatograms. (A) MHPG (10 pmol) standard solution. (B) Extract of mouse brain perfusate. (C) Extract of control perfusion fluid. Flow-rate, 1 ml/min. Mobile phase, 10 m*M* potassium phosphate buffer (pH 7.0). Analytical column, μBondapak C_{18}.

Fig. 5. Calibration curve for determining MHPG in extract of mouse brain perfusate. Ratio of detector responses for MHPG and external standard hydroquinone (HQ) plotted against MHPG injected on to the column. Normalized to 2.97 pmol hydroquinone. Slope = $1.53 \cdot 10^{-1}$ $pmol^{-1}$ MHPG; *y* intercept = $-4.05 \cdot 10^{-2}$.

MHPG, the *y* intercept is $-4.05 \cdot 10^{-2}$, and it is linear within the range of 2—21 pmol MHPG. A graph of the amount of MHPG extracted from perfusion fluid vs. the amount of MHPG originally added is shown in Fig. 6. This graph has a slope of $1.99 \cdot 10^{-1}$ $pmol^{-1}$ MHPG and a *y* intercept of 6.75. The scatter in this calibration curve represents the variance in the extraction procedure over a period of three months and 34 separate extractions. It was demonstrated that MHPG rapidly equilibrated with the red blood cells which comprised 33% of the total volume of the artificial blood. The loss of MHPG in the hematocrit was accounted for in Fig. 6 because the MHPG was added to the blanks before the red cells were separated from the serum by centrifugation.

Recently Langlais et al. [19] demonstrated that MHPG and other neurotransmitter metabolites can be measured in human cerebrospinal fluid by direct injection of the fluid on to the HPLC column without prior extraction procedures. This technique demonstrates the power of HPLC—EC although direct injection of biological samples may contribute to column deterioration when performed on a routine basis. Their MHPG detection limit (0.27 pmol) is supported by the value reported here (0.37 pmol). We have determined that electrochemical detection is approximately 14 times more sensitive with the catecholamine norepinephrine than with the metabolite MHPG. This difference in sensitivity is supported by the electrochemical studies of Sternson et al. [20].

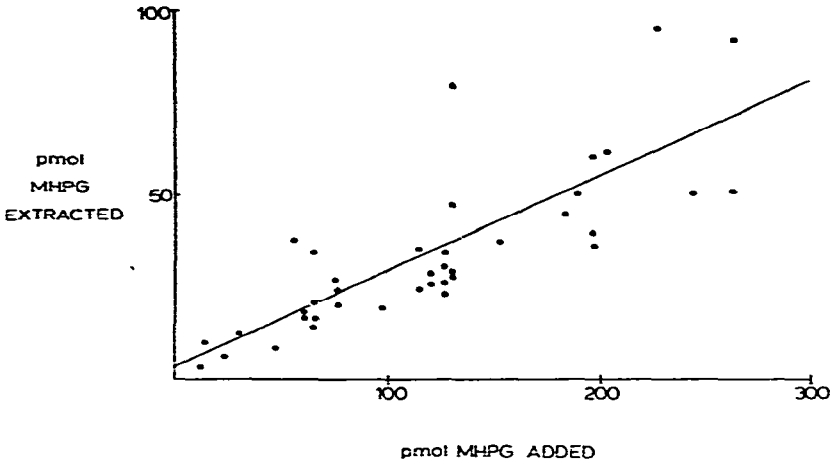


Fig. 6. Calibration curve for determining MHPG extraction efficiency from mouse brain perfusate. Amount of MHPG extracted plotted against MHPG added to control perfusion fluid samples. Slope = $1.99 \cdot 10^{-1}$ pmol^{-1} MHPG; y intercept = 6.75.

In conclusion, we have developed assays for determining the primary metabolite of norepinephrine, MHPG, in mouse brain and in the perfusate of a mouse brain by HPLC—EC. These techniques offer a sensitive alternative to gas chromatography with electron-capture detection and mass spectrometry, and can be used in conjunction with brain catecholamine determinations.

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REFERENCES

1 W.E. Bunney, Jr. and J.M. Davis, Arch. Gen. Psychiat., 13 (1965) 483.
2 J.W. Maas, Arch. Gen. Psychiat., 32 (1975) 1357.
3 J.J. Schildkraut, Amer. J. Psychiat., 122 (1976) 509.
4 J.W. Maas, S.E. Hattox, N.M. Greene and D.H. Landis, Science, 205 (1979) 1025.
5 J.W. Maas, S.E. Hattox, D.H. Landis and R.H. Roth, Brain Res., 118 (1976) 167.
6 S. Takahashi, D.D. Godse, J.J. Warsh and H.C. Stancer, Clin. Chim. Acta, 81 (1977) 183.
7 H. Dekirmenjian and J. Maas, Clin. Chim. Acta, 52 (1974) 203.
8 J.F. Towell and V.G. Erwin, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 422 (Abstract).

9 J.F. Towell and V.G. Erwin, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 39 (1980) 619 (Abstract).
10 J.F. Towell and V.G. Erwin, Brain Res., in press.
11 J.A. Zivin and J.F. Snarr, J. Appl. Physiol., 32 (1972) 658.
12 Calbiochem-Behring Corporation, personal communication.
13 S.E. Hattox and R.C. Murphy, Biomed. Mass Spectrom., 5 (1978) 338.
14 B. Tabakoff, R.F. Ritzmann and W.O. Boggan, J. Neurochem., 24 (1975) 1043.
15 S.G.A. Alivisatos and P.K. Seth, in R. Fried (Editor), Methods of Neurochemistry, Vol. 2, Marcel Dekker, New York, 1972, p. 205.
16 P.M. Ceasar, P. Hague, D.F. Sharman and B. Werdinius, Brit. J. Pharmacol., 51 (1974) 187.
17 V.E. Grimm and G. Sedvall, Life Sci., 22 (1978) 2017.
18 L.J. Felice, J.D. Felice and P.T. Kissinger, J. Neurochem., 31 (1978) 1461.
19 P.J. Langlais, W.J. McEntee and E.D. Bird, Clin. Chem., 26 (1980) 788.
20 A.W. Sternson, R. McCrecry, B. Feinberg and R.N. Adams, Electroanal. Chem. Interfacial Electrochem., 46 (1973) 313.