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Rapid assay of the monoamine content in small volumes of rat plasma

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Abstract

A method for the simultaneous measurement of serotonin catecholamines, and their metabolites in rat plasma by ultrafiltration and microbore liquid chromatography with electrochemical detection (LC-ED) in small volumes is established. Prior to the LC assay, sixteen plasma ultrafiltrates are readily prepared within 30 min in the present study. The present method, applying a dual-electrode detection technique, provides an additional reliable assignment or measurement of peaks by identifying the peaks on the basis of their redox ratios. In addition, the important early-eluting peaks and interfering peaks are eliminated in the cathodic chromatogram resulting in a reliable measurement of norepinephrine, epinephrine, and 3,4-dihydroxyphenylacetic acid. Isocratic separation of serotonin, catecholamines, and their metabolites by a microbore column is achieved within 15 min. Hence, theoretically, over 90 analyses can be performed in a working day. The limit of detection (signal-to-noise ratio = 3) of this method is *ca.* 0.2–0.5 pg per injection for all analytes. The required volume of the plasma samples can be less than 100 μl. Hence, the remainder of the plasma sample can be analysed for other substances. This rapid, simple, and sensitive method can thus be used as a research tool in the simultaneous measurement of rat plasma serotonin, catecholamines, and their metabolites.

1. Introduction

The measurement of plasma serotonin, catecholamines, and their metabolites is commonly used in the evaluation of the etiology of neuroendocrinology disorders [1,2] and in the study of 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 these purposes [5–8]. Liquid chromatography with electrochemical detection (LC-ED) is one of most popular and sensitive methods because it is rather simple, versatile, sensitive, and specific [9]. In general, the traditional LC-ED methods used for the determination of these compounds in plasma samples need extensive and time consuming clean-up procedures [10–13]. This is due to the large number of interfering substances in plasma appearing in traditional chromatography. Improved separations prior to LC are thus required for the measurement of these analytes in plasma samples. Furthermore, no simple and ideal LC

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method exists for the simultaneous determination of these compounds in small-volume plasma samples without tedious clean-up procedures [14–17].

Ultrafiltration (UF) techniques have been widely applied in plasma processing for drug monitoring assays [18,19]. In ultrafiltration the membrane is used as a selective barrier, enriching certain compounds and depleting others [20]. This is very attractive for the isolation of small and hydrophilic molecules and can be performed by discriminating against high-molecular-mass substances, such as proteins and suspended matters, on the basis of their size. In addition, the plasma volume required in ultrafiltration can be smaller than 100 μl [21]. In our previous study, ultrafiltration has been successfully applied in assaying human plasma serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC) [21]. Unfortunately, as in traditional LC-ED methods, early eluting peaks, such as norepinephrine (NE) and epinephrine (E), were largely masked by the solvent front and other early-eluting peaks. Reliable measurement of NE and E could not be performed. A dual-electrode electrochemical detector was used to solve this problem in this study. The principle of selective detection is as follows: the anode and cathode in the thin-layer flow cell are set at the appropriate potentials where analytes are oxidized (upstream) and reduced (downstream), respectively. Therefore, the reversible, irreversible, and quasi-reversible species can be discriminated electrochemically from one another on the basis of their redox properties. In the present study ultrafiltration was applied prior to LC assay in the pretreatment of plasma samples from portal hypertensive rats, an animal model which is known to have enhanced sympathetic activity [22].

2. Experimental

2.1. Apparatus and chromatographic conditions

The LC system consisted of a Beckman 126 pump (Beckman Instruments, Taiwan Branch, Taiwan), a CMA-200 microautosampler (CMA/Microdialysis, Stockholm, Sweden), two BAS-4C amperometric detectors (Bioanalytical Systems, West Lafayette, IN, USA), a microbore reversed-phase column (Inertsil-2 ODS 5 μm, 150 × 1.0 mm I.D., G.L. Sciences, Tokyo, Japan), a Beckman I/O 406 interface, and Beckman System Gold Data Analysis Software (Beckman Instruments). The potentials of the anodic and cathodic glassy carbon working electrodes were set at +0.75 V (upstream) and +0.05 V (downstream) with a silver/silver chloride electrode as reference, respectively. Glassy carbon working electrodes were maintained by polishing them once a week or when needed in accordance with the manufacturer's manual. The microbore column lifetime was also tested by frequently injecting standard samples during our routine work. Generally, the efficiency did not change over 800 injections of plasma ultrafiltrates.

2.2. Chemicals and reagents

NE, E, dopamine (DA), DOPAC, HVA, 5-HT, 5-HIAA, 3-methoxytyramine (3-MT, internal standard), ethylenediaminetetraacetic acid (EDTA), diethylamine, sodium 1-octanesulfonate (SOS), monosodium dihydrogen orthophosphate, and sodium citrate were purchased from Sigma (St. Louis, MO, USA). LC-grade acetonitrile and tetrahydrofuran (THF) were purchased from E. Merck (Merck-Schuchardt, Darmstadt, Germany). All reagents were of analytical quality unless otherwise stated.

2.3. Sample preparation and assay

Standard stock solutions of NE, E, DOPAC, DA, HVA, 5-HT, 5-HIAA, and 3-MT were prepared at a concentration of 2 ng/ml in 0.1 *M* perchloric acid and stored at −70°C in the dark and thawed at 4°C prior to preparation of a standard mixture. 3-MT was well separated from the other substances and could not be detected in rat plasma ultrafiltrates ($n = 9$) in our preliminary study. Therefore, 3-MT was chosen as the internal standard in this study. The internal

standard 3-MT and the standard mixture were prepared every day from a portion of these stock solutions after appropriate dilution with Ringer solution containing 10^{-7} *M* ascorbic acid in 0.1 *M* hydrochloric acid.

CCl_4-induced portal hypertension was performed by the method of Proctor and Chatamra [23]. Male Sprague-Dawley rats (100–150 g) were first given drinking water containing phenobarbital (0.33 mg/ml). After ten days, gastric feeding of CCl_4 was performed weekly for 12 weeks while the animals still were given phenobarbital-containing water. Both treatments were stopped one week before the experiment. Control rats were treated with phenobarbital only. On the day of sacrifice, rats (300–350 g) were decapitated between 9:00 and 10:00 a.m. Blood was collected into pre-chilled polypropylene tubes with heparin as an anticoagulant and centrifuged (10 min, 700 *g* at 4°C) immediately to separate the plasma. Frozen plasma samples were kept at −70°C prior to assay. After thawing at 4°C, 100 μl plasma sample was transferred into a Millipore Ultrafree-MC unit [Place, Ultrafree-MC with 10 000 nominal molecular mass cut-off (NMWC), Millipore Co., Bedford, MA, USA] and centrifuged at 15 000 *g* for 15 min. The Ultrafree-MC unit is a 400-μl sample cup with a regenerated cellulose membrane sealed to the bottom, which sits inside a 1.5-ml microcentrifuge tube. Under these conditions, typically, *ca.* 60% ($n = 9$, $58 \pm 2\%$) of the rat plasma sample volume was ultrafiltrated. A 50-μl aliquot of the plasma ultrafiltrate and 10-μl Ringer solution containing 1.4 ng 3-MT (as an internal standard) were mixed. Five microliter of the mixture was injected onto the LC-ED system.

Plasma 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 and corrected for the plasma recovery volume. The identity of the peaks in the plasma ultrafiltrate was confirmed by their retention times, the addition of standards, redox ratios, and a superimposed-alignment technique which was provided by Beckman (System Gold Data Analysis Software, version 6.01).

2.4. Mobile phase preparation

The mobile phase was slightly modified from our previous study [21] by adding 60 ml acetonitrile, 8 ml THF, 0.48 g sodium 1-octan sulfonate (SOS) (2.6 m*M*), 2.00 g monosodium dihydrogen orthophosphate (14.7 m*M*), 8.82 g sodium citrate (30 m*M*), 10 mg EDTA (0.027 m*M*), and 1 ml diethylamine in double distilled water. The solution pH was adjusted to 3.2 with 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 purging with helium for 20 min. The flow-rate was 60 μl/min maintaining a column pressure of *ca.* 10.3 MPa.

2.5. Statistical analysis

Results are expressed as mean ± S.D. Comparison between two groups was performed using the Student's t-test for unpaired data. A $p < 0.05$ was considered statistically significant.

3. Results and discussion

Figs. 1A and B show typical chromatograms of a standard mixture containing NE, E, DOPAC, DA, HVA, 5-HIAA, 3-MT (internal standard), and 5-HT. An analysis was completed within 15 min. All components under study were well resolved. The known amount (5 pg–20 ng) of analytes in the standard mixtures was linearly related with the anodic or cathodic chromatographic area obtained from the standard mixture. The standard curves and determination of correlations (r^2) for NE, E, DOPAC and DA, of anodic and cathodic responses were linear ($r^2 = 0.999$, Table 1). On the other hand, the limits of detection and the linear responses for 5-HIAA, HVA, and 5-HT on the anode ($r^2 = 0.999$, Table 1) were much better than those on the cathode (in the range of 100 pg–20 ng). Therefore, the concentrations of NE, E, DOPAC, and DA were determined from the cathodic chromatogram, while 5-HIAA, HVA, and 5-HT were deter-

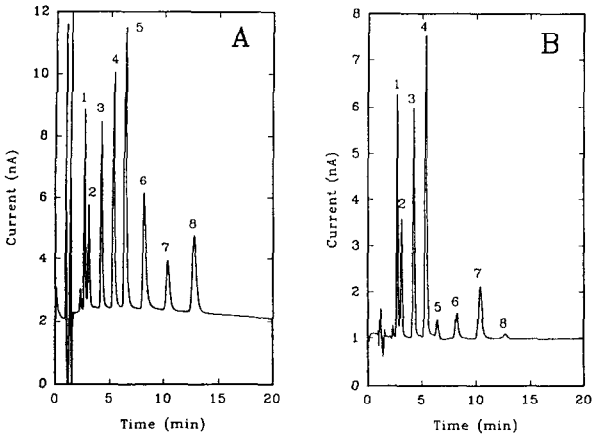

Fig. 1. Typical chromatograms of a standard mixture containing (1) NE, 222 pg; (2) E, 189 pg; (3) DOPAC, 199 pg; (4) DA, 248 pg; (5) 5-HIAA, 177 pg; (6) HVA, 208 pg; (7) 3-MT (internal standard), 213 pg; (8) 5-HT, 176 pg. (A) anodic current, (B) cathodic current: Applied potentials (*vs.* Ag/AgCl): anode (+) 0.75 V, cathode (+) 0.05 V.

mined from the anodic chromatogram in the further applications of the LC-ED system [24].

The precision of the assays was tested using a pooled plasma ultrafiltrate and a standard mixture in 0.1 *M* hydrochloric acid and a Ringer solution containing 10^{-7} *M* ascorbic acid. The intra-assay variability was assessed with 25 replicates at 1-h intervals and expressed as coefficients of variation (C.V., %). In the standard mixture 5-HIAA gave a high C.V. value (8.67%) while the others were satisfactory (≤5.41%) upon measurement. The standard mixture was also chosen for evaluating the inter-assay variability. C.V. values of all analytes in a pooled rat plasma ultrafiltrate were less than 4.65%. The inter-assay variabilities assessed with a standard mixture during 6 consecutive working days were less than 5.57% for the measurements of all analytes. The limits of detection (signal-to-noise ratio = 3) of the analytes per injection were 0.2–0.5 pg. The intra- and inter-assay variabilities were assessed and expressed as coefficients of variation (C.V.) and are shown in Table 2.

Figs. 2A and B show typical chromatograms of a rat plasma ultrafiltrate. Peaks of NE, E, DOPAC, DA, HVA, 5-HIAA, 3-MT, and 5-HT in Fig. 2 were identical to those in Fig. 1. However, there were several interfering peaks around NE, E, DOPAC and DA in the anodic chromatogram as shown in Fig. 2A. These interfering peaks were dramatically diminished in the cathodic chromatogram as shown in Fig. 2B. An additionally reliable assignment of the peaks was thus achieved by measuring the NE, E, DOPAC and DA peaks from the cathodic chromatograms. Occasionally, each peak was also verified by spiking of a standard mixture, and observing if the addition increased its peak height but did not change its shape and retention time. Redox ratios of the analytes were recorded and compared for the standard mixture and the plasma ultrafiltrate. In general, peaks were confirmed if

Table 1
Correlations of the known amount (range: 5 pg–20 ng) of analytes in standard mixtures with the anodic or cathodic peak area measured by the LC-ED

Substance	Anode		Cathode	
	Standard curve equation[a]	r^2	Standard curve equation	r^2
NE	$y = 6.325x - 47.619$	0.999	$y = 5.211x - 30.460$	0.999
E	$y = 4.022x - 42.805$	0.999	$y = 2.973x - 14.952$	0.999
DOPAC	$y = 8.604x - 67.275$	0.999	$y = 7.134x - 64.315$	0.999
DA	$y = 8.906x - 84.288$	0.999	$y = 7.683x - 99.549$	0.999
5-HIAA	$y = 10.72x - 35.047$	0.999	$y = 0.668x - 13.378$[b]	0.996
HVA	$y = 8.091x - 68.026$	0.999	$y = 0.970x - 18.753$[b]	0.997
5-HT	$y = 9.672x - 113.98$	0.999	$y = 0.325x - 4.269$[b]	0.996

[a] y = Peak area measured; x = amount of analytes in pg.
[b] Analyte amounts in the range of 100 pg–20 ng.

Table 2
Analytical precision on the stabilities of intra-assay ($n = 25$, at 1-h intervals) and inter-assay ($n = 6$, in six consecutive working days) of the LC-ED system

Sample	Coefficient of variation (%)							
	NE	E	DOPAC	DA	5-HIAA	HVA	3-MT	5-HT
Intra-assay								
Standard mixture in 0.1 *M* HCl and vitamin C	4.27	2.79	5.41	2.21	8.67	2.40	0.72	3.58
Rat plasma ultrafiltrate in 0.1 *M* HCl and vitamin C	2.03	3.48	1.34	4.65	1.29	2.02	1.43	3.58
Inter-assay								
Standard mixture in 0.1 *M* HCl and vitamin C	3.41	2.54	5.26	3.19	5.31	5.57	4.77	2.55

Standard mixtures consist of 50 pg catecholamines, serotonin, and their metabolites. NE, E, DOPAC, and DA were measured from the cathodic chromatograms, whereas 5-HIAA, HVA, and 5-HT were measured from the anodic chromatograms.

the variation of these redox ratios was below 10%. With a single electrode detector, only one point on the current–potential curve can be obtained for each chromatogram. Additional points of the current–potential curve can be determined by modification of the applied potential, but this is not practically. If the retention characteristics and current–potential behavior of a sample chromatographic peak compare well with those of a standard compound, peak identity confirmation can be achieved by the sequential performance of a dual-electrode detector.

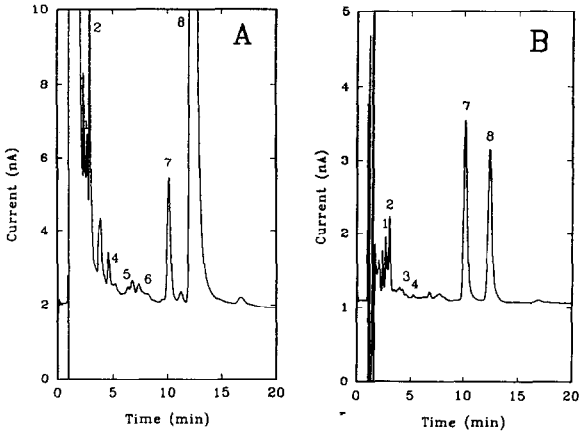

Fig. 2. Typical chromatograms of an ultrafiltrate of a plasma sample containing (1) NE; (2) E; (3) DOPAC; (4) DA; (5) 5-HIAA; (6) HVA; (7) 3-MT (internal standard); (8) 5-HT. (A) anodic current, (B) cathodic current.

In order to check the validity of the assays, pooled rat plasma samples ($n = 12$) with and without the addition of a known amount of a standard mixture (*ca.* 25–200 pg each) were ultrafiltrated and analyzed. The measured values of these analytes in pooled rat plasma samples, corrected by 3-MT recovery at each run, were consistent with the amounts added in both the anodic and cathodic chromatograms (Table 3). However, the redox ratios of these analytes for plasma ultrafiltrates are not exactly in agreement with those obtained for a standard mixture (Table 3). For instance, the redox ratios of authentic NE and E utilized in this study for the series dual electrode were 0.827 and 0.797, respectively. These values were also determined for plasma ultrafiltrates as NE (0.455) and E (0.824) eluted from the liquid chromatography column. Obviously, we were able to confirm the purity of E, whereas NE could have been co-eluted with some interfering peaks in the anodic chromatograms. In this case, those interfering peaks would increase the area measured in the

Table 3
Comparison of the redox ratios and amount of standard mixture added and the amount of standard mixture measured in plasma ultrafiltrates

	NE		E		DOPAC		DA		5-HIAA		HVA	5HT		
	Added (pg)	Measured[a] (pg)	Added (pg)	Measured[a] (pg)	Added (pg)	Measured[a] (pg)	Added (pg)	Measured[a] (pg)	Added (pg)	Measured[a] (pg)	Added (pg)	Measured[a] (pg)	Added (pg)	Measured[a] (pg)
($n = 3$)	51.0	52.0	55.0	53.1	50.0	49.6	70.0	67.3	99.5	96.3	55.0	68.9	54.5	57.1
	102.0	100.2	111.0	97.6	100.0	98.8	140.0	135.7	199.0	199.0	110.0	120.9	109.0	95.4
	204.0	200.7	222.0	190.8	200.0	195.0	280.0	272.8	398.0	408.9	220.0	229.2	218.0	201.2
	408.0	402.4	444.0	396.1	400.0	403.7	560.0	555.8	796.0	838.2	440.0	457.0	436.0	397.6
R.R.[b]	0.827	0.455	0.797	0.824	0.826	0.819	0.855	0.795	0.063	0.076	0.110	0.093	0.033	0.025

Anodic and cathodic potentials were +0.75 V and +0.05 V, respectively. Reatively close values in redox ratios and amounts indicate the accuracy in assaying analytes of plasma samples.
[a] Amount of standard mixture measured (amount measured from standard mixture spiked plasma samples – amount measured from plasma samples).
[b] R.R. = Redox ratios.

anodic chromatogram and decrease the redox ratio of the NE peak. Resolution of the NE peak could be achieved by modification of the LC mobile phase. However, the contaminants were usually not detected in the cathodic chromatograms. In general, clear peaks of NE, E, DOPAC and DA were obtained in the cathodic chromatogram in our routine assay. This might be due to the electrochemical irreversibility of those contaminants. Hence, measurement of NE, E, DOPAC and DA could also be made from the cathodic chromatograms, alternatively. In addition, a superimposed-alignment technique is also useful if chromatographic peaks slightly differ in their elution time between runs or if they coelute with other unknown interferences. The chromatograms are adjusted by alignment according to the differences between selected peaks and the internal standard, 3-MT, by the Beckman Gold System. Sometimes superimposition of the chromatograms is a valuable tool in confirming these peaks.

Table 4 shows the plasma concentration of biogenic amines and their metabolites as measured by the LC-ED system. In this study, the portal pressure of control Spragne-Dawley rats ($n = 7$) and CCl_4-treated rats ($n = 6$) ranged between 6 and 8 from 9 to 12 mmHg, respectively. Recently, Rodriguez-Puyol *et al.* [22] concluded that plasma NE and DA concentrations were similar in normal rats and rats with portal hypertension induced by CCl_4. In our study, there was also no difference in plasma NE, DA,

Table 4
Changes in plasma serotonin, catecholamines, and their metabolites (Mean ± S.D., ng/ml) in rats treated with CCl_4 measured by the LC-ED system

Compound	Concentration (mean ± S.D.) (ng/ml)	
	Control	CCl_4-treated
NE	8.254 ± 0.422	7.695 ± 1.251
E	21.247 ± 1.411	16.267 ± 2.074[a]
DOPAC	0.291 ± 0.056	2.638 ± 0.881[a]
DA	0.200 ± 0.066	0.327 ± 0.127
5-HIAA	1.257 ± 0.159	2.532 ± 0.403[a]
HVA	2.239 ± 0.724	1.850 ± 0.166
5-HT	554.669 ± 23.529	554.808 ± 89.009

[a] $p < 0.05$, compared to the control group by the Student's t-test (unpaired).

and HVA between control rats and in rats with portal hypertension. However, plasma E levels were significantly lower ($p < 0.05$), whereas plasma DOPAC and 5-HIAA were significantly higher ($p < 0.05$) in CCl_4-treated rats compared with control rats. In addition, the turn-over rates of serotinin and DA in plasma were higher in CCl_4-treated rats than in control rats (data not shown). Rat plasma 5-HT concentrations were higher than in our routine plasma assays. This is possibly due to the method of decapitation or phenobarbital-treatment effects. Further studies in this animal model should be conducted to investigate the mechanism of these differences.

The plasma pretreatment of the present method took less than 30 min for 16 samples, whereas the traditional alumina extraction procedure [25,26] took 4 h for 8–10 samples in our routine assays [27]. The variability of the recovery is still the main problem of the routine alumina extraction method in inter-laboratory comparison [28]. The present assay is advantageous in the simultaneous measurement of serotonin, catecholamines, and their metabolites, whereas only the catecholamines can be measured with the alumina extraction. Because of the very low hold-up volume (<5 μl) of the Millipore Ultrafree-MC units and the precision of the CMA-200 microautosampler, the method requires only 100-μl plasma samples. However, in our previous study a 30-μl plasma sample was sufficient to obtain data (data not shown) as precise and accurate as those obtained with a 100-μl sample in the ultrafiltration assay of the present study. Another advantage of this LC method are the very low of limits of detection (0.2–0.5 pg/per injection). The measurement of plasma biogenic amines in such small plasma volumes and with a low limit of detection has a great analytical potential when many physiological substances have to be analysed in small-animal experiments. Furthermore, a complete separation of the analytes within 15 min was achieved, and hence more than 90 analyses can be done in one working day by the automated LC-ED system. As was the case in this study, automated LC is advantageous when many plasma samples have to be analyzed in a short period of time.

4. Conclusion

The method presented in this paper is a rapid, reliable and convenient method compared to some rather complicated methods, such as alumina or liquid-phase extraction assays for the measurement of plasma serotonin, catecholamines, and their metabolites. We recommend this method as a suitable research tool for assaying biogenic amines in small blood volumes.

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