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Application of ultrafiltration method to measurement of catecholamines in plasma of human and rodents by high-performance liquid chromatography

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Abstract

In order to develop a reliable, simple and routine method using small sample volume to determine norepinephrine (NE) and epinephrine (E) concentrations in plasma of humans and rodents, we utilize the ultrafiltration (UF) method by Ultrafree-MC filter device and a high-performance liquid chromatography equipped with electrochemical detector (HPLC-ECD) to detect NE and E. Optimum UF and HPLC conditions were as follows: the filter nominal molecular weight limit size is 30,000, the pH of added phosphate buffer to each plasma sample for UF is 3.0, and the mobile phase is 0.1 M phosphate buffer (pH 3)/acetonitrile (98:2) containing 0.05% sodium disulfite and 0.001% EDTA 2Na. The plasma samples and 1.0 M phosphate buffer (pH 3) containing 3,4-dihydroxybenzylamine (DHBA), as an internal standard, was mixed and poured into the UF units. After the centrifugation for 60 min at 13,000 × *g* at 4 °C, the filtrate was directly injected into HPLC. The calibration curve of NE and E was linear for the concentrations studied (20–400 pg) with a correlation coefficient of >0.999. Intra-assay coefficients of variation for NE and E using this method were less than 3%. The method also correlated well with the well-established alumina method ($r = 0.954$). The present findings suggest that a newly-developed UF method with HPLC-ECD would apply successfully to measure plasma NE and E concentrations in humans and rodents.
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1. Introduction

Catecholamines (CAs), such as norepinephrine (NE), epinephrine (E) and dopamine, in biological fluids are routinely measured. For example, the levels of NE and E in plasma are widely used for the diagnosis of pheochromocytoma, neuroblastoma and heart failure, in addition to the evaluation of sympathetic nerve activity in humans as well as experimental animals [1,2]. However, the precise measurement of NE and E in human plasma is fairly difficult since (1) their low plasma concentrations (pg–ng range) except for certain disease states [1,3], and (2) the complicated protocols to extract CAs from plasma. Therefore, the improvement of the methods for the detection of CAs in plasma would be needed. In order to analyze CAs, there are two important processes to allow the improvement of their measurements: one is extraction to remove various endogenous compounds, which might interfere the analysis. For example, acid-washed aluminum adsorption method is commonly used to extract CAs and to remove other compounds in plasma samples [6–8]. There are several other methods available such as liquid-liquid extraction [9], solid-phase extraction using cation-exchange [10] and boronic acid gel [11]. The other to improve is the measurement process. So far, gas-chromatography [12], high-performance liquid chromatography (HPLC) [5–10] and radioenzymatic determination [13] have been established. Among them, HPLC with electrochemical detection (HPLC-ECD) is particularly suitable for CAs measurement because of its relative high sensitivity, selectivity and simplicity [14,15]. However,

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commonly-used HPLC-ECD together with alumina method still needs at least 0.5–1.0 ml of human plasma to analyze CAs [6,7,14].

Ultrafiltration (UF) is a simple method to separate samples based on their molecular weight by centrifugation. UF is now applied to many purposes such as concentrating and purifying protein or nucleic acid, buffer changes [16,17] and measuring protein binding potency [18]. Considering the ability of UF only to filtrate substrates having small molecular weights, UF would be applicable to collect CAs from plasma samples and to remove the substrates having large molecular weights including plasma proteins, which possibly interfere the detections of CAs. Indeed, Cheng et al. [19] have used UF to determine plasma CAs in rats. However, it is unavailable, thus far, to further develop this method especially for human plasma having relatively lower levels of CAs than those in rodents.

In the present study, we first tried to find out the optimum condition of UF method by verifying various influencing factors such as filter nominal molecular weight limit (NMWL) size, buffer pH and antioxidant added to filter unit since CAs are degraded by spontaneous oxidation, ultraviolet radiation and metal ions [4,5]. Second, we tried to measure plasma NE and E in human and rodents with the appropriate experimental conditions, found by the process of above-mentioned optimizations.

2. Experimental

2.1. Reagents

Norepinephrine (NE), epinephrine (E) and 3,4-dihydroxybenzylamine (DHBA) were obtained from Sigma (St. Louis, MO, USA). Sodium disulfite (SOD) was obtained from Kanto Chemicals (Tokyo, Japan). Human serum albumin (HSA) and 1-octansulfonic acid sodium salt (SOS) were obtained from Wako Pure Chemicals (Osaka, Japan) and ethylenediaminetetraacetic acid disodium salt (EDTA 2Na) was from Yoneyama Yakuhin (Kyoto, Japan). Reagent-grade water obtained from a Millipore Milli-Q system was used throughout the experiments. All other regents were of analytical grade of purity.

2.2. Apparatus and chromatographic conditions

Ultrafree-MC filter units (30,000, 50,000 and 100,000 NMWL) were purchased from Millipore Co. (Bedford, MA, USA). Each filter membrane is hydrophilic regenerated cellulose containing glycerol (non-sterile) and adsorption of protein to each membrane is low. The effective filtration area is 0.2 cm^2. HPLC was used Shimadzu LC-VP systems (Kyoto, Japan): isocratic pump (LC-10ADvp), autoinjector (SIL-10ADvp), column oven (CTO-10ADvp), chromatogram analyzer and recorder (C-R8A, Chromatopac). Detection of the CAs was performed by an electrochemical detection using an amperometric detector (Model ECD-100, Eicom, Kyoto, Japan). The potential of the glassy carbon working electrode was held at +0.70 V versus Ag/AgCl reference electrode. A reversed-phase column (EIKOMPAK SC-5ODS, Ø3.0 × 150 mm, Eicom, Kyoto, Japan) equipped with pre-column (Eicom, Kyoto, Japan) was used to separate CAs.

The mobile phase, which contains 0.98 l of 0.1 M phosphate buffer (pH 3), 20 ml of acetonitrile, 500 mg of SOS and 10 mg of EDTA 2 Na, was degassed prior to use. The flow-rate was 0.5 ml/min. The temperatures of pre-column and column were held at 25.0 °C. The recorder was set at plotter chart speed to 1 mm/min.

2.3. Preparation of working standards

One molar phosphate buffer (pH 3) was used for the stock solution at concentration of 1 mg/ml of NE, E and DHBA as an internal standard (IS). Stock solution was stored at the place protected from light at 4 °C and was used within 1 week. Working standards were prepared each day by taking aliquots from stock solution by diluting with 1.0 M phosphate buffer (pH 3) to make desired concentration solutions.

2.4. Comparison of filter NMWL size

The optimum filter NMWL sizes to measure CAs were tested with 30,000, 50,000 and 100,000. Firstly, the possible loss of CAs at filter unit was determined by filtering 300 μl of 1.0 M phosphate buffer (pH 3) containing CAs (3.33 ng/ml) with prechilled 30,000, 50,000 or 100,000 NMWL filter by the centrifugation (6,000 × *g* for 10 min at 4 °C). The collected filtrates were measured their volumes, and 80 μl of both phosphate buffer containing CAs before and after the filtration were injected into HPLC to compare their recoveries of CAs. Secondly, 200 μl of 4% HSA solution spiked with CAs (5 ng/ml) and 100 μl of 1.0 M phosphate buffer (pH 3) containing DHBA (40 ng/ml) was filtrated by each filter unit. After the centrifugation, the volume of filtrate was measured, and 80 μl of the each filtrate was injected into HPLC. Furthermore, the amount of CAs in filtrate was calculated (i.e. concentration multiplied by volume), and data was expressed as 100% of the recovery of CAs from 30,000 NMWL.

2.5. Effect of pH of phosphate buffer

The optimum pH of phosphate buffer for CAs amount in filtrate with UF method was examined. Each 1.0 M phosphate buffer (pH 3, pH 5, pH 7 and pH 9) containing DHBA was prepared just before the experiment. Two hundred microliters of 4% HSA spiked with CAs (5 ng/ml) and 100 μl of 1.0 M phosphate buffer (pH 3–9) containing DHBA (40 ng/ml) were added to the prechilled filter unit ($n = 3$). After the centrifugation, the volume

of filtrates was measured and 80 μl of the filtrates was injected into HPLC. From the HPLC data, the amount of CAs in filtrates was calculated. The recoveries of CAs at different pH were expressed as 100% of that at pH 3.

2.6. Time-dependent degradation of NE, E and DHBA in filtrates after UF and the effect of antioxidant

The stabilities of CAs in filtrates after UF were examined when SOD, an antioxidant, was added in phosphate buffer. Two-hundred microliters of the HSA solution spiked with CAs (5 ng/ml) and 0.1 ml of 1.0 M phosphate buffer (pH 3) containing SOD (0, 10, 500 and 800 μg/ml) were added to the prechilled filter unit ($n = 3$). After centrifugation, the obtained filtrates (80 μl) by UF were stored at 4 °C in the dark until analysis, and were measured 2 h (0 day), 1 day and 7 days after UF. The amounts of CAs in each filtrate were expressed as 100% of that in filtrate 2 h after UF.

2.7. Calibration curve and precision

Using UF method after optimization by the experiments described above, precision and accuracy for CAs concentrations in the filtrates obtained from 4% HSA solution spiked with CAs ranging from 20 to 400 pg (five points) were evaluated. The peak-height of CAs was measured and was expressed as the ratio to IS Calibration curve was constructed with these data. The intra-assay coefficients of variation were assessed with six independent experiments. The concentration of CAs in the HSA solution was 5.0 ng/ml. UF method was compared with acid-washed alumina method [20]. Briefly, NE concentrations in Syrian hamster plasma ($n = 14$) obtained by the UF method were compared with them obtained by alumina method.

2.8. Blood sampling

Venous blood samples from seven healthy volunteers (1.5 ml), who had been given informed consent, were collected into prechilled polypropylene vials containing EDTA 2Na (1 mg/ml of whole blood). Blood sampling of all rodents was performed under anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Blood samples from rats (0.4 ml) were collected from the right carotid artery using cannulation with heparin-rocked polyethylene catheter. Blood samples from hamsters (1.0 ml) and mice (0.2 ml) were collected from the descending aorta and the heart, and then were put into the tubes preloaded with EDTA 2Na (1 mg/ml of whole blood). The collected blood samples were immediately placed on ice and were centrifuged (6000 × g for 10 min at 4 °C). Each plasma sample was stored at −80 °C until analysis.

2.9. The measurement of CAs in humans and rodents

In the measurement of CAs in plasma of humans and rats, 0.2 ml of each plasma sample and 0.1 ml of 1.0 M phosphate buffer (pH 3) containing DHBA (40 ng/ml) and SOD (800 μg/ml), were poured into the prechilled filter units. In the measurement of CAs in plasma of hamsters and mice, 0.05 ml of each plasma sample and 0.2 ml of 1.0 M phosphate buffer (pH 3) containing DHBA (40 ng/ml) and SOD (800 μg/ml), were poured into the prechilled filter units. The filter units were then allowed to centrifuge at 13,000 × g at 4 °C for 60 min (30 min for plasma of hamsters and mice). Each filtrate was stored at 4 °C in the dark until measurement. CAs in samples were measured by HPLC and data was expressed as mean ± S.E.

2.10. Statistical analysis

All results were expressed as mean ± S.E. Calibration curve and correlation coefficient were calculated with Microsoft Excel 2000 (Microsoft Corporation, Seattle, USA). Statistical analyses were performed with one way analysis of variance (one way ANOVA). Significances of individual differences were evaluated by using the Scheffé test if ANOVA was significant. P-values <0.05 were considered statistically significant. These statistical analyses were performed the Stat View software (ABACUS, Berkeley, CA).

3. Results and discussion

UF method is very simple (only one-step operation) and fast processing of sample purification with high yield. The aim of this study was to evaluate the optimal condition of UF to measure CAs in plasma samples.

3.1. Effect of NMWL sizes on recoveries of CAs

It has been investigated which NMWL size is the most appropriate to measure CAs in plasma. The collected volume of filtrate in each filter was close to 100% (data not shown) and no significant differences of both the calculated amounts of CAs in between phosphate buffer, mounted on filters, and filtrates was observed (data not shown). These results suggest that each filter possesses the equal ability to filtrate without adsorption of CAs to filter membrane. Next, we applied 4% HSA solution, spiked with CAs, to each filter unit. As a result, the collected volume of filtrate obtained from each filter size was no significant difference. The recoveries of CAs in filtrate were slightly decreased if compared to those in filtrate of phosphate buffer; i.e. 91.9 ± 2.0% for NE, 92.0 ± 2.0% for E and 89.1 ± 1.5% for DHBA were found in filtrates of 4% HSA solution through the 30,000 NMWL. Comparing the recoveries of CAs between the filter units, the recoveries of NE were difference among the NMWLs

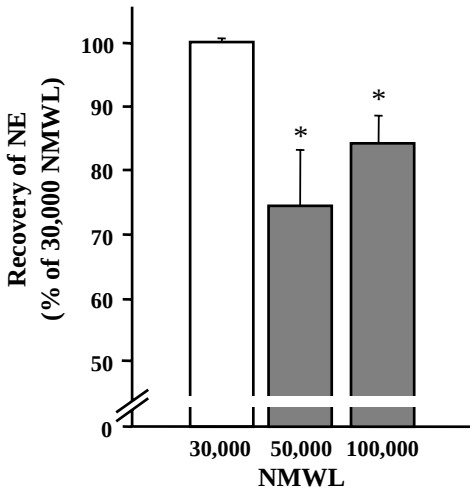

Fig. 1. Effect of the difference of filter NMWL sizes on recovery of NE in filtrates obtained from 4% HSA solution contained 5 ng/ml of NE ($n = 3$). Data represent the mean ± S.E. of three samples and are presented by the percentage (%) of NE recovery of 30,000 NMWL. $^{*}P < 0.05$ vs. 30,000 NMWL.

(Fig. 1), but not those of E and DHBA (data not shown). The recoveries of NE in 50,000 (62.9 ± 7.3%) and 100,000 NMWL (71.1 ± 3.6%) filter units were significantly lower than that in 30,000 NMWL filter unit. One of the possible reasons would be that the substances, having its molecular weight over 30,000, interfere the detection of NE due to its binding to NE in the filtrates. Indeed, albumin (MW approximately 69,000) and other plasma proteins have an ability to bind CAs [21]. Somehow, these results suggest that the choice of proper NMWL size is critical to measure NE in plasma although further studies should be necessary to understand this phenomena. We chose the 30,000 NMWL filter unit in the following experiments.

3.2. Effect of buffer pH for the recoveries of CAs

To avoid the concentration polarization, which effect on filtration, plasma was diluted with phosphate buffer. Because, it is well known that CAs are well solved in phosphate buffer. However, it is not clear whether or not pH of phosphate buffer affects the recoveries of CAs by UF method. Thus, we addressed this issue by using phosphate buffer having different pH. The volume of filtrates was not altered by pH of phosphate buffer. However, the collected amount of CAs and DHBA was differed by pH. As illustrated in Fig. 2, the recoveries of each compound at pH 7 (53–64%) and pH 9 (43–49%) were significantly lower than those at pH 3 (100%) and pH 5 (83–101%). Generally, acidic buffer can prevent the degradation of CAs [22]. So, this would be the possible reason why acidic buffer is appropriate rather than alkaline buffer. Based on these results, we chose the UF with 30,000 NMWL filter unit and 1.0 M phosphate buffer (pH 3) to analyze CAs in plasma in the following experiments.

3.3. Protective effect of SOD against oxidation of NE, E and DHBA on storage

It is well known that CAs in plasma are easily oxidized: that is, CAs in plasma are stable for 1 day at 20 °C, 2 days at 4 °C and up to 1 year at −70 °C [5]. To prevent such oxidation of CAs, ascorbic acid or SOD has been frequently used [4,5]. However, it is not clear which kind of antioxidants are appropriate and which concentration is proper to prevent the degradation of CAs in this method. Thus, we examined the time-dependent effect of an antioxidant, SOD on the amount of NE, E and DHBA in filtrates. The different SOD concentration did not show the significant effect on the volume of filtrates (data not shown). As shown in Fig. 3, when SOD was not added, the amount of NE, E and DHBA at 7 days after UF decreased to 52 ± 7, 42 ± 4 and 63 ± 2% of the control levels (day 0), even though they kept at 4 °C in the dark. However, SOD (500–800 μg/ml) could prevent the degradation of CAs until 7 days after UF, but not of low doses of SOD (0–10 μg/ml). Ascorbic acid is often used as a potent antioxidant for measurement of CAs. In our experimental condition, ascorbic acid interfered with the electrochemical measurement of NE, E and DHBA (data not shown), which was consistent with the previous report [18]. Thus, the use of SOD (500–800 μg/ml) was suitable against the degradation of CAs before starting the filtration.

3.4. Precision and accuracy

UF (30,000 NMWL) with 1.0 M phosphate buffer (pH 3) containing SOD (800 μg/ml) and DHBA was applied to measure CAs in 4% HSA solution containing the known amounts of CAs. The data were obtained by comparing the peak-height ratio (NE/DHBA or E/DHBA). The calibration curves were linear (standard curve equation: $y = 0.131x + 0.032$ for NE, $y = 0.113x - 0.004$ for E) in the concentration range investigated (20–400 pg), with correlations coefficient of more than 0.999. The intra-assay coefficients of variation for NE (1.4%) using the UF method, which were lower than those of using the previous method, UF and alumina method (3.4 and 9.0%) [19,24], and those of E (2.3%) was almost similar to the previous method (1.7 and 8.6%) [19,24]. When CAs in 4% HSA solution was measured, limit of detection (LOD, signal-to-noise ratio: 3) and limit of quantitation (LOQ, signal-to-noise ratio: 10) were about 4.0 and 13.3 pg for NE, 3.2 and 10.7 pg for E, respectively.

3.5. The comparison of UF method and alumina method

Concentrations of plasma NE in hamster were compared between alumina method and UF method. The data obtained by UF method nicely correlated with those obtained by alumina method ($r = 0.954$, Fig. 4). Thus, UF method used in this study is suitable to measure NE in experimental

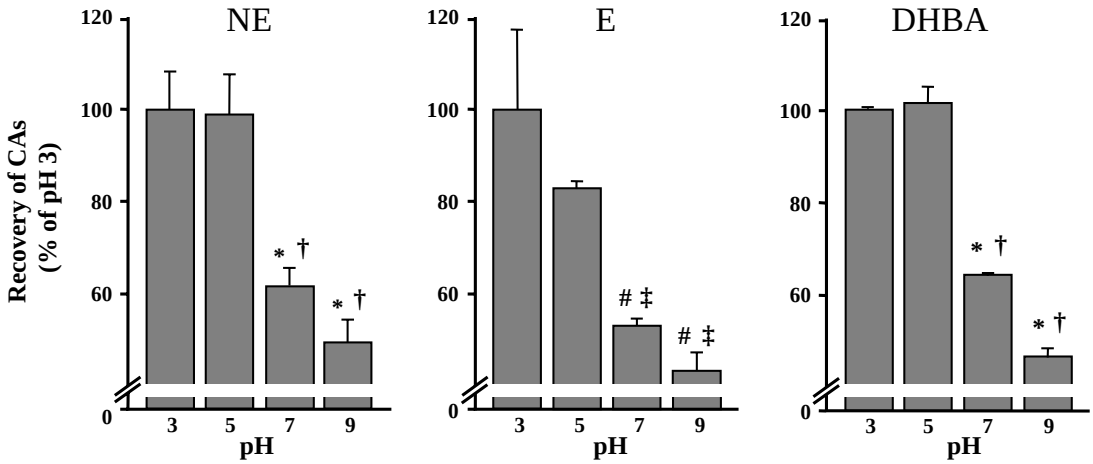

Fig. 2. Effect of buffer pH on recoveries of NE, E and DHBA in 4% HSA filtrates using 30,000 NMWL size. Data represent the mean ± S.E. of three samples and were presented by the percentage (%) of each recovery of pH 3. $^{*}P < 0.01$, $^{\#}P < 0.05$, significantly different from the values at pH 3. $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.05$, significantly different from the values at pH 5.

animals, which was similar to the alumina method described elsewhere [17].

3.6. Application of UF method

The concentrations of NE and E in four species (human, rat, hamster and mouse plasma) are measured by UF method. Compared to 4% HSA as shown in Fig. 5A and B, few unknown peaks were found in each samples. Thus, it was needed to modify slightly the mobile phase, gradient system and flow rate.

The mobile phase was composed of 0.1 M phosphate buffer (pH 3), acetonitrile, methanol (mobile phase A, 98:2:0 v/v; mobile phase B 100:0:0; mobile phase C, 93:2:5 v/v), 500 mg/l of SOS and 10 mg/l of EDTA 2Na. In analysis of the human plasma, total flow-rate (mobile phase A and B) was 0.5 ml/min from zero to 25 min, and gradually increased to 0.7 ml/min (25–60 min). The gradient ratio was started from mobile phase A: B (100:0). The mobile phase B was gradually increased to 100% by 15 min, and then maintained the rate A:B (0:100) until analysis is finished. On the other hand, in analysis of the rat, mouse and hamster plasma,

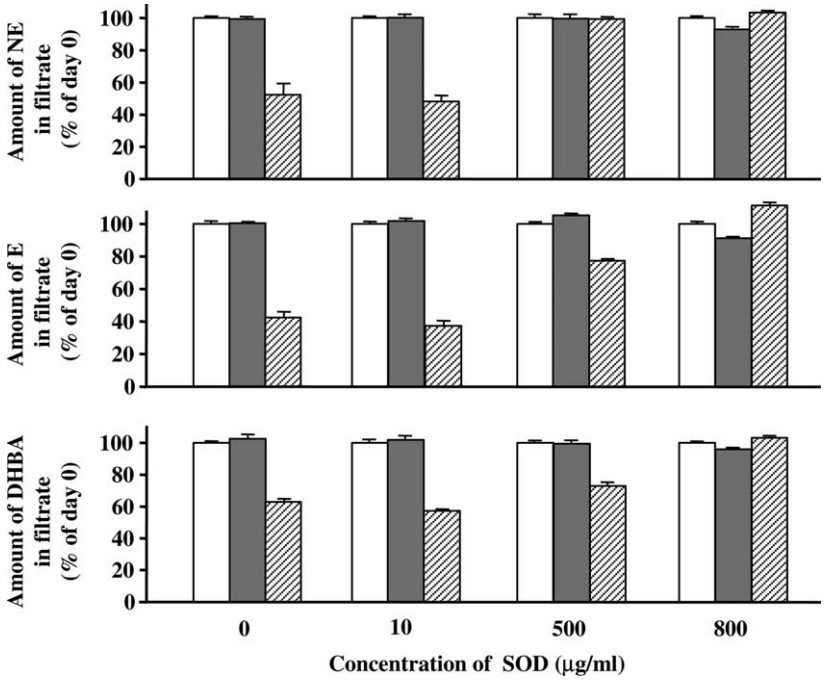

Fig. 3. Effect of different concentrations of SOD (0, 10, 500 and 800 μg/ml) in phosphate buffer for the stabilities of CAs in filtrates on storage, zero (□), 1 (■) and 7 (▨) days after UF. Data represent the mean±S.E. of three samples and are presented as the percentage (%) of each amount at zero day after UF.

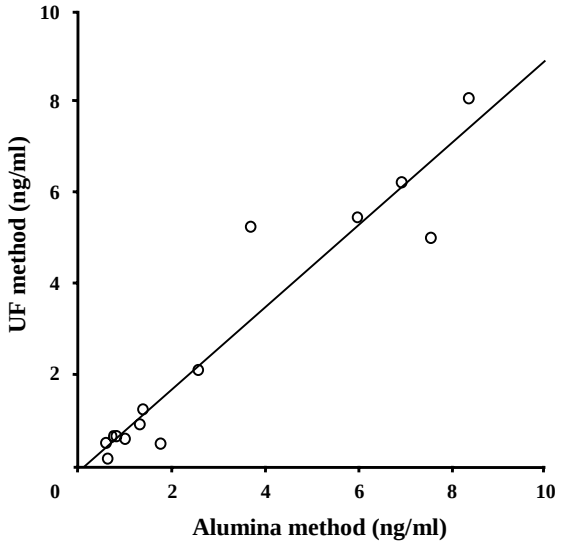

Fig. 4. Correlation of NE levels in plasma between the UF and alumina methods ($n = 14$). $r = 0.954$, $y = 0.902x - 0.110$.

the flow-rate was 0.5 ml/min and the mobile phase C was used.

A typical chromatogram of CAs and DHBA (known concentration) in 4% HSA was illustrated in Fig. 5A and that in human plasma was illustrated in Fig. 5B. A typical chromatogram of human plasma added only DHBA also illustrated in Fig. 5C. Consequently, the retention time of NE, E and DHBA in human plasma was approximately 12, 20 and 40 min, respectively. Plasma concentrations of the NE and E measured from seven healthy volunteers with UF were 374 ± 57 and 97 ± 25 pg/ml. Plasma concentrations of NE and E with UF were 475 ± 52 and 397 ± 70 pg/ml for rats ($n = 6$), 7482 ± 768 and 5284 ± 833 pg/ml for hamsters ($n = 17$), 4108 ± 305 and $29{,}025 \pm 5104$ pg/ml for mice

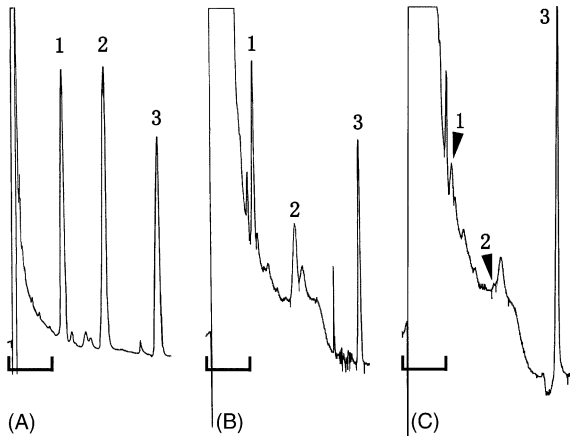

Fig. 5. Typical HPLC-ECD chromatograms of NE, E and DHBA: (A) 10 ng/ml of CAs in 4% HSA solution, (B) normal human plasma spiked with 4 ng/ml of CAs and 25 ng/ml of DHBA, (C) normal human plasma spiked with 25 ng/ml of DHBA. Concentrations of CAs were 375 pg/ml for NE and 118 pg/ml for E. Notation: (1) norepinephrine; (2) epinephrine; (3) DHBA. Scale bar expresses 10 min.

($n = 8$). In specially, the value of NE in this study was similar to the reported values [2,7,23,24]. Additionally, when CAs in human and other species sample were measured, LOD and LOQ were about 25 and 85 pg for NE, 20 and 67 pg for E, respectively. These LOD and LOQ levels of E are close to E level of healthy human plasma. Therefore, we must more study about measurement of E in human plasma. In this UF method, filter units having effective filtration area (0.2 cm^2) were used. If filter units having more effective filtration area were used, LOD and LOQ might be improved. However, considering running cost and quality of measurement, the filter units used in this UF method are better at the present moment. These data suggest that our method may be appropriate for the clinical or experimental assay of CAs using small plasma volume of humans, rats, hamsters and mice.

4. Conclusion

The present study demonstrated that UF method is a newly-developed analytical method to measure plasma CAs in various species with simplicity, accuracy and stability even if the volume of sample is fairly low (0.05–0.2 ml). Thus, this method would be expected to use for the measurement of CAs not only in experimental animals under normal condition or disease states but also in human as a routine assay.

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