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DETERMINATION OF DOPAMINE AND ITS METABOLITES IN SMALL VOLUMES OF RAT BRAIN DIALYSATES USING SMALL-BORE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

ANDERS CARLSSON*
Department of Analytical Chemistry, University of Uppsala, P.O. Box 531, S-751 21 Uppsala (Sweden)
and
T. SHARP, T. ZETTERSTRÖM and U. UNGERSTEDT
Department of Pharmacology, Karolinska Institutet, Box 60 400, S-104 01 Stockholm (Sweden)
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SUMMARY

A miniaturized liquid chromatographic system with electrochemical detection (LC–ED) was developed and applied to the analysis of dopamine and its metabolites in dialysate samples collected from the rat brain *in vivo*. An existing LC–ED system was down-scaled using a 1 mm I.D. small-bore column operated at a reduced flow-rate and with an injection volume of 1 μl. With the small-bore system the limit of detection for dopamine of *ca.* 0.06 pg was almost 50 times less than with the conventional system, which represents a two-fold improvement in concentration sensitivity. Miniaturization was accomplished with negligible loss in resolution by using a conventional commercial amperometric detector with minor modifications. The results indicate that a number of useful advantages could be realized by the combination of this small-bore LC–ED system and the *in vivo* brain dialysis method.

INTRODUCTION

Liquid chromatography (LC) with electrochemical detection (ED) is a widely used technique for determination of biogenic amines and their metabolites in biological samples[1,2]. The popularity of the method owes much to the selectivity and high sensitivity offered, making it possible to detect low concentrations of the parent amines and their metabolites with minimal sample preparation.

In many biomedical applications, including monoamine measurements in body tissue and fluids, the amount of sample available for analysis is limited. One limitation in the use of conventional LC systems is that they are not sufficiently sensitive to detect low concentrations of monoamines in very small volumes of sample. This is partly due to the sample dilution taking place on the column during separation[3]. The dilution can be approximated by the following equation

$$\frac{C_{max}}{C_0} = \frac{4V_s}{\pi d_c^2 \varepsilon (1 + k')(2\pi HL)^{1/2}}$$

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where C_{max} is the maximum concentration of solute at the end of the column, C_0 is the concentration of solute in the injected sample, V_s is the injection volume, d_c is the column inner diameter, ε is the total column porosity, k' is the capacity factor, L is the column length, and H is the theoretical plate height.

Recently, small-bore LC columns, with an I.D. of 1 mm or less, have received considerable attention[4,5]. According to the above equation, the solute concentration at the column outlet will increase *ca.* 21 times if a conventional (4.6 mm I.D.) column is replaced by a 1 mm I.D. column, while the rest of the parameters are kept unchanged. However, since the reduction in the eluting peak volume is inversely proportional to the square of the column I.D., extra-column band broadening must be minimized for the separation power of a small-bore column to be fully realized. This demands much of the injection and detection systems, as well as the connections to and from the column.

Most high-performance liquid chromatography (HPLC) detectors have to be miniaturized for use with small-bore columns. However, reducing the detector cell volume can adversely affect the concentration sensitivity[6], and the advantage obtained by reducing the column I.D. may be lost because of the detector design. Recently we reported a miniaturized amperometric detector, which is compatible with small-bore LC columns and can be used without loss in concentration sensitivity[7]. Furthermore, it is also possible to use a commercially available amperometric detector with miniaturized columns without sacrifice of sensitivity or resolution[8,9].

We have recently described a novel intracerebral dialysis method which enables constant monitoring of extracellular dopamine (DA) and its metabolites in different brain regions *in vivo*[10,11]. Essentially this method involves the constant slow (2 μl/min) perfusion of an implanted tube of small diameter dialysis membrane and the analysis of the perfusates using LC–ED. DA and its metabolites have been detected in basal perfusate samples using a reversed-phase ion-pair LC–ED system based on conventional equipment[12]. The method allows monoamine measurements in perfusates collected over 10–20 min periods. However, a shorter sampling time demands the routine detection of low or sub-picogram amounts of DA in very small perfusate volumes, which is beyond the capabilities of the conventional LC–ED system. The aims of the present study were to miniaturize the conventional LC–ED system using small-bore LC columns, and to investigate the possible application of this LC system to the determination of DA and its metabolites in small volumes (2 μl) of rat brain perfusate.

EXPERIMENTAL

Chromatographic equipment

The conventional chromatographic system on which the small-bore system was based consisted of a Waters Model 6000 A pump, a Rheodyne 7125 injector equipped with a 50-μl sample loop and a 250 × 4.6 mm I.D. Spherisorb S5 ODS 1 column (Chrompack, Middelburg, The Netherlands).

For the miniaturized LC system the necessary low, pulse-free flow was achieved using a Brownlee Labs MPLC Micro Pump. Samples were injected using a Valco CI 4 W injector with a 1-μl internal sample loop and separated on a 250 × 1 mm I.D. Spherisorb S5 ODS 1 column (Scantec, Partille, Sweden). Thus, both LC systems

used the same packing material and column length. The flow-rate for the small-bore system was 60 μl/min, which yields about the same linear velocity of mobile phase through the column as 1.2 ml/min on the conventional system. Under these conditions the pressure drop was *ca.* 15 MPa on both systems, indicating similar flow-resistances for the two columns. All separations were carried out at ambient temperature.

In both systems monoamines were measured by amperometric detection (BAS LC 2A or LC 4B controller) using a BAS TL-5 A thin-layer flow cell with either a glassy carbon or a CP-S carbon paste (conventional system only) indicator electrode maintained at 0.65 V *versus* silver/silver chloride (3 *M* sodium chloride). The manually prepared carbon paste electrode provides a lower detection limit when used with the conventional system (*ca.* 2.5 times)[12], owing to a lower noise level compared with the glassy carbon electrode. However, use of a carbon paste electrode was omitted with the miniaturized system because of practical problems. Detector output current was monitored using a Hitachi Model 561 stripchart recorder.

A number of measures were taken to minimize extra-column band broadening on the small-bore system. A short piece of 0.13 mm I.D. stainless-steel capillary connected the injector to the column. The column was connected to the detector using a 0.05 mm I.D. fused-silica capillary. These capillary tubes replaced the 0.25 mm I.D. tubing used with the conventional system. Furthermore, the detector dead volume of the small-bore system was reduced by replacing the 50-μm gasket, which defines the volume in the BAS TL-5 thin-layer flow cell, with a 25-μm polythene film that had a channel width of 3 mm. This provides a total detector volume of *ca.* 1 μl. The effective cell volume, defined as the space above the electrode surface[13], was less than 0.2 μl.

Reagents

Stock solutions (1 m*M*) of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), all obtained from Sigma, were prepared in 0.1 *M* perchloric acid (PCA) and diluted to the desired concentration prior to use. The mobile phase was prepared by mixing twelve parts (v/v) of HPLC-grade methanol with 88 parts of aqueous buffer containing 0.15 *M* sodium dihydrogen phosphate, 0.1 m*M* EDTA and 0.5 m*M* sodium octane-sulphonate (SOS). Since different batches of packing material showed small differences in selectivity, it was sometimes necessary to change the amount of SOS in the mobile phase to resolve DA adequately from interfering sample components. The final pH of the mobile phase was adjusted to 3.80. Before use the mobile phase was filtered through a 0.45-μm membrane filter (HVLP, Millipore). All solutions were prepared with water purified by a Millipore water purification system (Ion-Ex and Organex-Q cartridges).

Dialysis procedure

The brain dialysis procedure was carried out as previously described[10]. Short dialysis loops were stereotaxically implanted into the striatum or n. accumbens of the halothane-anaesthetized rat. The dialysis probes were connected to a microinfusion system (Microject CMA/100, Carnegie Medicine, Solna, Sweden) and continuously perfused at 2 μl/min with Ringer solution. The dialysate samples were

collected in small Eppendorf tubes, containing 10 µl of 1 *M* PCA, placed close to the outlet cannula to minimize dead volumes (*ca.* 2 µl). The samples were injected onto the liquid chromatographs without preparation on the day of collection.

RESULTS AND DISCUSSION

Down-scaling of a conventional LC–ED system

The small-bore LC–ED system described is essentially a down-scaled version of a validated conventional LC–ED system using reversed-phase ion-pair chromatographic conditions to separate dopamine and its metabolites DOPAC and HVA[12]. The two systems are based on the same mobile and stationary phase. Miniaturization was achieved by replacing the conventional column, pump and injector with (a) a small-bore column of equal length to the standard column (b) a syringe pump able to deliver the low uniform flow of mobile phase required by the small-bore column and (c) an injection port capable of handling small sample volumes. With these alterations the capacity factors and column efficiency remained on the whole unaffected. However, different batches of packing material showed small differences in selectivity and therefore minor changes in mobile phase composition were sometimes necessary to obtain a satisfactory resolution of the sample components. Further, the small difference in column efficiency encountered might be explained by the fact that the small-bore and conventional columns were packed by different procedures.

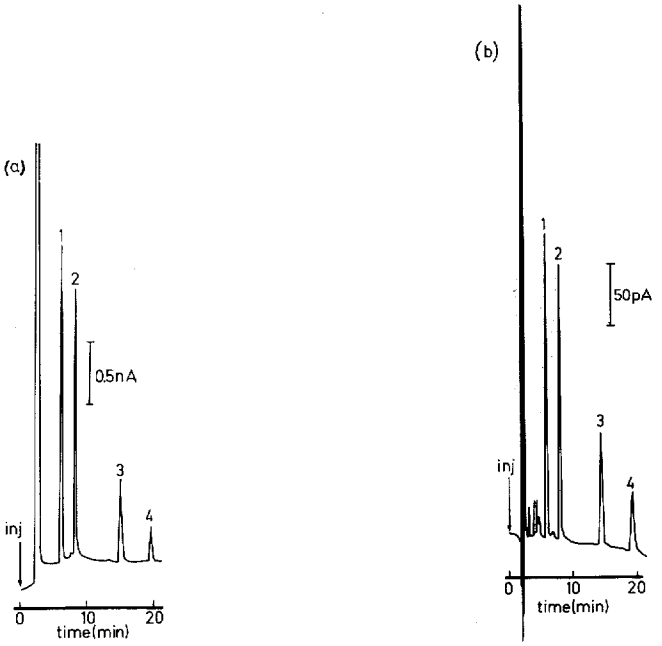

Fig. 1. Separation of a standard solution containing (1) $1 \cdot 10^{-7}$ *M* dopamine (DA); (2) dihydroxyphenylacetic acid (DOPAC); 5-hydroxyindoleacetic acid (5-HIAA); and (4) homovanillic acid (HVA). Mobile phase, 0.15 *M* sodium dihydrogen phosphate, 0.5 m*M* sodium octanesulphonate, 0.1 m*M* EDTA, 12% methanol (pH 3.80); detection at +0.65 V *vs.* silver/silver chloride (3 *M* sodium chloride). (a) Conventional 250 × 4.6 mm I.D. Spherisorb S5 ODS 1 column; flow-rate, 1.2 ml/min; injection volume, 50 µl. (b) Small-bore 250 × 1 mm I.D. Spherisorb S5 ODS 1 column; flow-rate, 60 µl/min; injection volume, 1 µl.

Chromatographic separation

Fig. 1 shows chromatograms obtained after injection of a standard solution containing $1 \cdot 10^{-7}$ *M* of DA, DOPAC and HVA, and the serotonin metabolite 5-HIAA, onto both the small-bore and conventional LC systems. On these systems serotonin elutes very close to 5-HIAA and is not completely baseline-separated. In the perfusate samples the amount of serotonin is very small and cannot be detected with these chromatographic conditions, so interference between serotonin and 5-HIAA is negligible. However, for analysis of brain tissue extracts or other samples where the amount of serotonin is much higher, it is possible to adequately resolve serotonin and 5-HIAA by decreasing the pH to 3.50. This change, however, extends the sample run-time, and a pH of 3.80 has been shown to be optimal for analysis of the perfusates. A small-bore LC–ED system optimized for determination of serotonin in brain perfusates is presently under study[14].

Detector response

For an equivalent concentration of analyte injected the current response obtained with the small-bore system was found to be 8–10 times less than with the conventional system (Fig. 1). Several factors contribute to this decrease in response. First, according to the equation given in the introduction, in order to obtain the same solute concentration at the small-bore column outlet, the injection volume should be reduced *ca.* 21 times, *i.e.* to 2.4 μl. Consequently, when an injection volume of 1 μl is used the solute concentration will be decreased correspondingly. In addition, the current response for an amperometric detector is flow-rate dependent. A lower response will be obtained as the flow-rate is decreased according to the relationships describing the behaviour of the thin-layer detector[15,16]. However, these relationships also predict a higher coulometric yield when the flow-rate is reduced. In the present study a coulometric yield of *ca.* 25% was obtained with the small-bore system, compared with *ca.* 4% for the conventional. It has been stated that this increase in coulometric yield will enhance the signal when using small-bore LC[8]. However, if the peak height is considered, a lower response is in fact theoretically predicted: this accords with the findings in the present study.

Sensitivity

Despite the smaller current response, it was found in this study and by Caliguri *et al.*[8] that the noise level was decreased when operating the detector at the low flow-rates applied with the small-bore column. In fact, the lowest detectable amount of DA with the miniaturized system was found to be *ca.* 0.06 pg, based on a signal that is twice the peak-to-peak noise, which is almost 50 times less than with the conventional system equipped with a glassy carbon electrode. This result is about the same as that obtained with the BAS detector reported by Caliguri *et al.*[8] and with our previously described miniaturized amperometric detector[7]. The increase in mass-sensitivity found with the miniaturized system indicates about a two-fold improvement in concentration-sensitivity compared with the conventional system equipped with a glassy carbon indicator electrode.

Although the improvement is small (about the same as that obtained by using a carbon paste electrode on the conventional system), it can be significant when, for instance, perfusates obtained from the f. cortex are analysed[12]. Fig. 2 shows calibra-

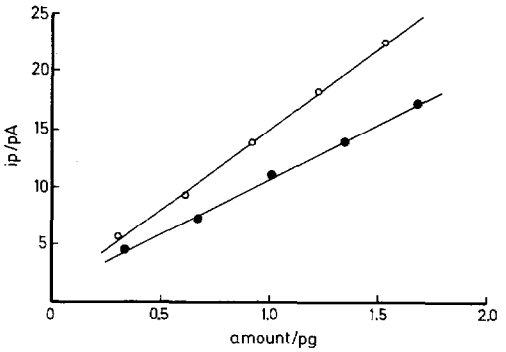

Fig. 2. Linearity of detector response using the small-bore column at low concentrations of (○) dopamine and (●) dihydroxyphenylacetic acid.

tion graphs obtained with the small-bore system for DA and DOPAC in the concentration range 2–10 n*M* (*ca.* 0.3–1.5 pg). The linearity is maintained to 10^{-5} *M* (higher concentrations have not been tested).

Peak resolution

When down-scaling a conventional system it is important that the separation efficiency, *i.e.* the number of theoretical plates, is not impaired. It is especially important in demanding separations to maintain both resolution and sensitivity. A critical detail in miniaturized systems is the extent of extra-column band broadening[4,5]. In our case, 6000 (DA) to 8000 (metabolites) theoretical plates were achieved with the small-bore column at a flow-rate of 60 μl/min. On the conventional system the corresponding values were 7000 to 12 000, obtained at a flow-rate of 1.2 ml/min. Different columns show somewhat different efficiencies and the data quoted are typical values. The slightly less efficient separation achieved with the small-bore column is most likely due to a less efficient column and not to extra-column dispersion. This is supported by column plate counts determined by the manufacturers. Furthermore, if extra-column band broadening has been significant the number of theoretical plates would have increased with increased capacity factor[17], and this was not the case since plate counts for 5-HIAA and HVA were essentially the same as for DOPAC. However, the lower efficiency of the small-bore column is not a large problem in our application. The difference for DA, which is most susceptible to interferences, is small and, as can been seen in Fig. 3, the resolution is adequate.

Reproducibility

For reliable quantitative measurements it is important that a reproducible system response is obtained and that sample injection can be performed with negligible cross-contamination. In the present study, it was found that a sample volume much larger than the volume of the sample loop (1 μl) was required to fill the loop completely, and that as the load volume was reduced the current response decreased (Table I). This finding is in accordance with results reported for a different brand of injection valve (Rheodyne 7520) designed for small-bore column chromatography[18]. The reproducibility was also impaired by decreasing the load volume as indicated by

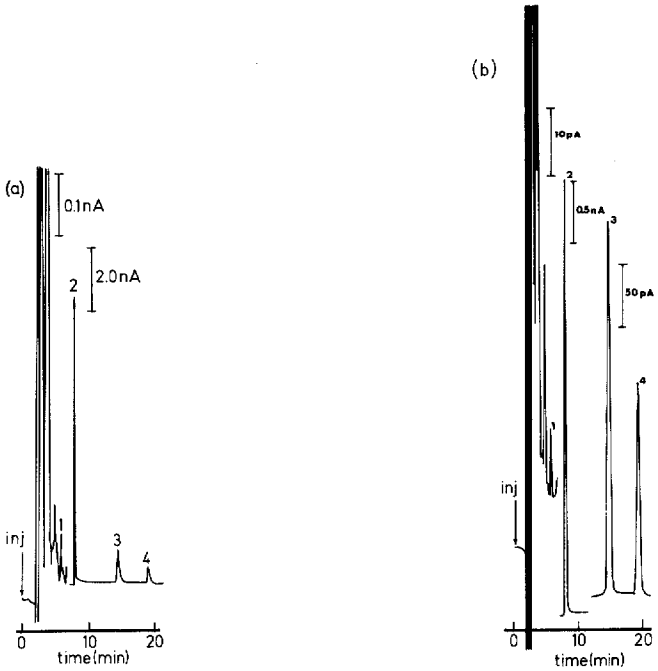

Fig. 3. Separation of dopamine and monoamine metabolites in a rat brain perfusate. The perfusate was collected at 2 μl/min from a dialysis probe implanted into the striatum of halothane-anaethetized rats. (a) Conventional system; (b) small-bore system. Compounds: 1 = dopamine (DA), 23 pg on conventional and 0.57 pg on small-bore; 2 = dihydroxyphenylacetic acid (DOPAC), 3.6 and 0.37 ng; 3 = 5-hydroxy-indoleacetic acid (5-HIAA), 1.8 and 0.26 ng; 4 = homovanillic acid (HVA), 1.5 and 0.20 ng. The samples were obtained from different rats. For conditions see Fig. 1.

the relative standard deviation (R.S.D.) values in Table I. Attempts to use load volumes of less than 2 μl gave a very poor reproducibility. In the present study, with a careful injection procedure using a load volume of 2 μl, a peak height R.S.D. of 0.04 was achieved for ten consecutive injections of a 5 n*M* DA standard solution, which is satisfactory considering the low concentration, small sample volume and manual injection procedure. It should be noted that when using a load volume of less than

TABLE I

INFLUENCE OF DIFFERENT LOAD VOLUMES ON RELATIVE RESPONSE AND PRECISION

A load volume of 100 μl was assumed to completely fill the 1-μl sample loop. Sample, 1.0 μM dopamine; N = 6 for all load volumes.

Load volume (μl)	*Relative response (%)*	*R.S.D.*
100	100	0.005
10	98	0.010
5	97	0.014
3	93	0.017
2	89	0.020

ca. 5 μl, in order to avoid systematic errors, it is mandatory to load the injector with the same volume of standard solution as samples when making up the calibration graph. A more general way to minimize errors associated with sample injection is to add an internal standard to the sample. However, in this application it is doubtful if that approach will improve the situation owing to the very small sample volumes used.

When using the small-bore system it is crucial to wash the injection port and the injection syringe thoroughly before each run. Minute residual amounts of sample in the injection port easily cause cross-contamination owing to the small sample volumes. It is also important to remove carefully any visible liquid left in the syringe prior to filling it with sample. With a careful washing it was possible to keep the cross-contamination to less than 0.1%, which is acceptable.

Perfusate analysis

Having developed the small-bore LC–ED system we investigated its potential for analysis of small volumes of rat brain perfusates. The perfusate samples were collected using a continuously perfused (2 μl/min) dialysis probe implanted into the striatum of halothane-anaesthetized rats[10]. Fig. 3 shows chromatograms obtained after injection of striatal perfusate onto the conventional and small-bore LC–ED systems. The dopamine peak corresponds to a perfusate concentration of 4.0 n*M* which is a typical level in this kind of sample[10,12]. The sensitivity provided by the small-bore system is sufficient to detect dasal levels of DA using an injection volume of 1 μl; however, at least 2 μl are required to load the sample loop with a satisfactory reproducibility. The reduced sample requirement realized with the small-bore LC–ED system enables the measurement of endogenous DA released into the perfusate over a sampling period of 1 min. This represents a 20-fold reduction in time sampling compared with that required using conventional LC.

Validation

To validate monoamine measurements obtained with the small-bore system, perfusate samples obtained from striatum of an anaesthetized rat under control con-

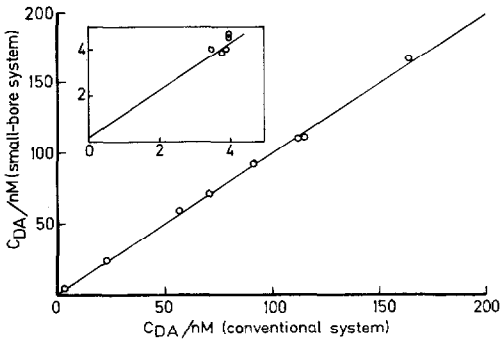

Fig. 4. Correlation plot of results obtained with the small-bore system *vs.* the conventional system for dopamine in perfusates collected at 2 μl/min from striatum of a halothane-anaesthetized rat before and after administration of amphetamine (2 mg/kg). The inset shows the control samples, obtained before the drug was administered, and the calculated regression line. Least squares fit: $y = 0.993x + 0.23$, $R^2 = 0.998$.

ditions, and after administration of 2 mg/kg of amphetamine, were divided and injected onto the conventional and small-bore systems. Fig. 4 shows the concentration of DA determined by the small-bore system plotted against the results obtained with the conventional system. A good correlation was achieved (R^2 = 0.998) with a slope close to 1 (0.993), demonstrating that the results obtained with the small-bore system are as reliable as those from the conventional system, despite only a fraction of the sample being required. Similar results were obtained also for DOPAC (R^2 = 0.994) and HVA (R^2 = 0.982).

CONCLUSIONS

By reducing the column I.D. from 4.6 mm to 1 mm it is possible to enhance the mass-sensitivity when using amperometric detection. However, when using small sample volumes great care must be taken when injecting the samples in order to minimize imprecision and cross-contamination. The higher mass-sensititivity obtained with the small-bore column makes possible a significant reduction in sample requirements. Owing to the very small sample volumes provided by the *in vivo* brain dialysis method, the combination with small-bore rather than conventional LC–ED methodology offers several advantages: (a) brain dialysates can be collected at time intervals as low as 1 min, allowing rapid measurement of DA release and metabolism; (b) by reducing the brain perfusion speed it is possible to concentrate the sample and thus facilitate detection in perfusates collected from brain regions containing very small amounts of monoamines; (c) using samples collected at 20-min intervals at a perfusion speed of 2 μl/min (commonly used procedure when using the conventional system for analysis) only a small fraction of the sample is required for analysis with the small-bore system. The remaining sample can then be used for determination of other compounds of interest, *e.g.* indoleamines, amino acids, purines and neuropeptides.

The application of small-bore LC–ED together with the intracerebral dialysis technique offers a very powerful combination. However, the small-bore LC–ED method is not restricted to this special application but can also be used for analysis of other kinds of sample, *e.g.* tissue extracts.

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