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Journal of Chromatography B, 663 (1995) 9–14

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Sensitive high-performance liquid chromatographic method for the determination of 5-S-cysteinyldopamine, 5-S-cysteinyl-3,4-dihydroxyphenylacetic acid and 5-S-cysteinyl-3,4-dihydroxyphenylalanine

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First received 22 September 1993; revised manuscript received 7 September 1994

Abstract

A new HPLC method for the determination of 5-S-cysteinylcatechols has been developed. The alumina adsorbed fraction of the supernatant of brain homogenate was injected onto a reversed-phase column and a citrate–phosphate buffer containing 1-nonyl sulphate was used as mobile phase (pH 2.1). Two dual-series working electrodes of a thin-layer cell were operating together, joined by a special coupler. The assay allows determination of the 5-S-cysteinylcatechols in the striatum, limbic system and mesencephalon of one guinea pig. Recoveries of the three 5-S-cysteinylcatechols were 59–76%, whereas the limit of quantitation was 0.04–0.10 pmol. The coefficient of variation was less than 0.76–1.10% and linearity was found up to a concentration of 500 pmol. By adding ascorbic acid to the samples, artifacts resulting in HPLC peaks were either reduced in size or deleted.

1. Introduction

Cysteinylcatechols have been indicated as markers for the in-vivo oxidation of dopamine (DA), 3,4-dihydroxyphenylalanine (DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC), to their quinone forms [1]. Determination of these cysteinylcatechols may lead to a better understanding of the neurodegeneration of the nigrostriatal neurons in Parkinson's disease (PD) and during non-pathological aging. Oxidation of dopamine (DA) and related brain catechols to quinones might contribute to the degeneration process in dopaminergic neurons [2].

The theory of quinone toxicity is based on the following observations. It is well known that catechols autoxidize easily in solution to semiquinones and then further to electron-deficient quinones, which form adducts with electron-rich groups, i.e. nucleophiles [3,4]. In the mammalian cell the strongest nucleophile is the thiol group, which is found in the amino acid cysteine and accordingly in cysteine-containing molecules, such as the tripeptide glutathione (GSH) and several proteins. The quinones of catechols have been demonstrated to be cytotox-

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0378-4347/95/$09.50
SSDI 0378-4347(94)00417-X

ic in vitro [5,6]. This toxicity is presumably related to the formation of covalent bonds between the quinone form of the catechols and nucleophilic groups on macromolecules of vital importance for cell functioning [6–8].

A method for the indirect detection of in-vivo formation of quinones of brain catechols has previously been presented [9]. This method was based on a reversed-phase HPLC method with electrochemical detection, and the tissue samples were prepared by alumina adsorption. Since the quinones formed during catechol autoxidation are too reactive to be detected easily, the interest was focused on the most likely in-vivo products of the quinones. These products appeared to be 5-S-cysteinyl-DA, 5-S-cysteinyl-DOPAC and 5-S-cysteinyl-DOPA, which have been detected in DA-rich brain regions in several mammalian species [9–11]. Experimental studies on the guinea pig support the theory of catechol oxidation in vivo [12–14].

The present paper describes a method which is faster and more sensitive than the previously published method [9] that allowed only one observation to be obtained by pooling striatal tissue from three to four guinea pigs.

2. Experimental

2.1. Chemicals

1,4-Dithio-DL-threitol (DTT) and diethylenetriaminepentaacetic acid (DTPA) were obtained from Fluka (Buchs, Switzerland). 1-Nonyl sulphate sodium salt and 2-propanol (PA grade) were obtained from Merck (Darmstadt, Germany). DOPAC, DA and α-methyl-DOPA hydrochloride were obtained from Sigma (St. Louis, MO, USA) and the 5-S-cysteinylcatechols were prepared as described for the enzymatic synthesis of 5-S-cysteinyl-DA [10], 5-S-cysteinyl-DOPAC [9] and 5-S-cysteinyl-DOPA [15]. All other chemicals used in this study (i.e. antioxidants, buffer substances, organic and inorganic acids etc.) were of analytical-reagent grade.

2.2. Animals

Female Dunkin–Hartley guinea pigs, 2.5–3 years old (HB Sahlins försöksdjursfarm, Malmö, Sweden), weighing fully 1000 g were used. The animals were kept, 5 per cage, under controlled environmental conditions (temperature 26°C; humidity 60–65%; light 5 a.m.–7 p.m., dark 7 p.m.–5 a.m.). Food was allowed ad libitum and so was the tap water, to which 200 mg/l ascorbic acid (AA) was added every other day. The animals were killed by decapitation during daytime and the brains were quickly removed and placed on an ice-chilled Petri dish. The striatum, the limbic system, the mesencephalon and the cerebellum were taken out and kept at −70°C until analysis.

2.3. Sample preparation

Striatal (100–180 mg) or limbic tissue (140–220 mg) was added to 1090 μl of an ice-chilled solution. This solution contained 0.1 *M* perchloric acid (PCA), 4.8 m*M* sodium bisulphite ($Na_2S_2O_5$), 4.7 m*M* DTPA, 0.78 m*M* AA and 5.5 m*M* DTT. It is of importance to add the different antioxidants and scavengers in the order mentioned above. The samples were ultrasonicated for 25 s and centrifuged (10 000 *g*, 4°C) for 10 min and the supernatants were withdrawn. A volume of 100 μl of α-methyl-DOPA solution (237 pmol/ml), 20 mg of acid-washed aluminum oxide [16] and 500 μl of 3 *M* Tris buffer (pH 8.6) were added to 900 μl of the supernatant in the order of mentioned. The samples were mixed for 5 min. The aluminum oxide was washed twice with 1.5 ml of distilled water. The catechols were eluted in 300 μl of mobile phase used for the analysis of 5-S-cysteinylcatechols at pH 2.3, and then kept at −20°C until analysis within a few days. The samples were chilled on ice-water during most of the preparative work. A ca. 400-mg amount of mesencephalic tissue was added to 2.18 ml of a solution identical to that used for striatal and limbic tissue. The samples were homogenized in an Ultra-Turrax and centrifuged (10 000 *g*, 4°C, for 10 min), and to 1800 μl of the supernatant 100 μl of α-methyl-DOPA

solution (237 pmol/ml), 20 mg of acid-washed aluminum oxide and 1000 μl of 3 *M* Tris buffer were added and then the samples were prepared as the striatal and limbic samples.

In a multiple-extraction test, guinea-pig striatum was extracted three times in the PCA solution described above. All extracts were collected and the catechol contents were determined in each extract by the method described in this paper. Part of the tissue catechols, corresponding to up to ca. 20% of the total amount, were found to remain in the pellets following the first extraction (data not shown). Most of this remaining amount was found in the two following extracts. However, since all comparable analyses in the literature have been performed on the first extract it was decided that also the present method should be based on this first extract.

Prior to sample preparation, a test for artifacts was performed. A 30-μl volume of a DA solution (50 μg/ml) was added to 1090 μl of the PCA solution before the addition of 150 mg cerebellum, and the samples were then prepared as the striatal and limbic samples. No 5-S-cysteinyl-DA was detected on the chromatogram. Reference samples containing 4.86 pmol of 5-S-cysteinyl-DA, 4.38 pmol of 5-S-cysteinyl-DOPAC and 8.60 pmol of 5-S-cysteinyl-DOPA pmol in 1000 μl of a 1:3 dilution of the PCA solution, were prepared in the same way as the supernatants of the tissue samples.

For DA, DOPAC and DOPA determinations in the striatum, 50 μl of the supernatant was added to 950 μl of the PCA solution used for ultrasonication and homogenization. Reference samples contained 65.2 pmol DA, 59.6 pmol DOPAC and 50.8 pmol DOPA in 1000 μl of a 1:3 dilution of the PCA solution. Both reference and tissue samples were prepared as the 5-S-cysteinyl-catechol samples except that a more concentrated solution of α-methyl-DOPA (2.37 nmol/ml) was used. For determinations in limbic structures, 100 μl of the supernatant was added to 900 μl of the PCA solution and reference samples contained the same amounts of catechols as in the striatal analysis. For determinations in the mesencephalon 200 μl of the supernatant was added to 800 μl of the PCA solution. Reference samples contained 32.6 pmol DA, 29.8 pmol DOPAC and 25.4 pmol DOPA in 1000 μl of a 1:3 dilution of the PCA solution.

2.4. Chromatography

The high-performance liquid chromatographic (HPLC) system used for analysis consisted of a pump Model 2150 (LKB, Bromma, Sweden), an injection valve Model C6W (Valco Europe, Schenkon, Switzerland) or Model 7010 (Rheodyne, Berkeley, CA, USA), a 200-μl loop in stainless steel, and a stainless steel column (250 × 4.6 mm I.D.) packed with Nucleosil RP-18, 5 μm (Macherey-Nagel, Düren, Germany). Detection was carried out by means of a thin-layer cell with dual-series working electrodes TL-5A (Bioanalytical Systems, BAS, West Lafayette, IN, USA) with a glassy-carbon working electrode, an Ag/AgCl reference electrode and an amperometric detector (LC-3, BAS). The detector potential was set at +0.75 V vs. the Ag/AgCl reference electrode and 5 or 10 nA/10 mV output was used. An integrator Model SP 4270 (Spectra-Physics, San José, CA, USA) was used to monitor the current signal at a chart speed of 0.25 cm/min. The mobile phase used for 5-S-cysteinyl-catechol analysis had a pH of 2.1 and consisted of 1.1 m*M* K_2HPO_4, 48.9 m*M* citric acid, 0.054 m*M* EDTA, 60 mg/l (0.244 m*M*) 1-nonyl sulphate, 9–10% (v/v) methanol and 1.7% (v/v) 2-propanol. The total amount of organic solvent needed could be slightly different depending on the age of the column. Likewise, the ratio of the two alcohols should be slightly changed, if necessary to obtain resolution of the peak for 5-S-cysteinyl-DOPA. The 5-S-cysteinyl-DA peak should not have a retention time much longer than 40 min in order to avoid too small HPLC peaks. The flow-rate was 1.25–1.40 ml/min. It should be mentioned that slightly different mobile phases were used in the pH test and the 1-nonyl sulphate test.

The HPLC equipment used for assaying the non-adduct catechols, was identical to that in the other system except for the use of an LDC minipump (Laboratory Data Control, Riviera

Beach, FL, USA), and a column length of 150 × 4.6 mm I.D. The mobile phase consisted of 12.5 m*M* K_2HPO_4, 37.5 m*M* citric acid, 60 mg/l (0.258 m*M*) 1-octyl sulphate (Merck, Darmstadt, Germany), 54 m*M* EDTA and 4–6% (v/v) methanol. The pH was 2.75 and the flow-rate was 1.0 ml/min.

In the validation of the new method the following data were considered. The three 5-S-cysteinylcatechols showed a recovery of 59% for 5-S-cysteinyl-DOPAC, 63% for 5-S-cysteinyl-DOPA and 76% for 5-S-cysteinyl-DA, and the coefficients of variation were 0.76, 0.93 and 1.10%, respectively. The limit of quantitation was 0.04 pmol for 5-S-cysteinyl-DOPAC and about 0.1 pmol for the two other adducts. Linearity was found up to 500 pmol, but was not examined at higher concentrations in view of the low endogenous levels. Control levels of the 5-S-cysteinylcatechols in the brain were 10–30 pmol/g wet tissue in the striatum and slightly lower in the limbic region, whereas only a few picomoles were found per mesencephalon.

3. Results and discussion

The need for a faster and more sensitive analysis method was obvious as with the old method [9] one observation could only be obtained by pooling the striatum from three to four animals; the analysis time was around 80 min, but could be as long as 110 min. The goal was to determine the 5-S-cysteinylcatechols in the striatum, limbic system and mesencephalon of only one animal. By decreasing the counter-ion concentration of the mobile phase, compared to the previous method, the HPLC peaks of interest were eluted earlier, and by decreasing the pH of the mobile phase peak separation could be accomplished. A low pH has now been used for an extended period of time without any sign of destruction of the column or electrode surface. The column is routinely replaced or repacked after 3–6 months of analysis, because of a gradual decrease in the extent of peak separation.

Three different pH values of the mobile phase were compared (2.10, 2.20 and 2.30). An increase in retention time was found with decreasing pH for 5-S-cysteinyl-DA and 5-S-cysteinyl-DOPA. Actually, the main artifact peak derived from DA and DTT (see below), and the 5-S-cysteinyl-DA peak changed elution order, comparing the mobile phases with the highest and the lowest pH. In a counter-ion study the 1-nonyl sulphate concentration was changed from 40 mg/l, to 50 and to 60 mg/l. The highest fractional increase in retention time was found for 5-S-cysteinyl-DA and 5-S-cysteinyl-DOPA.

The 1-nonyl sulphate concentration has to be 60 mg/l in order to separate unknown endogenous peaks from the peaks of interest, and the pH of the mobile phase should be about 2.1. These changes were the most useful tools in modifying peak separation. It should also be mentioned that 2.5–3 years old guinea pigs are used in the present method, since their brains have been found to contain higher amounts of the 5-S-cysteinylcatechols than the brains of younger animals [17].

Striatal tissue was taken out with as little adjacent tissue as possible, however, the most caudal and ventral tissue was occasionally left behind. The limbic system contained the septum, the olfactory tubercles and the amygdala. The amount of 5-S-cysteinylcatechols in the substantia nigra was too small for proper determinations. However, when the mesencephalon was taken out, the amounts of 5-S-cysteinylcatechols were high enough to allow determination.

The relative size of the 5-S-cysteinylcatechol peaks differs between the brain regions examined. In the striatum the 5-S-cysteinyl-DOPAC peak was much higher than the peak of the two other 5-S-cysteinyl adducts (Fig. 1). Also the tissue concentration was highest for the DOPAC derived adduct, it was intermediate for 5-S-cysteinyl-DA and lowest for 5-S-cysteinyl-DOPA. In limbic and mesencephalic tissue the three 5-S-cysteinyl-catechol peaks were more or less of the same size. The concentrations were lower in the limbic region than in the striatum, whereas the concentration was given as amount per animal for mesencephalon.

At least two artifact peaks appeared on the

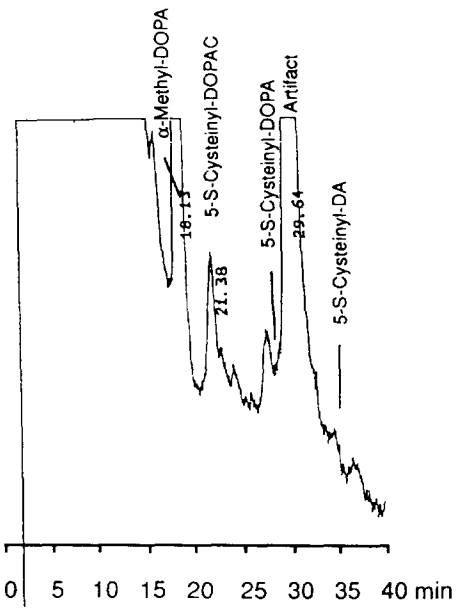

Fig. 1. Chromatogram of cerebellum, to which DA and DTT have been added. The sample was prepared and analyzed as described in the Experimental section, except for a lower content of 1-nonyl sulphate (0.234 m*M*) of the mobile phase. The peaks of the internal standard and main artifact are indicated as well as the expected elution place for the 5-S-cysteinyl catechols. The retention times of the artifacts and internal standard are indicated in minutes.

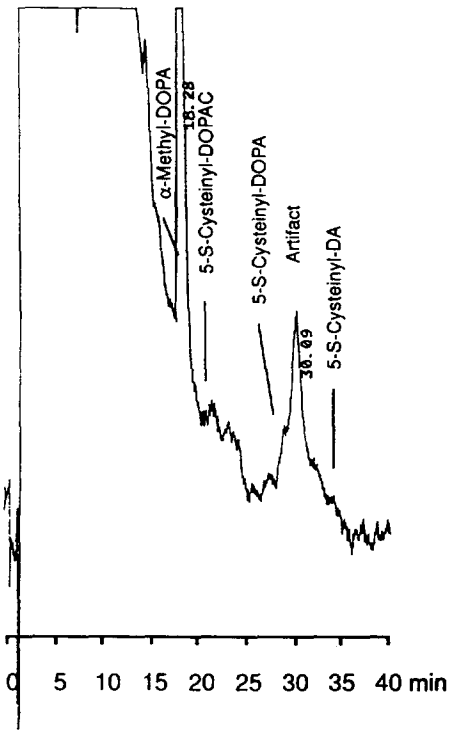

Fig. 2. Chromatogram of cerebellum to which DA, DTT and AA have been added. For further information see the legend of Fig. 1.

chromatogram of cerebellum samples to which had been added DA and DTT (Fig. 2). However, most attention has been paid to the main artifact peak. This peak appears by injecting an alumina-eluted sample of a solution of DA mixed with a solution of DTT. The size of this main artifact peak is dependent on the amount of DA and DTT in the samples, shown in tissue samples and in test solutions (data not shown). Accordingly, it was absent in DA-depleted striatum of reserpine treated animals [14] as well as in the cerebellum. If AA was added to the solution used for homogenization, the peak corresponding to the main artifact peak was extensively diminished and the additional artifact peak disappeared from the chromatogram (Fig. 2). In samples containing AA deficient cerebellum, to which DTT and striatal amounts of DA had been added, the artifact peaks formed were larger compared to cerebellum with normal AA levels (data not shown). This discrepancy disappeared when AA was added to the AA deficient tissue samples.

It is interesting to compare the above mentioned observations to the earlier reported findings, where striatal 5-S-cysteinyl-DA levels were significantly elevated in AA deficient animals and decreased again following AA level restoration [13]. Possibly, the same mechanism is responsible for the endogenous formation of 5-S-cysteinyl adducts as for the artificial formation of adducts.

Finally, it was found that an interfering system peak appeared on the chromatogram when 1- or 2-propanol was used as solvent. By adding methanol to the mobile phase and reducing the concentration of propanol, the system peak was eluted before all the peaks of importance. Propanol and methanol differ slightly in their effects on the retention times of the 5-S-cysteinylcatech-

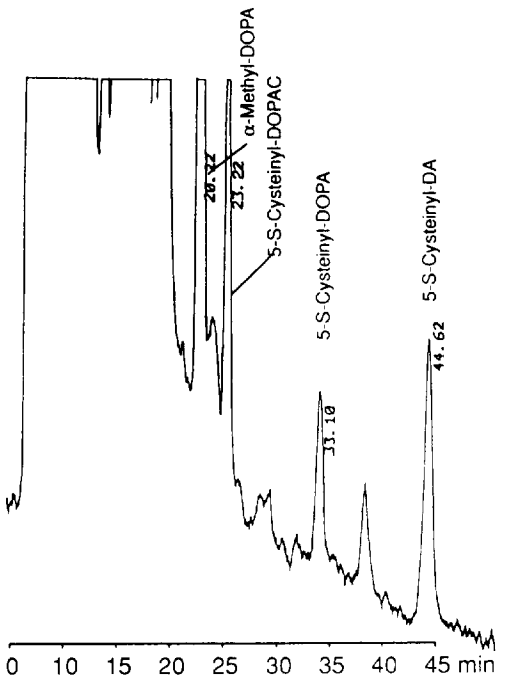

Fig. 3. Representative chromatogram of guinea-pig striatum analyzed by the present method (see the Experimental section). The tissue is derived from a single animal. The peaks of interest, and their corresponding retention times in minutes, are indicated in the figure.

ols. The 5-S-cysteinyl-DOPA and 5-S-cysteinyl-DOPAC peaks seem to be getting closer to each other, while the 5-S-cysteinyl-DOPA and 5-S-cysteinyl-DA peaks become more separated by propanol compared to methanol.

Acknowledgements

We wish to thank Drs. Torgny Fornstedt and Sören Lagerkvist for critical analytical advice. This study was supported by grants from the Swedish Medical Research Council (No. 155), the Upjohn Company, Adlerbertska forskningsfonden, Anna Ahrenbergs fond, Gun och Bertil Stohnes Stiftelse, Loo och Hans Ostermans fond för medicinsk forskning, Magnus Bergvalls stiftelse, Neurologiskt handikappades förbund, Stiftelsen Clas Groschinskys Minnesfond, Stiftelsen för Gamla Tjänarinnor, Stiftelsen Handlanden Hjalmar Svenssons forskningsfond, Stiftelsen Sigurd och Elsa Goljes Minne, Svenska Läkaresällskapet, Svenska Sällskapet för Medicinsk forskning, Wilhelm and Martina Lundgrens vetenskapsfond, Åhlén-stiftelsen, and the Medical Faculty, University of Göteborg.

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