ELSEVIER

Journal of Chromatography B, 660 (1994) 158–163

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Microbore high-performance liquid chromatographic method for the measurement of dopamine and its metabolites: recommendations for optimal sample collection and storage

Adrian J. Carter

Department of Pharmacology, Boehringer Ingelheim KG, D-55216 Ingelheim, Germany

First received 23 March 1994; revised manuscript received 11 May 1994

Abstract

A microbore high-performance liquid chromatographic method with electrochemical detection was developed for the measurement of small quantities of dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid, in microdialysis samples from rat striatum. The limit of detection of dopamine was in the low nmol/l range (10 fmol/40 μl dialysate). Standard mixtures of dopamine and its metabolites were stored in tubes made of polypropylene, soda–lime glass or borosilicate glass. Dopamine and homovanillic acid were stable in all three types of container for 24 h providing they were kept at an acidic pH. However, 3,4-dihydroxyphenylacetic acid was only stable in tubes made of polypropylene or borosilicate glass. Basal levels of dopamine in the dialysate obtained from rats which had been chronically implanted with a microdialysis probe in the anterior striatum were stable for one to three days following probe implantation. However, the levels of 3,4-dihydroxyphenylacetic acid and homovanillic acid in the dialysate decreased rapidly with time after the first day. This indicates that the synthesizing capacity of the neurons is compromised by the microdialysis probe after day one, and that microdialysis experiments in freely moving animals should be confined to the day immediately following probe implantation.

1. Introduction

Dopamine (DA) is an important neurotransmitter in the central nervous system. Its effects are mediated by an interaction with either D_1-like (D_1 and D_5) or D_2-like (D_2, D_3 and D_4) receptors [1]. The major dopaminergic tract in the brain originates in the substantia nigra and sends projections to the striatum; indeed, 80% of all DA is found in this area [1]. Microdialysis is a technique which allows continual perfusion of discrete areas of the brain with minimal exposure of the brain tissue to the perfusion medium by means of a special probe [2–4]. The technique has been used in the past to determine the effects of various centrally acting drugs on dopamine release and metabolism in the striatum of rats [5,6]. However, because microdialysis is a method which involves animal experimentation, attempts have been made in the past to chronically implant microdialysis probes and thereby reduce the number of animals used [7–10].

Microbore and capillary high-performance liquid chromatography (HPLC) techniques have been developed for the measurement of biogenic amines in microdialysis samples [11–13]. Tem-

0378-4347/94/$07.00
SSDI 0378-4347(94)00256-5

perature, pH and length of storage have been shown to have dramatic effects on the stability of biogenic amines [14–16]. The aim of this study was to develop an efficient microbore HPLC method based on commercially available microbore HPLC columns for the measurement of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in microdialysis samples, and to determine the optimal type of tube for sample storage. In addition, the effect of chronic implantation of microdialysis probes on the levels of DA, DOPAC and HVA in the striatum over a 4-day period was determined.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Merck-Hitachi (Darmstadt, Germany) L-6200 intelligent pump, a refrigerated Gilson 231-401 autosampler (Abimed, Düsseldorf, Germany) with a Rheodyne injection valve (5 μl loop) and a Bioanalytical Systems LC-4C amperometric electrochemical detector purchased from Axel Semrau (Sprockhövel, Germany). The electrochemical detector was equipped with a glassy carbon working electrode and a Ag/AgCl reference electrode. The potential was set to 750 mV, the range to 5 nA, the filter to 0.1 Hz and the offset to 1–10 nA. An online degasser from VDS Optilab (Berlin, Germany) was also used to ensure that the mobile phase was free of air. Microbore HPLC columns (SepStik, 100 × 1 mm I.D.) filled with octadecylsilane-bonded silica (3 μm) were purchased from Bioanalytical Systems (West Lafayette, IN, USA). Three different sorts of tube, all purchased from K. Ziemer (Mannheim, Germany), were used for storing acidified standard solutions: 12 × 32 mm, polypropylene tubes (0.3 ml), 5 × 30 mm soda–lime (also called soft or AR) glass tubes (0.1 ml) and 12 x 32 mm borosilicate (Type IA) glass tubes (0.1 ml).

2.2. Reagents

Chloroacetic acid was purchased from J. T. Baker (Groß-Gerau, Germany), EDTA (ethylenedinitrilo tetraacetetic acid, disodium salt) as Titriplex III, acetonitrile (Lichrosolv) and tetrahydrofuran (Lichrosolv) from E. Merck (Darmstadt, Germany), NaOH from Fluka (Neu-Ulm, Germany), and octylsulphate, sodium salt from Aldrich (Steinheim, Germany). Standard DA and DOPAC were obtained from Research Biochemicals International (Natick, MA, USA), and HVA and 5-hydroxyindoleacetic acid (5-HIAA) from Serva (Heidelberg, Germany). All other reagents were at least of reagent grade. Purified water was prepared by means of a Millipore Q-System.

2.3. HPLC procedure

The mobile phase was prepared freshly each week: 9.87 g (0.1 mol/l) chloroacetic acid, 0.1953 g Titriplex III (0.525 mmol/l) and 0.137 g octylsulphate, sodium salt (0.59 mmol/l) were dissolved in purified water. The pH was adjusted to 3.2 by adding 32% NaOH (ca. 7.0–7.3 ml). The solution was then made up to 1000 ml and filtered through a 0.45-μm membrane filter from Schleicher and Schüll (Dassel, Germany). A volume of 990 ml was withdrawn to which 52 ml acetonitrile (4.95%) and 8.3 ml tetrahydrofuran (0.79%) were added. The mobile phase was then degassed by stirring for ca. 10 min under vacuum. The mobile phase was pumped at a flow rate of 80–90 μl/min. A pulsation-free chromatogram was achieved without the need of a splitting system or microheads with the Merck–Hitachi intelligent pump. Peak identification was performed with external standards and concentrations were calculated on the basis of peak areas. The injection volume was 5 μl.

2.4. Microdialysis procedure

The microdialysis procedure used in these experiments was identical to that previously described [17]. The microdialysis probe (CMA/10, 3 mm) was implanted in the anterior striatum

with the stereotaxic coordinates +2.5 mm rostral, +2.5 mm lateral and −6.5 mm ventral relative to the bregma and dura [18]. The probes were secured by means of a guide cannula, 2 small metal screws and a small amount of Technovit 3040 cold-curing resin from Kulzer (Wehrheim/Taunus, Germany). On the following day, approximately 18–24 h following implantation, the microdialysis probe was perfused with a physiological Ringer solution (147 mmol/l NaCl, 2.3 mmol/l $CaCl_2$, 4 mmol/l KCl, pH 6.0) at a flow-rate of 2 μl/min for 80 min before collecting samples. Thereafter, samples were collected every 20 min (40 μl) until a total of 17 had been collected. The microinjection pump was then switched off and the procedure repeated on days 2 and 3. Aliquots (40 μl) of standards and microdialysis samples were immediately acidified with 5 μl perchloric acid (0.25 mol/l) such that the pH of the mixture was between 1 and 2.

2.5. Data calculation

HPLC data were collected and stored with a Nelson Analytical Series 900 interface from ESWE Analytik (Sinsheim, Germany). Peak integration was carried out with Nelson 2600 chromatography software on a Hewlett-Packard Vectra personal computer.

3. Results and discussion

The method described in this paper provides an excellent separation of DA from its metabolites, DOPAC and HVA, in standard mixtures and microdialysis samples (Fig. 1). The lower nmol/l range sensitivity achieved with this method is comparable with previously published microbore systems [11,12], although not quite as good as the upper fmol/l sensitivity achieved by capillary HPLC methods [13]. Nevertheless, the present method uses commercially available microbore columns without the need for special HPLC pumps. It was also possible to use a splitting system consisting of a piece of fused-silica tubing such that only about 10% of a normal flow-rate of approximately 1 ml/min passes through the microbore column. This is useful for HPLC pumps which cannot deliver low small flow-rates without problems of pulsation. However, careful attention must be given in both systems to degassing; a better baseline was always obtained when a degasser was connected on-line.

Comparison of the system described in this paper with a classical HPLC method previously applied in our laboratory for measuring DA in microdialysis samples [17] highlighted the three main advantages of the new microbore technique. Firstly, because the mass sensitivity of microbore techniques is greater, a much smaller volume of sample (5 μl) than was previously required (20–40 μl) can be injected onto the column. Consequently, slower microdialysis perfusion rates can be used to increase recovery or a normal sample can be split for simultaneous determination of several different neurotransmitters. Secondly, the analysis time was shortened from approximately 25 min to 14 min. More samples can therefore be measured over the same period of time. Thirdly, at a flow rate of 80 μl/min, the use of mobile phase is reduced to less than 5% of the previous system (flow rate = 1.9 ml/min). This is of considerable benefit to the environment because of the organic solvents present in the mobile phases.

In an elegant study, Palazzolo and Quadri [16] defined the optimal conditions for the long-term storage of biogenic amines for subsequent analysis by HPLC with electrochemical detection. On the basis of their recommendations, all standards and microdialysis samples in the present study were acidified immediately with perchloric acid such that the pH of the resultant mixture was between 1 and 2. The concentrations of DA, DOPAC and HVA in a standard mixture stored in tubes made of three different materials were measured every hour for 24 h. A representative plot of the concentrations against storage time (Fig. 2) revealed that the level of DOPAC in soda–lime glass tubes decreased rapidly from an initial concentration of 40 pmol/40 μl to less than 3 pmol/40 μl after 24 h. This represented a

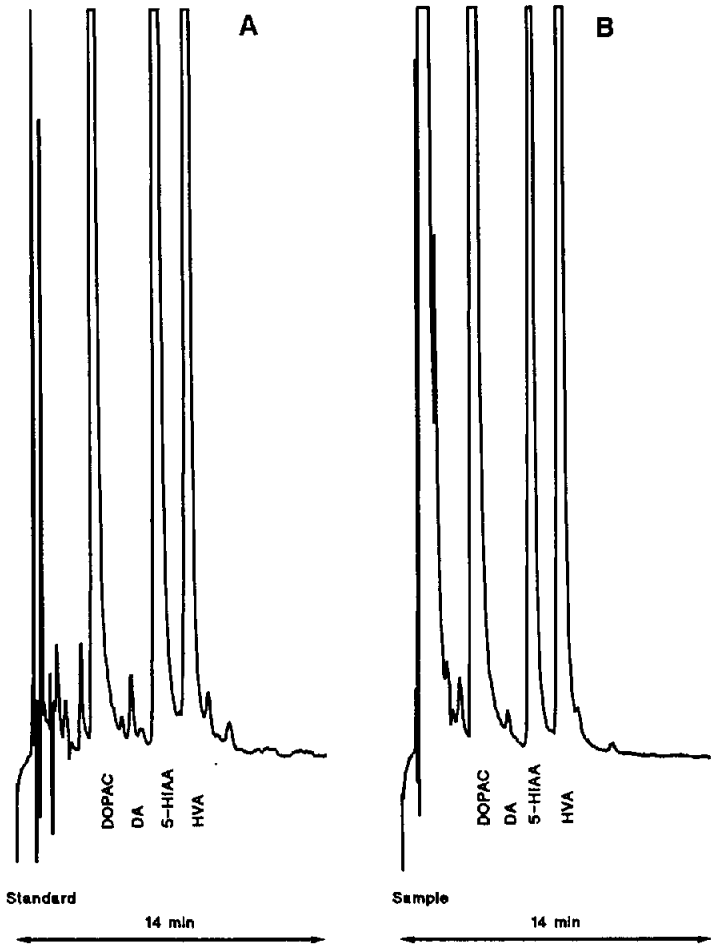

Fig. 1. Microbore HPLC separation of (A) a standard mixture of DA (0.48 pmol/40 μl), DOPAC (40 pmol/40 μl), 5-HIAA (24 pmol/40 μl) and HVA (32 pmol/40 μl) and (B) a typical baseline microdialysis sample. The chromatogram is plotted on a vertical scale of 50 mV and the elution time was 14 min. The microdialysis sample was found to contain DA (0.15 pmol/40 μl), DOPAC (81.76 pmol/40 μl) and HVA (41.96 pmol/40 μl).

loss of more than 90%. In contrast, no clear decrease in the concentrations of DA and HVA was observed in any of the tubes (Fig. 2). No additional peaks were observed in these chromatograms. These results were reproducible and show that tubes made of soda–lime glass are not suitable for storing acidified standard mixtures containing DOPAC even for short periods of time.

The mean ± S.D. baseline concentrations of DA, DOPAC and HVA in the dialysate from a microdialysis probe implanted in the anterior striatum 18-24 h previously were 0.17 ± 0.09, 86.37 ± 22.29 and 51.07 ± 13.13 pmol/40 μl respectively. The concentration of DA in the dialysate remained stable on days 2 and 3 following probe implantation, whereas the levels of DOPAC and HVA rapidly decreased: the levels on day 3 were 6.43 ± 5.13 and 18.35 ± 2.50 pmol/40 μl, respectively. Robinson and Camp [8] have previously reported a similar phenomenon: they demonstrated a marked time-dependent decrease in the basal concentrations of DOPAC and HVA. Osborne et al. [7] have also

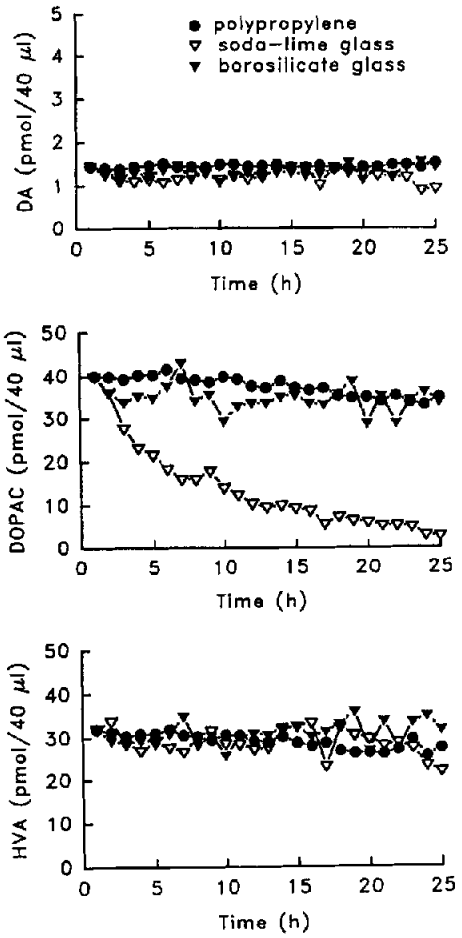

Fig. 2. Effect of time on the stability of acidified standard mixture of DA, DOPAC and HVA stored in tubes made of polypropylene, soda–lime glass or borosilicate glass. Each point represents one injection. ● = Polypropylene; ▽ = soda–lime glass; ▼ = borosilicate glass.

shown a time-dependent decrease in the extracellular concentrations of DOPAC under similar conditions. Although the perfusion rates in our study (2 µl/min) and the study of Robinson and Camp (1.5 µl/min) are comparable, the latter study also perfused the microdialysis continually overnight at a rate of 0.3 µl/min in between experiments. One criticism of this work is that this overnight perfusion might have caused a washout or exhaustion of DOPAC in the extracellular space. However, the present study illustrates that even in the absence of overnight perfusion, there is still a time-dependent reduction in DOPAC and HVA levels.

In summary, this paper describes a microbore HPLC method for the measurement of DA and its metabolites in microdialysis samples from the striatum of freely moving animals. The results demonstrate that acidified standard mixtures and samples are best stored in tubes made of polypropylene or borosilicate glass. In addition, microdialysis studies in awake, freely moving rats should be confined to the day following probe implantation.

Acknowledgements

The author is very grateful to Hans Hermann Dechent and Ute Müller for their expert technical assistance.

References

[1] N. Weiner and P.B. Molinoff, in G.J. Siegel, B.W. Agranoff, R.W. Albers and P.B. Molinoff (Editors), *Basic Neurochemistry*, Raven Press, New York, 1994, p. 261.
[2] B.H.C. Westerink, G. Damsma, H. Rollema, J.B. de Vries and A.S. Horn, *Life Sci.*, 41 (1987) 1763.
[3] H. Benveniste, *J. Neurochem.*, 52 (1989) 1667.
[4] U. Ungerstedt, in T.E. Robinson and J.B. Justice (Editors), *Microdialysis in the Neurosciences*, Elsevier, Amsterdam, 1991, p. 3.
[5] T. Zetterström, T. Sharp, C.A. Marsden and U. Ungerstedt, *J. Neurochem.*, 41 (1983) 1769.
[6] A. Imperato and G. DiChiara, *J. Neurosci.*, 4 (1984) 966.
[7] P.G. Osborne, W.T. O'Connor, J. Kehr and U. Ungerstedt, *J. Neurosci. Methods*, 37 (1991) 93.
[8] T.E. Robinson and D.M. Camp, *J. Neurosci. Methods*, 40 (1991) 211.
[9] D.M. Camp and T.E. Robinson, *J. Neurochem.*, 58 (1992) 1706.
[10] J. Georgieva, J. Luthman, B. Mohringe and O. Magnusson, *Brain Res. Bull.*, 31 (1993) 463.
[11] S.A. Wages, W.H. Church and J.B. Justice, *Anal. Chem.*, 58 (1986) 1649.
[12] W.H. Church, J.B. Justice and L.D. Byrd, *Eur. J. Pharmacol.*, 139 (1987) 345.
[13] V.F. Ruban, *J. Chromatogr.*, 619 (1993) 111.

[14] A.S. Welch and B.L. Welch, *Anal. Biochem.*, 30 (1969) 161.
[15] C.F. Saller and A.I. Salama, *J. Chromatogr.*, 309 (1984) 287.
[16] D.L. Palazzolo and S.K. Quadri, *J. Chromatogr.*, 518 (1990) 258.
[17] A.J. Carter and R.E. Müller, *Eur. J. Pharmacol.*, 200 (1991) 65.
[18] L.J. Pellegrino, A.S. Pellegrino and A.J. Cushman, *A Stereotaxic Atlas of the Rat Brain*, Plenum Press, New York, 1979.