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Short communication

Simultaneous measurement of monoamines, their metabolites and 2,3- and 2,5-dihydroxybenzoates by high-performance liquid chromatography with electrochemical detection. Application to rat brain dialysates

J.M. Duine, F. Floch, C. Cann-Moisan*, P. Mialon, J. Caroff[1]

Physiology Laboratory, Medicine Faculty, B.P. 815, 29285 Brest Cedex, France

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Abstract

A reversed-phase chromatographic method with electrochemical detection was developed for the simultaneous determination of 2,3- and 2,5-dihydroxybenzoates, indicators of in vivo hydroxyl free radical formation, monoamines (NE, DA, 5-HT) and their metabolites (MHPG, DOPAC, HVA, 3MT, 5-HIAA). Linearity was observed from 10 pg to 10 ng injected. Reproducibility is correct (C.V. about 9%) except for 3MT and 5-HT. The limit of detection for almost all products was about 20 pg injected on the column. An application of this method in the study of the neurotoxicity of high pressure oxygen in rat is described. The limit of quantification for all compounds was 5 ng/ml except for HVA (10 ng/ml). Some basal levels DA, 5-HT, 5-HIAA, HVA, DOPAC, 3MT, 2,5-DHBA and 2,3-DHBA in microdialysates coming from striatum of normoxic restrained rats are given.

Keywords: Monoamines; Dihydroxybenzoates

1. Introduction

Many free radical species are formed during oxidative stress, but the hydroxyl radical (HO°) is generally considered as one of the most toxic. In the brain and the heart, HO° formation has been related to monoamines due to both their autooxidation and their deamination in the presence of transition metals such as iron and copper [1–3].

Chromatographic methods (HPLC–ED) are proposed in the literature for the determination of monoamines and their metabolites [4]. In vivo formation of HO° is difficult to detect due to its very short lifetime. Thus indirect methods have been used to monitor this process. One method involves the administration of salicylate as the hydroxyl trap. Salicylate is not toxic, reacts fast and yields two stable products 2,3- and 2,5-dihydroxybenzoate (2,3- and 2,5-DHBA) which can be then measured by HPLC–ED [5].

Up to now, two methods in parallel were necessary to quantify monoamines and DHBA. As salicylic acid is a partial trapper of HO°, it does not fully

*Corresponding author.

[1]In memoriam of Jehan Caroff, who suddenly died during the preparation of this paper. He was a lovable man, a great analyst and all the members of our group greatly miss him.

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deplete the area of study of HO°. Should an analytical method for all these compounds be available, it could be possible to quantify both the monoaminergic system activity and its contribution to HO° formation. Patthy et al. [6] have proposed a method in which only dopamine, serotonin and their metabolites were determined. However, in oxidative stress, it is often interesting to quantify epinephrine and norepinephrine in brain and in peripheral tissue such as muscle.

In this paper, a method is proposed for the simultaneous determination of dihydroxybenzoates, bioamines and their main metabolites. This method has the advantage of requiring sample volumes as low as 5 μl which are sufficient for analysis. This makes it suitable for microdialysis. Sensitivity, linearity and reproducibility were verified before being applied to microdialysis. This paper also reports the values for monoamines, their metabolites and 2,3- and 2,5-DHBA in perfusates of rat striatum under normoxia and an application of this method to the neurotoxicity of high pressure oxygen in rat.

2. Experimental

2.1. Reagents and chemicals

Salicylic acid, sodium acetate, citric acid, sodium chloride, calcium chloride, potassium chloride, magnesium chloride, ethylenediaminetetraacetate sodium salt (EDTA), sodium dihydrogenphosphate, sodium octyl sulfate (SOS) and acetonitrile were of analytical reagent grade (Merck, Darmstadt, Germany).

Norepinephrine (NE), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3MT), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 2,3- and 2,5-DHBA were purchased from Sigma (St Louis, MO, USA).

All stock solutions were prepared in 0.1 *M* perchloric acid and stored at −80°C. External standards were prepared just before analysis in the perfusion liquid for microdialysis. The composition of this liquid was as follow: 125 m*M* NaCl, 1.26 m*M* $CaCl_2$, 2.5 m*M* KCl, 1.18 m*M* $MgCl_2$, 0.2 m*M* NaH_2PO_4, 5 m*M* salicylic acid ajusted at pH 7.3 with 0.1 *M* NaOH. The composition of the mixture standard was: 0.15 μ*M* NE, 0.22 μ*M* MHPG, 0.16 μ*M* 2,3-DHBA, 0.15 μ*M* DOPAC, 0.13 μ*M* 2,5-DHBA, 0.25 μ*M* DA, 0.26 μ*M* 5-HIAA, 0.55 μ*M* HVA, 0.25 μ*M* 3MT and 0.13 μ*M* 5-HT.

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a 420 pump (Kontron Instruments, St-Quentin-en-Yvelines, France), an autosampler 465 (Kontron) equipped with a cooling plate maintained at 4°C and a 7110 Rheodyne injection valve fitted with a variable volume injection loop (5 μl in our experiments). Separation was achieved on a Nucleosil ODS column (250×4.6 mm ID, 5 μm) protected by a BrownLee RP 18 guard column (30×4.6 mm ID).

The mobile phase was 95% 50 m*M* sodium acetate, 10 m*M* citric acid, 0.15 m*M* EDTA, 0.43 m*M* SOS and 5% of acetonitrile adjusted to pH 3.4 with glacial acetic acid and filtered through 0.22 μm. The flow-rate was 0.8 ml/min.

Electrochemical detection was accomplished using an amperometric detector (model 460, Waters, Millford, MA, USA) with a glassy working electrode and a Ag/AgCl reference electrode at a potential of +700 mV. A microcomputer (Kontron) controlled the chromatographic system.

3. Results and discussion

The chromatographic conditions were modified from a method used for the measurement of 3,4-dihydroxyphenylethyleneglycol (DOPEG) in plasma [7].

The major analytical difficulty was to establish chromatographic conditions for a standard mixture containing acids and amines. Two parameters were mainly studied: pH and SOS concentration (ion pairing agent) of the eluent. Retention times of acids depend particularly upon pH and those of amines upon SOS concentration. Thus, during this study, were tested pH from 3.3 to 3.6 and SOS from 0.32 to 0.43 m*M*. The best separation was obtained at pH 3.4 and 0.34 m*M* SOS.

Under these conditions, the chromatogram (Fig. 1A) shows all the components of the standard

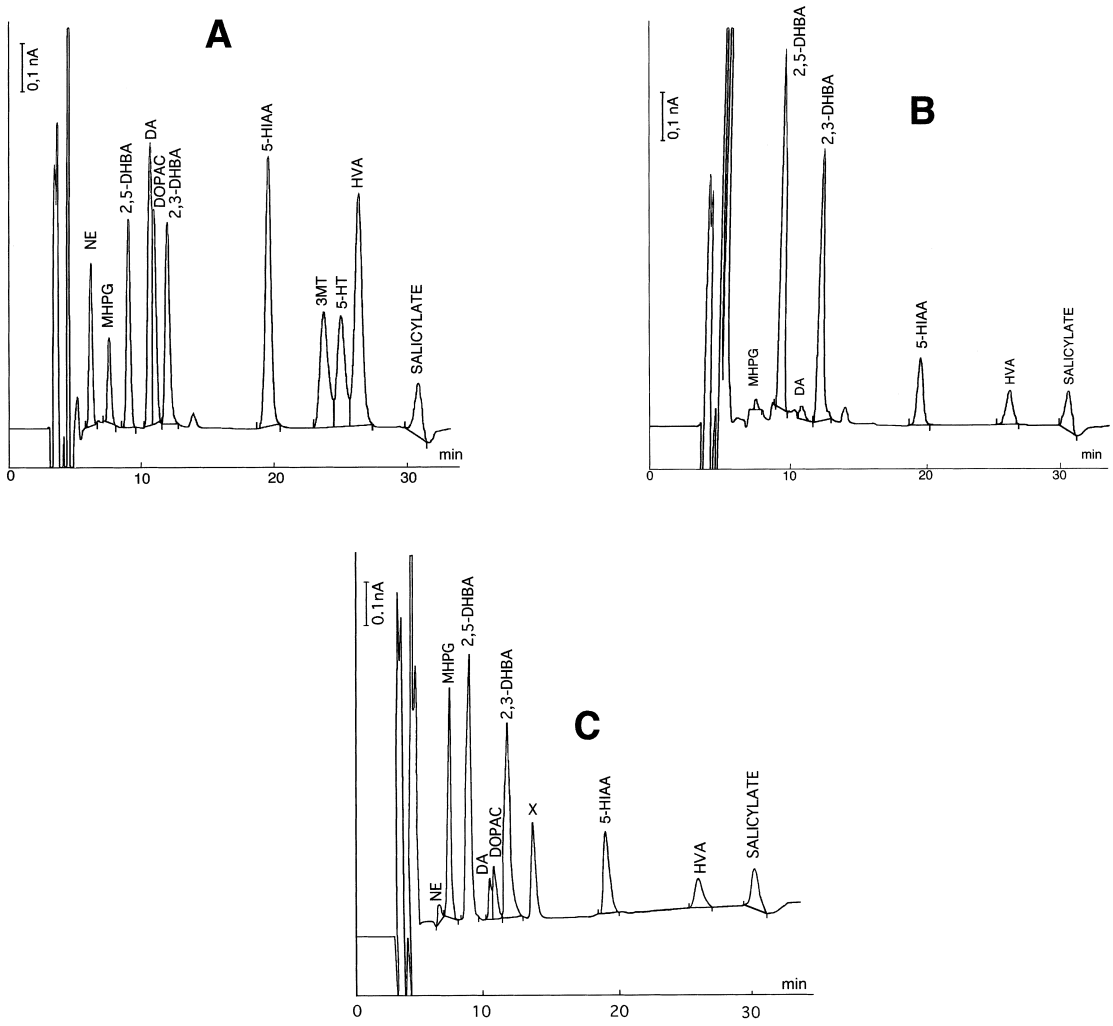

Fig. 1. Separation of some bioamines, metabolites and dihydrobenzoates under the following chromatographic conditions: column Nucleosil 5 μm, 250×4.6 mm ID, mobile phase: 5% acetonitrile and 95% 50 m*M* sodium acetate, 10 m*M* citric acid, 0.15 m*M* EDTA and 0.43 m*M* SOS, pH 3.4, flow-rate 0.8 ml/min. (A) Chromatogram of a standard prepared in salicylate solution. The amounts of compounds injected are expressed in pg: NE 127 pg; MHPG 182 pg; 2,3-DHBA 115 pg; DOPAC 126 pg; 2,5-DHBA 114 pg; DA 237 pg; 5-HIAA 248 pg; HVA 501 pg; 3-MT 255 pg and 5-HT 252 pg. (B) Chromatogram of a microdialysis perfusate obtained in rat striatum under normoxia. (C) Chromatogram of a microdialysis perfusate obtained in rat striatum under hyperbaric hyperoxia.

mixture with good resolution and an analysis time of less than 30 min. As can be seen, salicylic acid does not interfere with the analysis.

To validate the method, the linearity, reproducibility and sensitivity were verified under the above chromatographic conditions with 5 μl injected.

Calibration curves for all the components were established from 10 pg to 10 ng injected: MHPG, $y=0.12x-3.06$; NE, $y=0.42x-2.98$; DOPAC, $y=0.33x+22.95$; DA, $y=0.39x-3.92$; 5-HIAA, $y=0.45x-20.23$; 2,3-DHBA, $y=0.51x-18.57$; HVA, $y=0.24x+2.12$; 2,5-DHBA, $y=0.31x+1.98$; 3MT, $y=0.09x-0.56$; 5-HT, $y=0.23x+3.02$; ($r=0.999$ for all).

The inter-assay and intra-assay coefficients of variation (C.V.) were calculated ($n=10$): inter-assay C.V. was between 8.3 and 9.5% for all the components except for 3MT and 5-HT respectively 17.2

and 21%. The high variability of these components may be explained by their instability or more likely by their long retention times under these conditions. The intra-assay C.V. was between 3.5 and 7% for all the components except for 3MT and 5-HT, 8.3 and 9% respectively. The detection limits for these compounds (three times the noise) were as follows: 11 pg or 5.7 n*M* for 5-HT, 13 pg or 15.3 n*M* for NE, 15 pg or 19.5 n*M* for 2,3-DHBA, 15 pg or 17.8 n*M* for DOPAC, 20 pg or 18 n*M* for MHPG, 20 pg or 21 n*M* for DA, 22 pg or 24.9 n*M* for 2,5-DHBA, 22 pg or 21.6 n*M* for 3 MT, 25 pg or 26 n*M* for 5-HIAA, 50 pg or 55 n*M* for HVA. The sensitivity of this assay is sufficient to determine each of these products in rat brain dialysate as the limits of quantification was about 5 ng/ml for all the components (10 ng/ml for HVA).

This method was established to study the effects of high pressure oxygen on monoamines and hydroxyl radicals in rat striatum: these compounds are thought to play a role in high pressure oxygen neurotoxicity [8] but no direct demonstration has been available. In a preliminary study performed in normoxia at atmospheric pressure, this method was applied to striatum perfusates obtained by microdialysis in normal Sprague Dawley rats. The rats were stereotaxically implanted [9] with a CMA guide canula (CMA Carnegie, Solna, Sweden) within striatum (0.2 mm anterior, 2.5 mm lateral to bregma and 3. 5 mm under the brain surface). Two days after implantation, a CMA 12/4 canula was inserted in the guide, then the rats were inserted in a restrainer, their legs being free above the ground. They stayed in this restrainer, which is needed in our small hyperbaric chamber filled with oxygen, during dialysis. The chromatogram (Fig. 1B) shows good separation of all components. In 8 normoxic rats, the perfusate concentrations were as follows: 50 to 200 n*M* for DA, 10 to 60 for 5-HT, 200 to 1000 for 5-HIAA, 400 to 2500 for HVA, 500 to 4500 for DOPAC, 50 to 2600 for 3MT, 350 to 1500 for 2,5-DHBA and 20 to 500 for 2,3-DHBA. The range of the results and the inter-animal variability are comparable to those reported in the literature despite contention [10–13]. In Fig. 1C a chromatogram obtained from a rat exposed to high oxygen pressure is shown.

In conclusion, this method enables simultaneous determination of dihydroxybenzoates, indicators of HO° free radical formation, monoamines and their main metabolites with sufficient sensitivity. The use of an autosampler fitted with a cooling plate is interesting in terms of stability and reproducibility when samples are numerous, as in microdialysis. We hope that this method will be found useful in oxidative stress studies.

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