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Short communication

Oxalic acid stabilizes dopamine, serotonin, and their metabolites in automated liquid chromatography with electrochemical detection

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Abstract

Use of antioxidative agents is required in automated LC assay of microdialysis samples, due to rapid degradation of the monoamine neurotransmitters and their metabolites. Addition of oxalic acid prevented degradation of dopamine, serotonin, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxyindoleacetic acid efficiently: after a 24-h incubation at room temperature the decreases in peak heights were less than 10%. The long-term stability of the analytes, however, was still enhanced when acetic acid and L-cysteine were included in the solution. Using this antioxidative solution, the monoamine neurotransmitters and their metabolites could be determined with an automated LC assay even at room temperature.

Keywords: Oxalic acid; Dopamine; Serotonin

1. Introduction

Microdialysis is a technique widely used to monitor extracellular levels of neurotransmitters and their metabolites in the central nervous system [1,2]. Samples of extracellular fluid are collected by perfusing a dialysis tube implanted in brain tissue of a fully conscious, freely moving animal with a solution that resembles cerebrospinal fluid. Analysis of the neurotransmitters of interest, such as dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT), and their metabolites in perfusate samples is most commonly carried out using reversed-phase high-performance liquid chromatography (HPLC) coupled with oxidative electrochemical detection (LC–EC) [1–3].

Due to rapidly occurring spontaneous oxidative degradation of the analytes, the measurement of monoamine neurotransmitters in dialysate samples using an automated LC assay requires the use of protective agents. Acidified solutions of ascorbic acid (AA) have proved to be efficient stabilizers of catecholamines [4–6], while L-cysteine (Cyst) is commonly added to prevent degradation of indole-based compounds [6–8]. AA, however, often interferes with the analysis by eluting during the same time range as the analytes and by disturbing EC.

The aim here was to find a novel antioxidative agent that would stabilize DA, 5-HT, and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) in dialysates for at least

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24 h without interfering with the LC–EC analysis.We replaced the commonly used AA [4–6] with oxalic acid (HOx), and tested the stability of the analytes in the presence of this as well as various other potential antioxidative agents. A preliminary report of the present experiments was published in abstract form [9].

2. Experimental

2.1. Chemicals and reagents

DA hydrochloride, 5-HT hydrochloride, DOPAC, HVA, and 5-HIAA were purchased from Sigma (St. Louis, MO, USA). HOx dihydrate was from J.T. Baker (Deventer, The Netherlands), Cyst from Acros Organics (Geel, Belgium) and L-(+)-AA from Merck (Darmstadt, Germany). All chemicals used in the study were of high quality. Water was purified using an Elgastat UHQ system (Elga, High Wycombe, UK).

Modified Ringer's solution was used as the perfusion fluid in the microdialysis experiments, and it consisted of 147 m*M* NaCl, 1.2 m*M* $CaCl_2$, 2.7 m*M* KCl, and 1.0 m*M* $MgCl_2$. The pH of the solution was 5.9. Stock standard solutions of DA, 5-HT, DOPAC, HVA and 5-HIAA (10 m*M*) were prepared by dissolving the substances in 0.01 *M* HCl and kept frozen at −70°C. Working standards were made with appropriate dilutions of the stock solutions with Ringer's solution immediately prior to use.

2.2. Chromatography

The HPLC system consisted of a Varian 9001 solvent delivery system, a Varian 9100 autosampler (Walnut Creek, CA, USA), and ANTEC electrochemical amperometric detector (ANTEC Analytical Technology Leyden, Leyden, The Netherlands). Separation was performed on a 250 mm×4.6 mm I.D. inert reversed-phase analytical column (Inertsil ODS-3V, particle size 5 μm; GL-Sciences, Tokyo, Japan). The flow-rate was set at 1.20 ml/min and the detector potential was +780 mV vs. Ag/AgCl reference electrode. The circulating mobile phase was continuously degassed (Degasys DG-1310; Uniflows, Tokyo, Japan). All the samples were injected according to an automated sequence in a volume of 20 μl. The sample vials were tightly capped in order to prevent evaporation. Integration of the chromatograms was performed with Varian Star Chromatography Software (Version 4.0).

For the mobile phase buffer 6.90 g of $NaH_2PO_4 \cdot H_2O$ (50 m*M*), 37 mg of Na_2EDTA (0.1 m*M*) and 702 mg of 1-octanesulfonic acid sodium salt monohydrate (2.3 m*M*) were dissolved in UHQ water. After the pH was lowered to 3.0 with phosphoric acid, the final volume was adjusted to 1 l with UHQ water. A total of 790 ml of the buffer was then mixed with 210 ml of acetonitrile, and the mixture was filtered through a 0.22-μm hydrophilic polypropylene filter (GH-Polypro; Gelman Sciences, Ann Arbor, MI, USA) and degassed under vacuum.

2.3. Stability experiments with standard solutions

The series of stability experiments was initiated by testing for possible interference in the chromatography caused by potential antioxidative solutions. A total of 13 solutions containing different concentrations of AA, HCl, acetic acid (HAc), HOx and Cyst (see Table 1) were mixed with Ringer's solution (1:7) and analyzed in the HPLC system per se. The solution containing AA interfered with the analysis and was omitted from further testing.

The other solutions were tested for their ability to stabilize the analytes. The potential antioxidative mixtures were added to working standard solutions (1:7) containing DA, 5-HT, DOPAC, HVA and 5-HIAA (2.4, 1.2, 24, 12 and 2.4 pmol/60 μl, respectively). The standard solutions were made immediately prior to use by diluting the deep-frozen (−70°C) stock solutions in Ringer's solution. These were then repeatedly analyzed once per hour for 24 h at room temperature (22±1°C).

The long-term efficacy of the most promising antioxidative solution (1.0 m*M* HOx, 3.0 m*M* Cyst, and 0.1 *M* HAc; No. 13 in Table 1) was tested: a standard solution containing all the analytes was analyzed in six repetitions after incubation of 0, 24, 48 and 72 h at room temperature. In order to evaluate the role of the different components of the most promising antioxidative mixture, a solution without Cyst and a solution containing HOx alone

Table 1
Compositions of the antioxidative mixtures tested[a]

No.	AA	HCI	Hac	Hox	Cyst	pH	Comment
1	0	0	0	0	0	5.9	See Fig. 1 Ringer
2	0.1 m*M*	0	0	0	0	5.0	Interference with chromatography
3	0	0.1 *M*	0	0	0	2.0	See Fig. 1 HCl
4	0	0	0.1 *M*	0	0	2.8	See Fig. 1 HAc, pH 2.8
5	0	0	0.1 *M*	0	0	3.2	See Fig. 1 HAc, pH 3.2
6	0	0	0.1 *M*	0	0	3.6	See Fig. 1 HAc, pH 3.6
7	0	0	0.1 *M*	0	0	4.0	See Fig. 1 HAc, pH 4.0
8	0	0	0	0.1 m*M*	0	5.0	DA unstable
9	0	0	0	0.1 m*M*	1.0 m*M*	5.0	DA unstable
10	0	0	0	1.0 m*M*	0	3.6	Acceptable, see Fig. 1 HOx
11	0	0	0	1.0 m*M*	3.0 m*M*	3.6	Acceptable
12	0	0	0.1 *M*	1.0 m*M*	0	3.2	Acceptable
13	0	0	0.1 *M*	1.0 m*M*	3.0 m*M*	3.2	Preferred, see Fig. 1 Antioxidant

[a] HCl refers to hydrochloric acid, HAc to acetic acid, HOx to oxalic acid, Cyst to L-cysteine, and AA to ascorbic acid; pH is the final pH when antioxidant is mixed with modified Ringer's solution (1:7). In solution No. 4 the pH was adjusted with HCl, and in solution Nos. 6 and 7 with 2 *M* NaOH.

were included in the test. Statistical evaluation of the data between different time points was conducted with analysis of variance (ANOVA) followed by one-sided Dunnet's test with time at 0 h as a control.

2.4. Microdialysis

After the experiments with standard solutions, the performance of the most promising antioxidative mixture (No. 13 in Table 1) was tested in a microdialysis experiment. Microdialysis was performed as previously described [10], except that the rats were allowed to recover from the surgical procedure for 6–7 days, and the dummy needles of the guide cannulas were replaced with microdialysis probes (CMA/12 Microdialysis Probe, membrane length 2 mm; CMA Microdialysis, Sweden) at least 24 h prior to initiating the perfusion. Thirty-min samples were collected from the nucleus accumbens of freely moving rats by perfusing the probes with modified Ringer's solution at a flow-rate of 2 μl/min. A 10-μl aliquot of the antioxidative mixture was added beforehand in vials in which 60-μl dialysate samples were collected. All the dialysate samples were then pooled together, and after brief vortexing 20-μl aliquots of this mixture were assayed in nine repetitions with HPLC immediately after the experiment, and then reanalyzed after 20 h.

3. Results and discussion

The screening results of the entire range of potential antioxidative mixtures are summarized in the comments section of Table 1. The solution containing AA was discarded from further testing due to interference with the analysis, and the other mixtures were evaluated for their ability to preserve DA, 5-HT, DOPAC, HVA and 5-HIAA when added (1:7) to standard solutions in modified Ringer's solution.

The results of the 24-h stability experiments showed that the need for an antioxidative is clear: in modified Ringer's solution, which was used as the perfusion fluid in the microdialysis experiments, DA and DOPAC degraded within 2 h at room temperature (Fig. 1). The rapid degradation of DA, which is due to the well-known chemical instability of catecholamines in alkaline solutions, was not observed in solutions with final pH of $\leq$3.6. However, as demonstrated previously [4,8,11], increasing the acid concentrations tends to increase the degradation of indole-based compounds. In the present study the height of the 5-HIAA peak decreased markedly over time in the solution of HCl (0.1 *M*, final pH 2.0 when one part of antioxidative mixture was mixed with six parts of Ringer's solution, Fig. 1). This pH-dependency was also clearly seen in the series of HAc solutions (0.1 *M*, final pH 2.8, 3.2, 3.6 and 4.0),

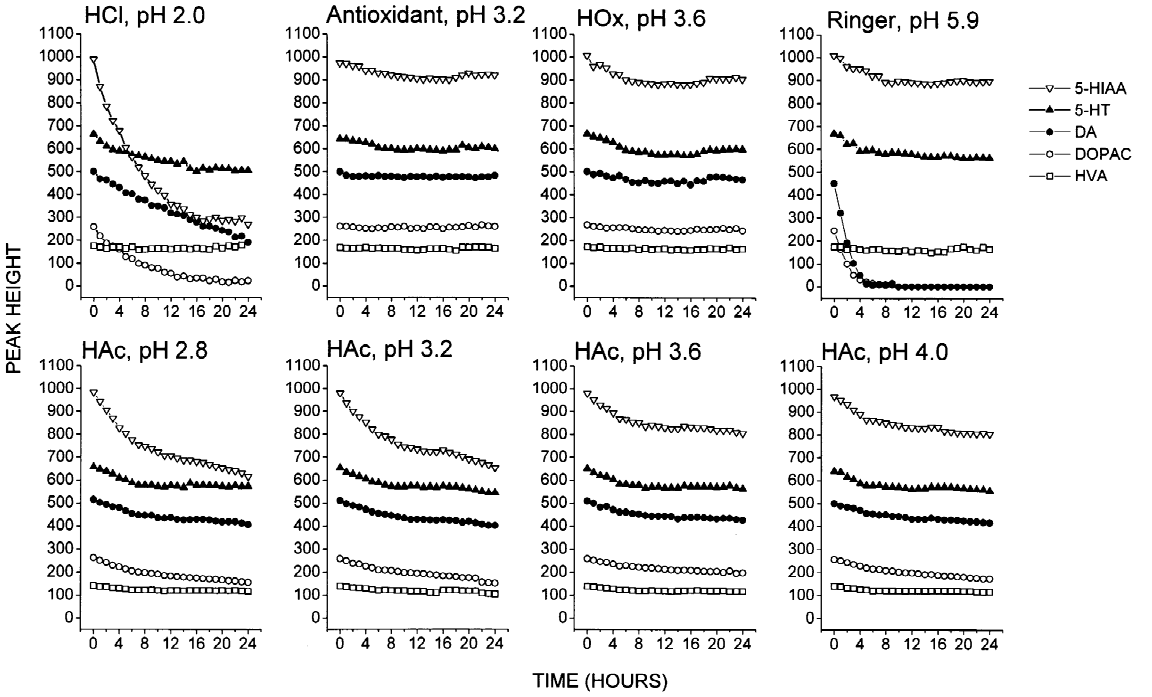

Fig. 1. Effects of selected potential antioxidative solutions on the stability of DA, 5-HT, DOPAC, HVA and 5-HIAA. Ringer refers to the solution with no preservatives (No. 1 in Table 1), HCl refers to 0.1 *M* hydrochloric acid (No. 3), HAc to 0.1 *M* acetic acid (Nos. 4–7), HOx to 1.0 m*M* oxalic acid (No. 10), and Antioxidant to the preferred antioxidative mixture (No. 13), which contains 1.0 m*M* oxalic acid, 3.0 m*M* L-cysteine, and 0.1 *M* acetic acid.

in which the stability of 5-HIAA correlated with the pH of the solution (Fig. 1). DOPAC also showed a tendency to degrade, especially in the solution of HCl (Fig. 1).

In contrast, addition of 1.0 m*M* HOx even alone prevented the degradation of DA, 5-HT, DOPAC, HVA and 5-HIAA in the 24-h experiment (Fig. 1). The final acidic pH of the solution (3.6) evidently contributed to the stability of DA, but as DA degraded less in the HOx solution than in the HAc solution of the same pH (Fig. 1), it seems likely that other types of interaction were responsible for the antioxidative potential of HOx. The rapid degradation of the analytes in the absence of protective agents is due to oxidation by air dissolved in the samples: catecholamines are converted to their corresponding quinones and indoleamines to quinoneimines, identical to EC oxidation reactions on the electrode surface. Metallic cations, especially Fe^{3+}, are known to accelerate oxidation reactions, and consequently it may be hypothesized that the protective effect of HOx may be based on its ability to eliminate metallic traces from the solution by forming stable complexes with them [12]. Since these associations do not, however, readily occur under acidic pH conditions, Verbiese-Genard et al. [4] suggested that another metal chelator, EDTA, protects catechol and indole solutions by acting as a competitive agent that interferes with oxidation of indole derivatives. Similarly, Fleming and Bensch [13] hypothesized that the stabilizing effect of EDTA on ascorbate can, at least in part, be attributed to its direct interaction with ascorbate, possibly through hydrogen bonding with a hydroxyl group or amine. Thus it can be speculated that HOx, which resembles EDTA at least with regard to its ability to form complexes with metal ions, may also share its mechanism of action in protecting catechol- and indoleamines from oxidative degradation.

Although the addition of 1.0 m*M* HOx already

protected all the analytes satisfactorily, the overall performance of the analysis was still enhanced when Cyst (3.0 m*M*) and HAc (0.1 *M*) were added to the solution (Fig. 1). This mixture is referred to as ''Antioxidant'' and was utilized throughout the remaining experiments. Since the advantages of the Antioxidant relative to HOx alone were small, yet considered significant, the exact role of the addition-

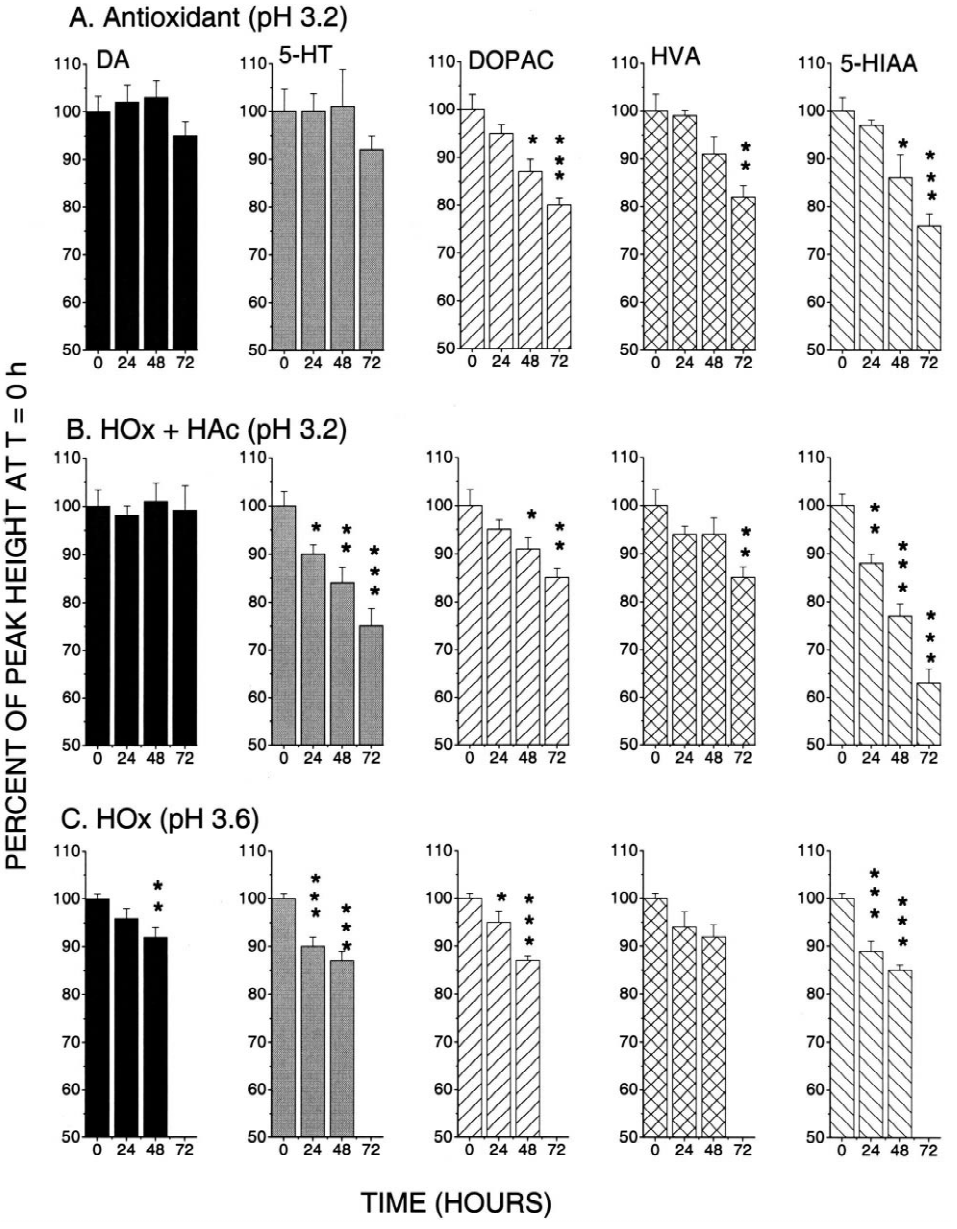

Fig. 2. Effects of the Antioxidant solution (A), a solution without Cyst (B) and a solution containing HOx alone (C) on the long-term stability of DA, 5-HT, DOPAC, HVA and 5-HIAA. Mixtures of standard solutions and the antioxidative solutions were analyzed in six repetitions at each timepoint. The data are given as means of the percentages of peak heights at timepoint 0 h±S.E.M. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with time 0 h, one-tailed Dunnet's test.

al components were further examined in a long-term experiment.

Fig. 2A shows the results of the long-term stability test with the Antioxidant solution. DA and 5-HT were stable for at least 48 h, and even after 72 h at room temperature only a nonsignificant trend towards decrease in peak height was observed. The height of the HVA peak showed a slight decrease after 48 h, but the difference after time 0 h was statistically significant only at 72 h. DOPAC and 5-HIAA were stable for at least 24 h. The effect of Cyst on the stability of the analytes is evident when Fig. 2A–C are compared: the indole-based compounds are most stable in the Cyst-containing Antioxidant solution (Fig. 2A). This additional protection for indole-based compounds provided by Cyst is probably due to its ability to remove oxygen from the solution by auto-oxidation to L-cystine [7,11]. Even in the presence of HOx alone, the degradation of the analytes did not exceed 10% in 24 h or 15% in 48 h (Fig. 2C). Increasing the acidity of the solution seemed to primarily stabilize DA (Fig. 2B vs. Fig. 2C).

The Antioxidant solution also preserved the dialysate samples for at least 20 h after sample collection (Fig. 3), confirming the suitability of the solution in microdialysis experiments. In Fig. 4, a typical chromatogram of a microdialysis sample stabilized with the Antioxidant is shown.

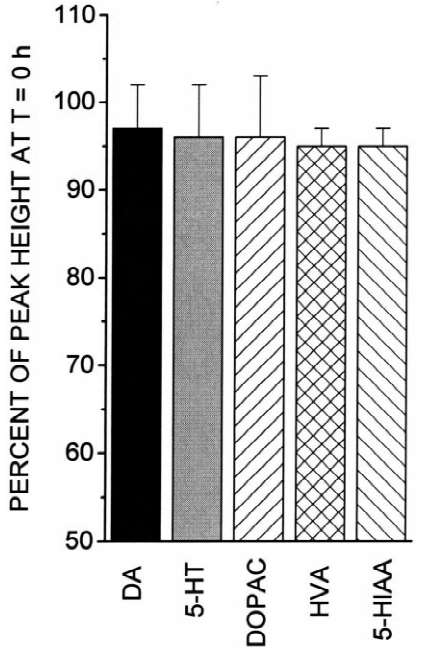

Fig. 3. The relative degradation of DA, 5-HT, DOPAC, HVA and 5-HIAA in dialysate samples after 20-h incubation at room temperature. A mixture of a pooled dialysate sample and the Antioxidant solution was analyzed in nine repetitions immediately after the microdialysis experiment, and reanalyzed after 20 h. The data are given as means of the percentages of peak heights at timepoint 0 h±S.E.M.

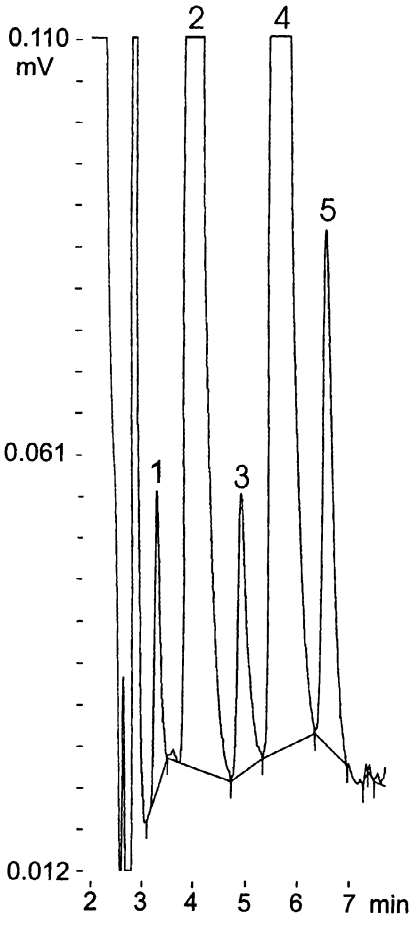

Fig. 4. Chromatogram of a dialysate sample from nucleus accumbens: 1, DA; 2, DOPAC; 3, 5-HT; 4, 5-HIAA; 5, HVA. The concentrations of DA and 5-HT were 60 and 30 fmol/60 μl dialysate, respectively.

4. Conclusions

The results of this study indicate that even alone HOx stabilizes DA, 5-HT, DOPAC, HVA and 5-HIAA efficiently. In a solution with Cyst and HAc it prevents the oxidative degradation of DA and 5-HT for as long as 3 days. Moreover, these solutions do not cause any interference with the analysis, at least when using the method described here. Together, these properties make HOx an ideal antioxidative agent that allows an automated LC–EC determination of monoamine transmitters and their metabolites in a large number of dialysate samples even at room temperature.

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