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Sensitive and specific method for the simultaneous determination of natural and synthetic catecholamines and 3,4-dihydroxyphenylglycol in microdialysis samples

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Abstract

The relatively new technique of microdialysis provides new possibilities for investigating in vivo the functioning of the sympathetic nervous system. The small sample volumes obtained, however, are a great challenge for analytical chemists. We report here a HPLC method for measuring in one run both natural and synthetic catecholamines [dopamine, (nor)epinephrine, α-methylnorepinephrine, isoproterenol and epinine] and the intraneuronal metabolite 3,4-dihydroxyphenylglycol in small microdialysis samples after derivatization with the fluorogenic agent 1,2-diphenylethylenediamine. No prior clean-up step is necessary. *N*-Ethylmaleimide is necessary for preventing an inhibitory action on derivatization occurring in in vivo microdialysis samples. The method can handle large numbers of samples, is sensitive (on-column detection limits 30 to 200 fg) and reproducible (RSD 1 to 7%). Recovery characteristics of the commercial microdialysis probe used (CMA/20) were extensively investigated both in vitro and in vivo at various perfusion rates; for practical purposes a rate of 2 μl/min and sampling at 10-min intervals was found to be workable and to give good and reproducible recoveries (50 to 70%).

Keywords: Catecholamines; 3,4-Dihydroxyphenylglycol

1. Introduction

The availability of sensitive methods for measuring the catecholamines norepinephrine (NE), epinephrine (E) and dopamine (DA) in plasma has increased our understanding of the sympathetic nervous system. The development of the isotope dilution method made it possible to unravel the contributions from spill-over and clearance to a measured concentration, both overall and in various organs and tissues [1,2]. The method, however, also has its drawbacks, both from a theoretical and from a practical viewpoint [3]. With the introduction of the microdialysis technique, in which a small catheter with a semipermeable membrane at its end is introduced into the interstitial space of an organ or tissue, it has become possible to actually measure as well as to influence the concentration of compounds in the

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interstitial space [4]. The slow perfusion rate with microdialysis (in the order of 0.5 to 5 μl/min) means however that the amount of dialysate which can be collected in a reasonable time is very small, thus leading to a need for highly sensitive assay methods with a large through-put.

It would be advantageous to be able to measure at the same time not only the natural catecholamines NE, E and DA, but also some synthetic catecholamines which are used sometimes in the study of the sympathetic nervous system (e.g., isoproterenol, ISO) or used as internal standards (e.g., α-methylnorepinephrine, AMN, and epinine, EPI). Likewise, simultaneous measurement of 3,4-dihydroxyphenylglycol (DHPG), an important intraneuronal metabolite of NE, would be desirable [5]. Since dialysates are relatively clean samples, it may be possible to avoid the time-consuming extraction procedures necessary for plasma and urine samples.

So far, catecholamine measurements of microdialysis samples are, apart from some radioenzymatic methods [6–8], mostly based on high-performance liquid chromatography (HPLC) with electrochemical detection [9–13]; in most reports a prior clean-up procedure was deemed necessary. An alternative, more specific method based on derivatization with the selective fluorogenic agent 1,2-diphenylethylenediamine (DPE) has been used successfully for measurement of both natural and synthetic catecholamines in plasma and urine [14–17]. Its sensitivity and selectivity would make it ideally suitable for measurements in microdialysis samples, as has already been proposed [18]. In one report, such a method has already been used [19]. For the measurement of DHPG in microdialysis samples, one separate method has been reported employing HPLC with electrochemical detection [20].

Here we describe a sensitive and selective method in which natural and synthetic catecholamines as well as DHPG in microdialysate samples are simultaneously measured after direct derivatization, without an extraction step, with the fluorogenic agent DPE followed by HPLC separation and fluorimetric detection.

The method has been optimized and some unexpected and hitherto unnoticed problems have been solved. The simple derivatization procedure and the short chromatographic runs (15 min) allow for a high through-put. We also report here an extensive evaluation of in vitro and in vivo recovery experiments using the new method.

2. Experimental

2.1. Reagents

Bicine, NE, E, DA, AMN, ISO, EPI and DHPG were obtained from Sigma (St. Louis, MO, USA), ethylenediaminetetraacetic acid (EDTA), *N*-ethylmaleimide (NEM) and hydrochloric acid from Merck (Darmstadt, Germany), potassium ferricyanide (PFC) from Aldrich (Bornem, Belgium), L-glutathione from Fluka (Buchs, Switzerland), acetic acid and acetonitrile from Baker (Deventer, The Netherlands), Ringer's solution from Braun (Melsungen, Germany) and Ringer's lactate from Baxter (Uden, The Netherlands). DPE was prepared as reported previously [14].

All water used had been purified by a Milli Q-Plus system (Millipore, Bedford, MA, USA).

2.2. Apparatus

The instrumentation for chromatography consisted of a Spectra System P4000 pump (Thermo Separation, San Jose, CA, USA), a Kontron 460 autosampler (Kontron, Milan, Italy) and an FP920 fluorescence detector (Jasco, Tokyo, Japan) operated at excitation and emission wavelengths of 350 and 480 nm, respectively. Data acquisition was performed using an SP4500 Labnet interface and a PC1000 system v3.0.1 from Thermo Separation. Separations were performed on a 3 μm Allsphere ODS-2 (100 mm×2.1 mm I.D.) column (Alltech, Deerfield, IL, USA) at ambient temperature.

For some preliminary experiments, an electrochemical detector (Antec, Leiden, The Netherlands; operated at 600 mV) was used.

2.3. Microdialysis samples

CMA/20 microdialysis catheters (Carnegie Medicine, Stockholm, Sweden; membrane 10 mm×0.5 mm, M_r 20 000 cut-off) were inserted into heart, vein and artery of pigs, and in freshly obtained blood samples from pigs and humans. CMA/60 catheters

(membrane 30 mm×0.6 mm, M_r 20 000 cut-off) were inserted into subcutaneous fat and muscle of humans. Catheters were perfused with Ringer's lactate solution at a rate of 2 μl/min using a CMA/100 microperfusion pump; 10-min fractions (20 μl) were collected into microvials containing 20 μl of 0.08 *M* acetic acid containing 2% (w/v) of disodium-EDTA (HAc/EDTA) which usually also contained 100 pg of internal standard AMN. Samples were stored at −80°C until assay.

2.4. Assay method

To the samples or standards were added subsequently 40 μl of acetonitrile, 10 μl of 20 m*M* NEM in 1.75 *M* bicine buffer containing 1% (w/v) of disodium-EDTA (pH 7.50), 20 μl of 0.1 *M* DPE in 0.1 *M* HCl, and 4 μl of 20 m*M* PFC in water. Samples were then incubated for 2 h at 37°C in the dark. After cooling to ambient temperature 6 μl of a 80 m*M* solution of glutathione in water was added, and the sample was put into the storage compartment of the autosampler which was kept at 4°C. The autosampler injected 20 μl into the chromatographic system. For elution, 0.05 *M* sodium acetate buffer, pH 7.0–methanol–acetonitrile were used in the proportions (60:20:20, v/v/v) (mobile phase A) and (30:10:60, v/v/v) (mobile phase B). A linear gradient was used starting with A–B (70:30) at time 0 to 100% B at 14 min; flow-rate was 200 μl/min.

All assays also contained blank samples and three different standard concentrations in triplicate. Standard mixtures containing (per ml) 5 ng of NE, AMN and E each, 10 ng of DA, ISO and DHPG each, and 25 ng of EPI were prepared in HAc/EDTA. Derivatizations of 5 (10,20) μl of standard mixture to which were added 15 (10,0) μl of HAc/EDTA and 20 μl of Ringer's solution were used for constructing a standard line.

3. Results and discussion

3.1. Electrochemical method

Preliminary experiments showed that measurement of catecholamines by direct injection of microdialysis samples into a chromatographic system equipped with an electrochemical detector leads to a large number of peaks completely obscuring the peaks of interest. Thus extensive clean-up of microdialysis samples will be necessary in order to reliably measure catecholamines using an electrochemical detector.

3.2. Derivatization with DPE

The alternative method of derivatizing samples with the fluorogenic agent DPE without prior clean-up procedures was carried out essentially as described previously for extracts of plasma and urine, except for smaller volumes and a different optimal pH of the bicine buffer [14–17]. To 20 μl of a mixture of catecholamines and DHPG in HAc/EDTA and 20 μl of Ringer's solution were added subsequently 40 μl of acetonitrile, 20 μl of 0.1 *M* DPE in 0.1 *M* HCl, 10 μl of 1.75 *M* bicine buffer containing 1% (w/v) of disodium-EDTA (pH 7.50), and 4 μl of 20 m*M* PFC in water. The order of addition of the various reagents has to be strictly adhered to, as has been described previously [18]. After 1 h at 37°C in the dark the vials were placed into the autosampler, and 20 μl was injected into the chromatographic system. Fluorescence observed was equal to the fluorescence observed when samples were first extracted as described previously and then derivatized, except for DHPG [14]. DHPG can be derivatized with DPE but is lost to a great extent during the extraction procedure necessary for plasma and urine samples [21]. A longer period of derivatization did not result in any difference in fluorescence of the catecholamines, but did increase the fluorescence of DHPG. The optimal time of incubation for catecholamines and DHPG was found to be 2 h.

When the thus established derivatization procedure was executed on catecholamines and DHPG standards dissolved not in Ringer's solution or HAc/EDTA, but in real microdialysis samples, we noticed that the fluorescence of DA, NE, AMN and DHPG was greatly inhibited. The fluorescence of E, ISO and EPI on the other hand was much more as expected (Table 1). This phenomenon occurred in microdialysis samples from pigs and humans as well as in microdialysis samples from freshly obtained blood, albeit to different extents. Apparently, real microdialysis samples contained (an) unknown compound(s) which inhibited the derivatization reaction.

Table 1
Relative fluorescence of compounds in in vivo microdialysis samples, as a percentage of the fluorescence of the same amount of the compounds in Ringer's solution[a]

Compound	−NEM −GLUT	+NEM −GLUT	+NEM +GLUT
3,4-Dihydroxyphenylglycol	8±5	120±10	102±5
Norepinephrine	12±2	108±7	103±4
α-Methylnorepinephrine	47±3	102±5	100±7
Epinephrine	96±3	102±3	103±4
Dopamine	6±1	102±5	102±5
Isoproterenol	84±3	102±1	99±2
Epinine	74±9	95±4	96±6

[a] Data are means±SD of experiments with three different standard concentrations. Samples were derivatized with or without *N*-ethylmaleimide (NEM) and with or without addition of glutathione (GLUT) at the end of the derivatization.

Longer incubation, incubation at higher temperatures or the addition of extra amounts of PFC did not solve the problem. The apparent inhibition of the DPE derivatization reaction for some, but not all, catecholamines is reminiscent of the report by Nohta et al. [22] who noticed that in erythrocytes and platelets samples compounds are present which interfere with the fluorescence derivatization reaction of NE and DA, but not E. They concluded that the sulfydryl-reagent NEM can prevent this interference, and we found that the same applies for microdialysis samples: including 20 m*M* of NEM in the bicine buffer was sufficient to overcome the inhibitory action of the unknown compound(s) on the derivatization reaction (Table 1). During microdialysis, apparently these compounds cross the membrane and enter the dialysate.

As can be seen in Table 1 the fluorescence peak of DHPG was now higher than expected. This was caused by a small peak arising from the added NEM, which had nearly the same retention time as DHPG. Adding 6 μl of a 80 m*M* solution of glutathione in water at the end of the derivatization procedure got rid of this interfering peak, and resulted in optimal and complete fluorescence for all catecholamines and DHPG as compared to derivatization in Ringer's solution or HAc/EDTA (Table 1).

In the chromatograms all peaks of interest are clearly separated (Fig. 1). Although it is possible to change the elution conditions in such a way that the

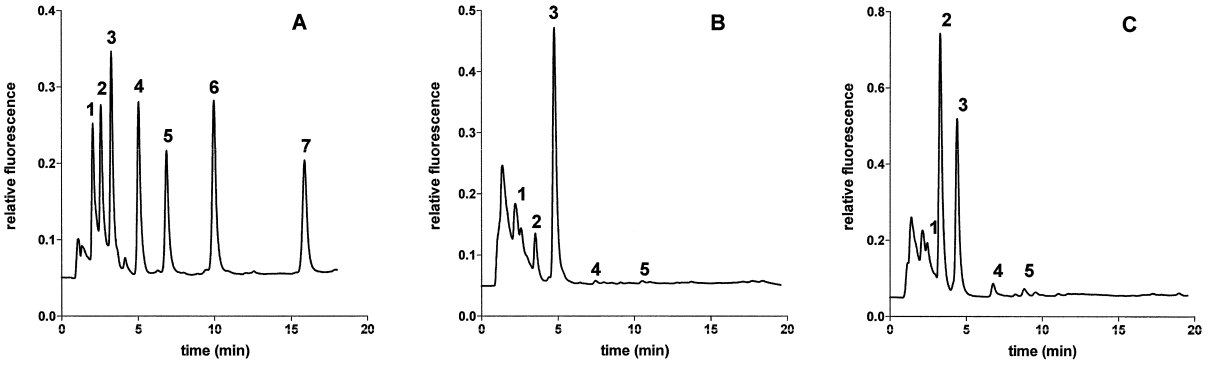

Fig. 1. Chromatograms of microdialysis samples. (A) Standard mixture. On-column amounts: 8.33 pg for NE, E and AMN, 16.66 pg for DHPG, DA and ISO, and 41.67 pg for EPI. (B) Pig myocardium sample. On-column amounts: DHPG 1.69 pg; NE 2.77 pg; E 0.22 pg; DA 0.19 pg. (C) Pig myocardium sample (after occlusion of coronary artery). On-column amounts: DHPG 2.61 pg; NE 33.55 pg; E 1.42 pg; DA 2.09 pg. Peaks: 1=DHPG, 3,4-dihydroxyphenylglycol; 2=NE, norepinephrine; 3=AMN, α-methylnorepinephrine; 4=E, epinephrine; 5=DA, dopamine; 6=ISO, isoproterenol; 7=EPI, epinine.

DHPG and NE peaks, which ride on the descending part of the front peak, come completely clear from the front, this will lengthen the runs considerably, with great loss of sensitivity for the later-eluting peaks. At low concentrations of DHPG and NE, automatic quantification of the chromatographic peaks by a computer program is not good enough, and careful delineation by hand is necessary. This can however be done reproducibly, certainly if performed by the same person. Since the superposition thus does not appreciably interfere with reliable quantification of DHPG and NE, we have left the elution conditions as stated. With small variations in the percentage of acetonitrile, however, the elution times of the peaks can be easily influenced.

3.3. Reproducibility and detection limit

Two mixtures of microdialysis samples enriched with different amounts of catecholamines and DHPG were used for determining the intra- and inter-assay variabilities. Results are presented in Table 2.

Taking an S/N ratio of 3 as the detection limits, the minimal on-column detectable amounts were 30 fg for NE, E and AMN, 50 fg for ISO, 80 fg for DHPG and DA, and 200 fg for EPI. With standard assay conditions, this amounts to 10 pg for NE, E and AMN, 15 pg for ISO, 25 pg for DHPG and DA, and 60 pg for EPI per ml of dialysate.

3.4. In vitro and in vivo recoveries

For relating measured dialysate concentrations to actual interstitial concentrations, one must know the relative recovery, i.e., the ratio between concentrations in dialysate and surrounding medium. Furthermore, from a practical point of view, it is important to know the absolute recovery, i.e., the amount of a compound entering the dialysate in a defined period of time [11]. We have determined relative and absolute recoveries of NE, E, DA, ISO, AMN and DHPG in vitro with CMA/20 microdialysis probes in triplicate by inserting them in standard mixtures of the above-mentioned compounds (concentrations 4 to 12 ng/ml) in Ringer's solution and perfusing the probes with Ringer's solution at flow-rates of 0.5, 1, 2, 4 and 8 μl/min. The standard mixtures were kept at 37°C and 1 h after starting perfusion four consecutive 20 μl samples were collected and assayed. Results (Fig. 2) show that both relative and absolute recoveries of all compounds are similar and reproducible. The absolute recoveries are represented here as a percentage of the amount found in the dialysate per min at the highest flow-rate of 8 μl/min. It can be concluded that a flow-rate of 2 μl/min, which gives good relative (53–66%) and absolute (69–77%) recoveries and allows for sampling at 10 min intervals, is appropriate for experiments. The interprobe variability is quite small: on average 3±2% (NE, E and

Table 2
Intra- and inter-assay variabilities at two different concentrations (C1–C2)[a]

Compound	DHPG	NE	E	DA	ISO	AMN	EPI
C1 Intra-assay							
Mean	1673	325	969	1891	1818	978	5019
RSD (%)	2.3	3.3	1.3	3.1	0.8	2.5	2.5
C1 Inter-assay							
Mean	1699	361	959	1918	1830	976	5168
RSD (%)	4.7	6.1	2.9	1.3	2.1	1.6	2.3
C2 Intra-assay							
Mean	175	57	169	381	345	212	1025
RSD (%)	7.6	3.3	0.9	1.8	2.4	2.5	3.3
C2 Inter-assay							
Mean	159	58	172	378	336	221	1036
RSD (%)	7.3	1.5	6.3	1.6	6.5	5.8	5.1

[a] Intra-assay: $n=6$; inter-assay: $n=6$. All concentrations in pg per ml microdialysate.

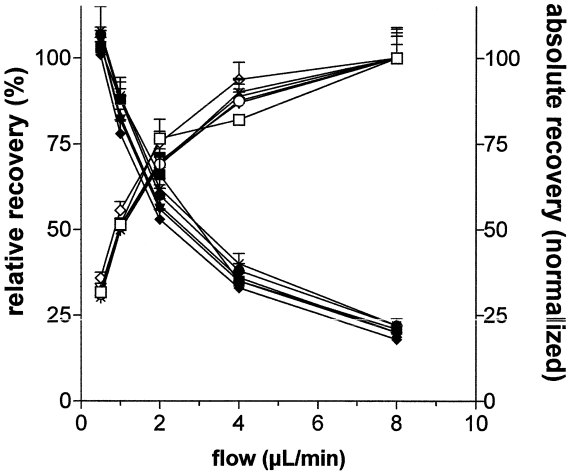

Fig. 2. Relative (closed symbols) and absolute (open symbols) recoveries of the compounds using the CMA/20 microdialysis probe at various perfusion flow-rates. For abbreviations see Fig. 1. (– ■ –) DHPG; (– • –) NE; (– ▲ –) E; (– ☆ –) DA; (– ♦ –) ISO; (– ▼ –) AMN.

AMN 2±1%, DA 4±2%, ISO 3±1%, DHPG 7±5%).

In vivo recovery was determined in two different ways. First, since characteristics of AMN are quite similar to those of the other catecholamines, we perfused the microdialysis probes inserted in the pig heart with Ringer's lactate solution containing a known concentration of AMN and measured the concentration of AMN in the collected dialysate samples. This retrodialysis method, employed in four pig experiments, gave a recovery for AMN of 52±8% (n=286), in close agreement with the results of the in vitro recovery determination.

Second, with probes inserted into the coronary artery of pigs, we compared the concentrations of NE and E in these dialysate samples with the concentrations in the plasma samples obtained at the same time from the same artery (midway between the 10-min microdialysis period) during periods of elevated NE and E concentrations. Results showed a recovery of 51±4% for NE (n=19) and somewhat higher recoveries for E (68±3%; n=17).

The recovery experiments show that catecholamines and DHPG can reliably be measured in this way and that they can reproducibly give a good indication of the interstitial concentrations. The recovery compares favorably with the scarce results (on NE only) given in the literature with different, laboratory-made microdialysis probes (23–41%) [8,11,23]. As a compromise between high relative but low absolute recovery at a perfusion rate of 0.5 μl/min, and low relative but high absolute recovery at 8 μl/min, a perfusion rate of 2 μl/min is satisfactory, with a sufficiently rapid sampling period of 10 min.

4. Conclusions

The method described is sensitive, simple and rapid. With the use of an autosampler a large number of samples can be processed. For our hospital setting, it was not practical to develop an automated on-line method; we preferred a manual derivatization procedure, which is time-consuming but not labor-intensive. If desired, our method can be adapted to an on-line method, as has been described previously [18,19], provided NEM is also included in the derivatization mixture. The addition of NEM is essential for obtaining good and reproducible derivatizations, and thus reliable measurements, of the compounds in question. The inhibition of the derivatization seen in the absence of NEM was never seen with plasma and urine samples, probably because the extraction procedures always used with such samples also remove these inhibitory substances.

The fact that the intraneuronal metabolite DHPG can also be quantitated at the same time is a great advantage, as is the possibility of measuring various unnatural catecholamines which provides flexibility both in choosing internal standards and in determining concentrations of infused synthetic substances like isoproterenol. The O-methylated metabolites cannot be measured with the present method. For measurement of these compounds, HPLC with electrochemical detection is the method of choice, which does not need derivatization, but is not selective enough to be used without extensive pre-purification of microdialysis samples.

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