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Monitoring of blood catecholamines by microdialysis and microbore LC with a dual amperometric detector

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Abstract

The accuracy of in vivo microdialysis for monitoring blood catecholamines and their metabolites in Lan-Yu mini-pigs was evaluated. To prevent blood clots and irritation, a microdialysis probe was secured in a Y-shaped tube. The tube was connected to an arterio-venous shunt, in a mini-pig, for in vivo experiments. Perfusates were injected onto a microbore LC equipped with a dual electrochemical detector (the upstream electrode was set at an oxidizing potential and the downstream electrode was set at a reducing potential. The typical large offscale peak or interfering peaks on the anodic chromatograms were mostly eliminated on the cathodic chromatograms, thereby providing reliable measurements of early eluters. Early eluates, such as norepinephrine and epinephrine, with reversible redox behaviour could be detected at the downstream reducing electrode. A comparison of the present method and a conventional blood-drawing method showed good correlation ($r = 0.775–0.983$ for all analytes). © 1997 Elsevier Science B.V.

Keywords: Catecholamine; Electrochemical detector; Liquid chromatography; Microdialysis

1. Introduction

Measurement of catecholamines and their metabolites in blood or plasma is a common and important research strategy for evaluating the etiologies of neuroendocrinological disorders [1,2], as well as the role of the autonomic nervous system in various physiological or pathophysiological conditions, in animal models [3,4]. Many analytical procedures have been devised for measuring catecholamines and their metabolites in blood or plasma [5–8]. Liquid chromatography with electrochemical detection (LC-EC) is one of the most popular, due to its simplicity, high sensitivity, versatility, and specificity [9]. Since its introduction, microdialysis coupled with LC has

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been applied to the study of the effects of various drugs on neurotransmitters and/or other chemical components in the extracellular fluid of the central nervous system [10,11]. The reliability and capability of microdialysis technique to measure drugs, catecholamines, and their metabolites in the brain and tissues have been well-documented [12,13]. However, it has been only recently that the microdialysis technique has been applied to the peripheral system, such as adipose tissue and blood vessels. Indeed, in vivo microdialysis is useful for determining blood neurotransmitters, mediators, and drug concentration.

Wester et al. were the first to report the measurement of blood serotonin (5-HT) in the rat artery using microdialysis [14]. However, Yoshioka et al. reported that the measurement of free 5-HT concentration in the blood, by direct placement of a microdialysis [14]. However, Yoshioka et al. reported that the measurement of free 5-HT concentration in the blood, by direct placement of a microdialysis probe in a vessel, may be inaccurate as 5-HT may be released from platelets which adhere to or aggregate on the probe membrane [15]. The usefulness of microdialysis techniques in blood vessels has been ill-defined and equivocally interpreted. Microdialysis techniques have not yet been applied to the simultaneous monitoring of blood catecholamines, and their metabolites. In our pilot study, we failed to measure these substances by direct placement of a probe either in the arteries or veins of rats, cats, or mini-pigs. Data obtained from the microdialysis assay and conventional blood-drawing assay did no correlate well. Some problems occurred such as blood-flow changes at the time of probe implantation, blood clots, and platelet adhesion or aggregation on the surface of the probes. This paper describes a modified, successful microdialysis method for the simultaneous determination of catecholamines and their metabolites in blood.

2. Materials and methods

2.1. Chemicals and reagents

Norepinephrine (NE), epinephrine (E), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (serotonin or 5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), disodium ethylenediaminetetraacetic acid (Na_2-EDTA), diethylamine, sodium 1-octanesulfonate (SOS), sodium dihydrogen orthophosphate, sodium citrate, 3-methoxytyramine (3-MT, as an internal standard), and PX were purchased from Sigma (St. Louis, MO, USA). LC grade acetonitrile and tetrahydrofuran (THF) were purchased from Merck (Merck-Schuchardt, Darmstadt, Germany). All reagents were of analytical quality, unless otherwise stated.

Standard stock solutions of NE, E, DA, DOPAC, HVA, 5-HT, 5-HIAA, and 3-MT were prepared at a concentration of 2 ng ml^{-1} in 0.1 M perchloric acid, and stored in the dark at $-70°C$. The solutions were thawed in an ice bath prior to preparation of a standard mixture. The internal standard 3-MT and the standard mixture were prepared fresh daily from a portion of the stock solutions, after appropriate dilution in 0.1 M hydrochloric acid containing 10^{-7} M ascorbic acid.

2.2. Chromatographic conditions

The mobile phase consisted of 80 ml acetonitrile, 0.42 g SOS (2.2 mM) 2.00 g monosodium dihydrogen orthophosphate (14.7 mM), 8.82 g sodium citrate (30 mM), 10 mg EDTA (0.027 mM), and 1 ml diethylamine in double distilled water [18]. The solution was adjusted to pH 3.5 by concentrated orthophosphoric acid, and the final volume was adjusted to 1 l. The mixture was filtered with a 0.22 μm nylon filter under reduced pressure and degassed by helium gas for 20 min. The flow-rate was 60 μl min^{-1} at a consistent column pressure of ca. 9.5 MPa.

The microbore column LC system was comprised of a Beckman 126 pump (Beckman Instruments, Taiwan Branch, Republic of China), a CMA-200 microautosampler (CMA/Microdialysis, Stockholm, Sweden), two BAS-4C amperometric detectors (Bioanalytical Systems, West Lafayette, IN, USA), a microbore column (GL Sciences, Inertsil-2, 5 μm ODS, 150 mm × 1.0

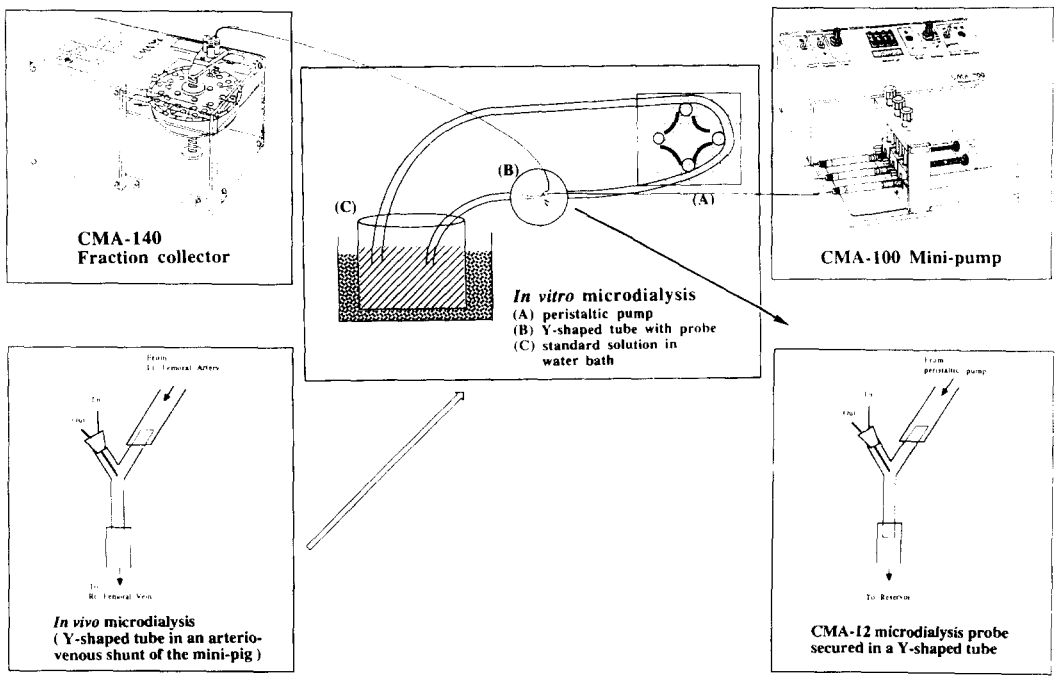

Fig. 1. A diagram of the instrument used in in vivo and in vitro microdialysis experiments: a CMA-100 mini-pump, a CMA-12 probe secured in a Y-shaped tube and a CMA-140 microfraction collector.

mm, I.D., Tokyo, Japan), a Beckman I/O 406 interface, and Beckman System Gold Data Analysis Software (Beckman Instruments). Potentials for the anodic and cathodic glassy carbon working electrodes were set at +0.75 V and +0.05 V, respectively to a silver/silver chloride electrode as Ref. [18]. Five microliters of dialysates or ultrafiltrates were analyzed for NE, E, DA, DOPAC, HVA, 5-HT, and 5-HIAA via the microbore LC-EC system. Performance of each MD probe was calibrated by dialyzing a known amount of the standard mixture, and determining recoveries of all analytes. Plasma ultrafiltrate (or blood dialysate) concentrations of NE, E, DOPAC, DA, HVA, 5-HT, and 5-HIAA were calculated by determining each peak area ratio relative to the internal standard 3-MT.

2.3. In vitro microdialysis

An in vitro microdialysis experiment was a pilot study for an in vivo experiment. The instrument used in the in vitro microdialysis experiment, is shown in a streaming standard mixture in Fig. 1. The microdialysis sampling system included a CMA-100 mini-pump, a CMA-140 fraction collector, and CMA-12 microdialysis probes (20000 Da MWCO, 4 mm in length) from Carnegie Medicine A.B. (CMA/Microdialysis, Stockholm, Sweden). The CMA-12 microdialysis probe was positioned and then glued into one arm of a Y-shaped tube (as shown in Fig. 1). Two Tygon tubings (5 mm I.D.), which were used as artificial blood vessels, were connected to the remaining two arms of the Y-shaped tube and equipped with a peristaltic pump. Various levels of standard mixtures were placed in large beakers and kept at 4°C. These solutions were forced, by the peristaltic pump through the Tygon tubing and the Y-shaped tube, at a constant flow velocity (30 cm s^{-1}), that approximated the artery blood-flow

velocity of mini-pigs. The microdialysis probe was perfused with Ringer's solution at 1 μl min^{-1} by means of a CMA-100 mini-pump. Perfusates were collected every 15 min during the course of each experiment in vials containing 5 μl of 10^{-7} ascorbic acid and 0.1 M hydrochloric acid, and analyzed by microbore column liquid chromatography (LC) with a dual electrochemical detector.

2.4. In vivo microdialysis

A male Lan-Yu mini-pig (42 kg) was anaesthetized with pentobarbital (25 mg kg^{-1}, intraperitoneally), heparinized (200 U kg^{-1}, intravenously) and ventilated with pure oxygen through an endotracheal tube. The rate and tidal volume were set to achieve a $PaCO_2$ of 30–35 mm Hg. A polyethylene catheter (PE 90) was placed in the right femoral artery for monitoring of systemic arterial pressure (SAP) and heart rate (HR). The instrument for the in vivo microdialysis experiments, except that the heart of the mini-pig replaced the peristaltic pump. In addition, each free end of the Tygon tubing, which was connected to the Y-shaped tube with a CMA-12 microdialysis probe, was also connected to a smaller polyethylene catheter (12 gauge) to facilitate catheterization of the vessels. By catheterizing the left femoral artery and the right femoral vein, an arterio-venous shunt was made. The pressure gradient between the femoral artery and vein forced the blood to flow from the artery to the venous site.

To evaluate the availability of this assay, Picrotoxin (PX) was administered (10 mg kg^{-1}, i.v.) 90 min after microdialysis probe insertion. The microdialysis probe was perfused continuously with Ringer's solution at 1 μl min^{-1}. Collection of the dialysate began immediately after completion of the arterio-venous shunt. Successive 15 μl samples were collected at 15 min intervals in vials containing 5 μl of internal standard in 10^{-7} M ascorbic acid and 0.1 M hydrochloric acid. Blood samples were also drawn at 15 min intervals following probe insertion, and collected into pre-chilled polypropylene tubes, with Na_2EDTA as an anticoagulant. The blood samples were then centrifuged ($1000 \times g$, 20 min at 4°C to separate the plasma. Plasma samples were immediately assayed by ultrafiltration and microbore LC-EC, according to published procedure [16,17].

3. Results and discussion

Fig. 2A and B show typical anodic and cathodic chromatograms, respectively, of a standard mixture containing serotonin, catecholamines, and their metabolites. Fig. 3A and B show typical anodic and cathodic chromatograms, respectively, of a dialysate from mini-pig blood. An analysis was completed within 15 min. All components under study were well resolved. The identity of each peak was confirmed by retention time, standard addition, redox ratio, and a superimposed-alignment technique provided by Beckman System Gold Data Analysis Software. The retention times of each component in Fig. 3A and B were identical to the retention times in Fig. 2A and B. Several offscale and interfering peaks around NE and E in the anodic chromatogram (Fig. 3A) diminished dramatically in the cathodic chromatogram (Fig. 3B).

The standard calibration curves (range between 0.5 pg and 20 ng) and correlations (r) for NE, E, DOPAC and DA were linear ($r > 0.999$) on both the anodic and cathodic chromatograms. The detection limits and linear responses ($r > 0.990$) for 5-HIAA, HVA, and 5-HT showed significant benefit on the anodic chromatogram when compared to the cathodic chromatogram. Thus, concentrations of NE, E, DOPAC, and DA were determined by cathodic chromatograms, while 5-HIAA, HVA, and 5-HT were determined by anodic chromatograms in their applications [17,18].

The precision of the assay was tested using a pooled plasma ultrafiltrate and a standard mixture (containing approximately 50 pg of each analyte) in 0.1 M hydrochloric acid and 10^{-7} ascorbic acid (Table 1). The intra-assay variabilities were assessed with 25 replicates at 1 h intervals and expressed as relative standard deviations (R.S.D., %). In the standard mixture, all analytes showed satisfactory R.S.D. values ($\leq 7.2\%$).

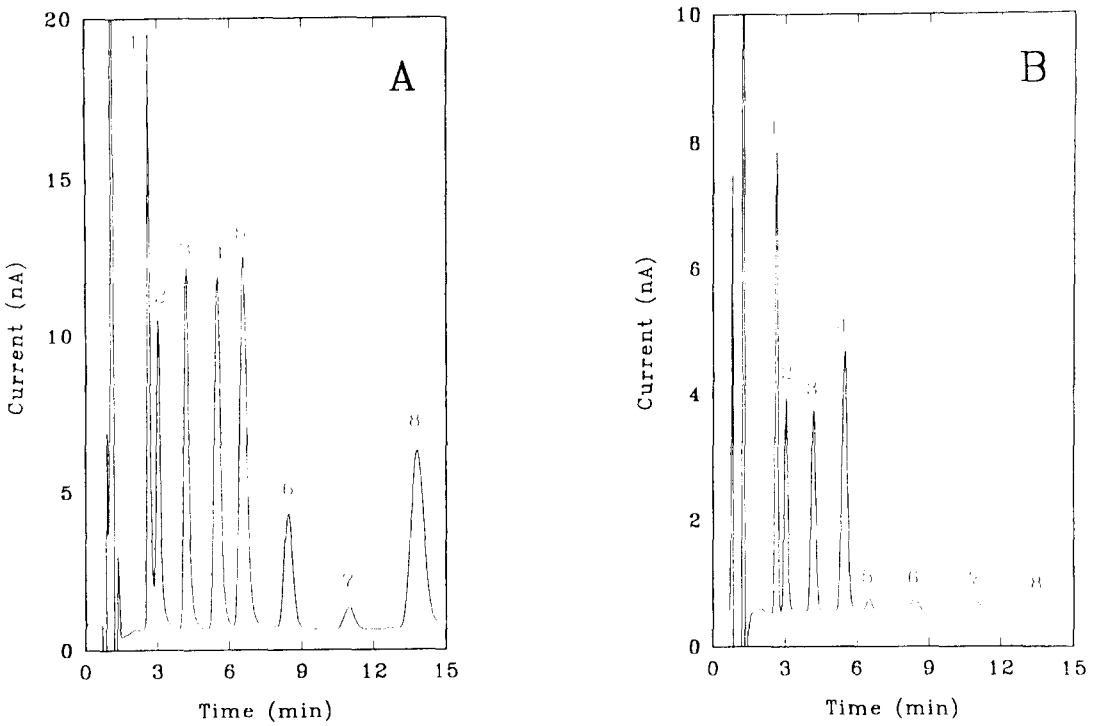

Fig. 2. Typical chromatograms of a standard mixture containing (1) NE, 151 pg; (2) E, 141 pg; (3) DOPAC, 136 pg; (4) DA, 121 pg; (5) 5-HIAA, 114 pg; (6) HVA, 130 pg; (7) 3-MT (as an internal standard), (8) 5-HT, 110 pg. (A) Anodic current, (B) cathodic current; applied potentials (vs. Ag/AgCl): anode (+) 0.75 V, cathode (+) 0.05 V.

R.S.D. values for all analytes in plasma ultrafiltrates or pooled blood perfusates were less than 6.3%. The inter-assay variabilities, assessed with the above standard mixture over 6 consecutive working days, were less than 5.5%. The detection limits (signal-to-noise ratio = 3) of analytes were between 0.1 and 0.5 pg per injection.

Percent recovery (R) for a given compound is defined as the ratio of the concentration in the dialysate (C_{out}) to the concentration in the medium (C_m) surrounding the probe ($R = C_{out}/C_m \times 100\%$). The difference in percent recovery between analytes is due to temperature variations, the membrane surface variations, properties of analytes, the surrounding medium, flow velocity, the perfusing medium and perfusing speed. The microdialysis probe (CMA-12, 4 mm) was perfused with Ringer's solution at 1 μl min^{-1} by means of a CMA-100 mini-pump. Dialysates (n = 5) were collected every 15 min and analyzed by LC assay. In general, the in vitro recoveries of all analytes in standard mixtures were between 25 and 31% in flow-off mode, and 46–62% in flow-on mode (30 cm s^{-1} flow velocity, Table 2). The in vitro recovery values doubled (as shown in Table 2) at a flow velocity of 30 cm s^{-1}. This may be the result of a continuously flowing stream through the probe, creating a steady gradient of analytes. In addition, the hydrostatic pressure between probe membranes may force analytes to penetrate the probe membrane. Thus, in vitro microdialysis in standard solutions gave greater recoveries than conventional microdialysis under unstirred conditions.

The in vitro recovery for each microdialysis probe was tested (before and after experiments), using a standard mixture. The consistent recovery variation (< 5%) between probes ensured that relative recovery could refer to particular conditions. Probes may reflect blood content or stan-

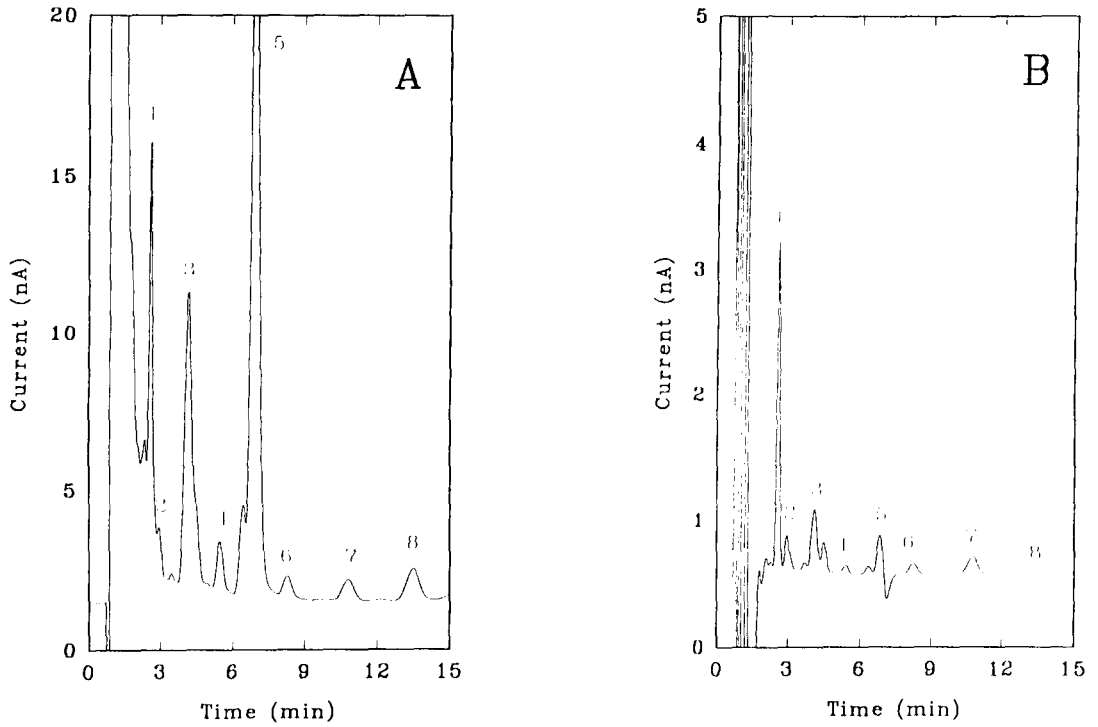

Fig. 3. Typical chromatograms of an in vivo microdialysate of a blood sample containing (1) NE; (2) E; (3) DOPAC; (4) DA; (5) 5-HIAA; (6) HVA; (7) 3-MT (as an internal standard), and (8) 5-HT. (A) anodic current, (B) cathodic current; applied potentials (vs. Ag/AgCl): anode (+) 0.75 V, cathode (+) 0.05 V.

dard mixture concentrations, under fixed conditions, with reasonable accuracy (Table 2). Typical microdialysis experiments measure the in vitro recovery of probes, to calibrate or estimate the concentration in the dialysate obtained by in vivo experiments, theoretically or experimentally [20]. The in vivo recovery of a substance can be affected by its diffusion behavior in the medium, as well as its clearance. The 'Internal standard' method or 'No-net-flow' method can be used to calibrate (or estimate) probe recovery in vivo [20].

The measured dialysate concentrations ($n = 5$) were compared with the corresponding known concentrations of standard mixtures (Table 2). The results indicated that dialysate concentrations of all analytes represented the accuracy of the assay in standard mixtures. The concentration-recovery profiles of all analytes in dialysates obtained from standard mixtures (range ca. 20–200 ng ml^{-1} for each analyte) are shown in Table 2.

The concentration-time profiles of all analytes, in dialysates from an arterio-venous shunt in a mini-pig and plasma ultrafiltrates, are shown in Fig. 4. Comparison of the present microdialysis method with ultrafiltration resulted in the following correlations (r): NE (0.896), E (0.775), DA (0.983), DOPAC (0.985), 5-HIAA (0.778), HVA (0.934), and 5-HT (0.173). Blood-flow velocity and higher in vivo temperature may explain the greater recovery obtained in vivo temperature may explain the greater recovery values. Large changes in probe recovery may occur during the course of an in vivo experiment [20]. However, our data supports the accuracy of in vivo microdialysis for the determination of blood catecholamine from dialysates. However, our data also showed that blood 5-HT could not be accurately measured by in vivo microdialysis assay. Sakurai et al. reported an initial increase of 5-HT concentration in the jugular vein of anaesthetized

Table 1
Analytical precision of intra-assays[a]

	Relative Standard Deviation						
	Ne	E	DOPAC	DA	5-HIAA	HVA	5-HT
Intra-assay							
Standard mixture	2.20	2.56	4.42	3.15	7.23	3.11	3.25
Pig plasma ultrafiltrate	3.15	3.27	5.12	5.08	6.33	3.49	3.92
Blood dialysate	4.11	4.50	3.93	4.77	5.26	4.06	3.34
Inter-assay							
Standard mixture	3.87	2.14	4.98	3.04	5.53	4.15	3.25

The standard mixture consists of 50 pg catecholamines, serotonin, and their metabolites. In addition, the NE, E, DOPAC, and DA were measured on cathodic chromatograms, whereas the 5-HIAA, HVA and 5-HT were measured on anodic chromatograms. Intra assays ($n = 25$, at 1 h intervals) of a standard mixture, pig plasma ultrafiltrates, and pig blood dialysates and the inter-assay ($n = 6$, over 6 consecutive working days) of microbore LC-EC system.

Table 2
Microdialysate concentrations and recoveries obtained from various standard mixtures (range ca. 20–200 ng ml^{-1}) and analyzed by microbore LC-EC system

	NE		E		DOPAC		DA		5-HIAA		HVA		5-HT	
Flow	Add.	Mea.	Add.	Mea.	Add.	Mea.	Add.	Mea.	Add.	Mea.	Add.	Mea.	Add.	Mea.
Off	19.2	5.4 28%	20.9	6.0 29%	21.6	5.8 27%	14.5	16.8 27%	5.2	17.0 31%	4.3	14.5 25%	3.9 27%	
On[a]	19.2	10.4 54%	20.9	11.1 53%	21.6	10.8 50%	14.5	7.7 53%	16.8	10.1 60%	17.0	8.8 52%	14.5 52%	7.5
On	38.4	20.7 54%	41.8	20.9 50%	43.2	21.6 50%	29.0	15.4 53%	33.6	20.8 60%	34.0	19.0 52%	29.0 52%	17.7
On	96.0	52.8 55%	104.5	48.1 46%	108.0	54.0 50%	72.5	37.7 52%	84.0	47.0 56%	85.0	50.2 59%	72.5 61%	44.2
On	192.0	109.4 57%	209.0	98.2 47%	216.0	123.1 57%	145.0	78.3 54%	168.0	100.8 60%	170.0	103.7 61%	145.0 62%	89.9

Dialysates were collected at 15 min intervals ($n = 5$) by Ringer's solution (1 μl min^{-1}). Standard solutions were pumped through Tygon tubings and a Y-shaped tube, via a peristaltic pump set at 30 cm s^{-1} flow-velocity.
Add, amount of standard mixture added (ng ml^{-1}).
Mea, amount of standard mixture measured (ng ml^{-1}).
[a]When on, the peristaltic pump flow was set at 30 cm s^{-1}.

rats [19]. Yoshioka et al. reported that free serotonin in the blood stream was not measurable by in vivo microdialysis techniques in dogs [15]. Platelet aggregation on or adhesion to the probe membrane may cause fluctuations in 5-HT levels in the vicinity of the probe environment. In addition, the use of heparin as an anticoagulant, or the insertion of a microdialysis probe into the blood stream, may alter the coagulation system associated with the platelets [15]. The activated platelets would subsequently induce aggregation to cause the release of stored 5-HT. Under these circumstances accurate determination of circulating 5-HT content in the blood stream would be difficult using in vivo microdialysis. However, Tsai et al. used a perfusion medium containing

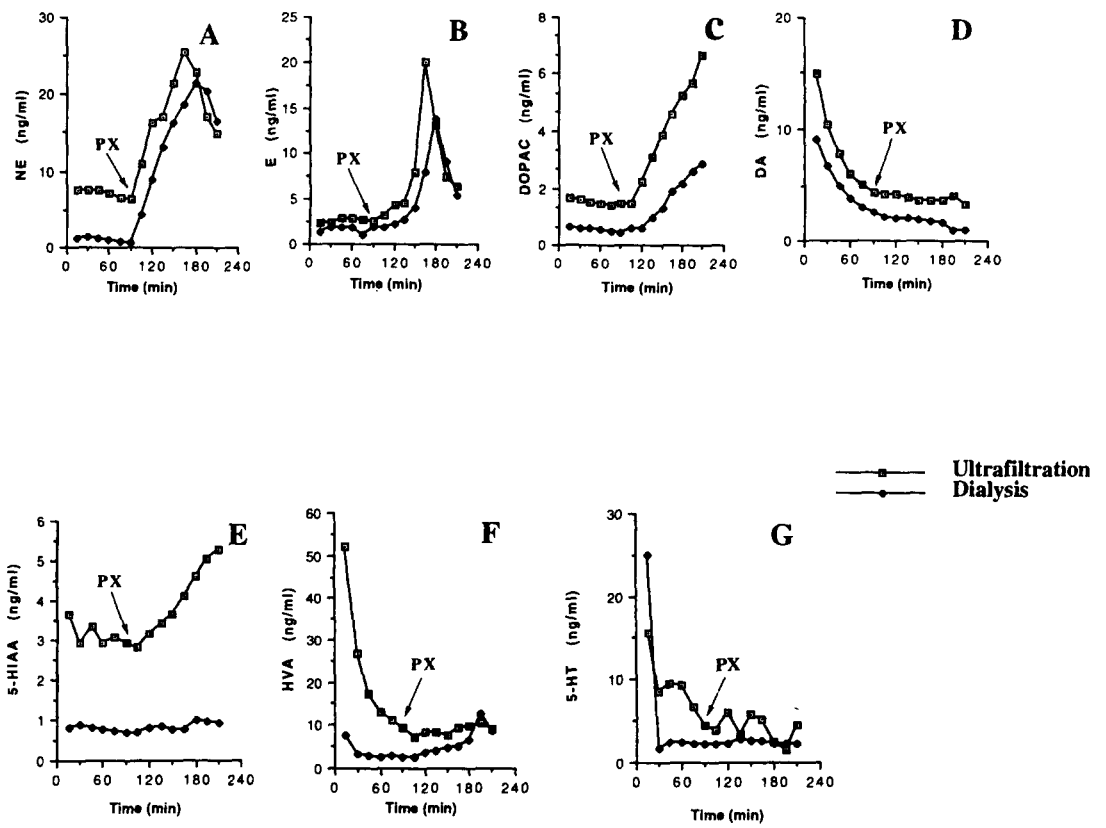

Fig. 4. Time-profiles and correlations for blood and plasma serotonin, catecholamines, and their metabolite concentrations in a mini-pig, measured by in vivo microdialysis and ultrafiltration methods, respectively. PX: picrotoxin was administered 90 min after microdialysis probe insertion.

citric acid and sodium citrate to investigate blood 5-HT in rats [21]. Further investigations using the above perfusion medium may prove valuable for measuring blood 5-HT. Combining a microbore LC-EC system equipped with a dual amperometric detector, with in vitro and in vivo microdialysis sampling techniques reduces the early solvent front, and interfering peaks. Blood catecholamines, their metabolites and 5-HIAA can be measured using an in vivo microdialysis technique. However, measurement of blood 5-HT may be inaccurate due to aggregation or activation of platelets. Further studies will be needed to solve the problem of in vivo 5-HT measurement.

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