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Journal of Chromatography B, 729 (1999) 89–95

JOURNAL OF CHROMATOGRAPHY B

Determination of angiotensin metabolites in human plasma by fluorimetric high-performance liquid chromatography using a heart-cut column-switching technique

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Received 9 December 1998; received in revised form 22 March 1999; accepted 22 March 1999

Abstract

Fluorimetric column-switching HPLC method with naphthalene-2,3-dialdehyde (NDA) was developed for the determination of endogenous angiotensin (ANG) metabolites in human plasma. After one-step extraction to clean up the ultrafiltered plasma sample on the reversed HPLC system, the zone of the retention time of each ANG analyte was subjected to the NDA-derivatization. After putting into a first Phe-ODS (for ANG (3–4) and (5–8) determinations) or ODS column (for ANG I and II determinations), the heart-cut of the retention time of the NDA-ANG was separated on a second ODS column with a mobile phase containing 5 m*M* ion-pair reagent. Complete separation and good detection were accomplished within 2 h. Good linearity of the regression equation for all ANG analytes with the correlation coefficient of >0.993 as well as good reproducibility (C.V.$<4.0\%$). Good agreement of the range of ANG II plasma level between the present (25–47 fmol/ml in plasma) and the radioimmunoassay methods (28–52 fmol/ml in plasma) indicated that the column-switching method could be applicable for the determination of endogenous smaller ANGs as well as for ANG I or II in plasma.

Keywords: Angiotensin

1. Introduction

Renin–angiotensin (RA) system is well known to be the most important pressor system in the body. In the circulatory system, angiotensins (ANGs), in particular ANG II and III play diverse physiological roles such as activation of vasoconstriction, and promotion of aldosterone release from the adrenal as well as in the localized RA systems [1]. In a series of our studies regarding the angiotensin I-converting enzyme (ACE, EC 3.4.15.1) inhibition by natural or synthetic peptides [2,3], we have revealed that the ANG (5–8) and (3–4) have strong in vitro ACE inhibitory activity. Thus, to know the metabolic behavior of these ANGs in human plasma is of interest in connection with the vital pressor mechanism.

The determination of ANG I, II and III in human blood was conventionally carried out by radioimmunoassay (RIA) using the ^{125}I-labeled corre-

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00128-0

sponding ANGs [4], but other smaller ANGs could not be subjected to the assay due to there being no possibility of obtaining their antibodies. At the aim of determining the smaller ANGs in human plasma, we have established a fluorometric high-performance liquid chromatographic (HPLC) method using naphthalene-2,3-dialdehyde (NDA) [5]. However, this proposed HPLC method required duplicate HPLC separation procedures to prepare the assaying ANG fraction from plasma prior to fluorometric derivatization with NDA. Until now, there have been many reports with respect to the analyses of drugs in plasma using column switching techniques [6–8], although no application was reported for endogenous ANGs as an analyte. Therefore, the aim of this study was to improve the NDA-HPLC method with the heart-cut column switching technique for rapid determination of ANGs in human plasma with minimal pre-fractionation procedures.

2. Experimental

2.1. Reagents and materials

ANG I and II were purchased from Sigma (St. Louis, MO, USA), and ANG (5–8) and (3–4) from Kokusan Chemical Works (Tokyo, Japan). Working standard ANG solution was prepared daily prior to the HPLC analysis. Naphthalene-2,3-dialdehyde (NDA) as a fluorogenic reagent was obtained from Fluka (Tokyo, Japan). Acetonitrile (CH_3CN) was of HPLC grade (Wako, Osaka, Japan). Deionized water was prepared by Milli-Q system (Millipore Japan, Tokyo, Japan). Sodium 1-octanesulphonate (SOS) with ion-pair reagent grade was obtained from Wako. All other chemicals were of analytical-reagent grade and used without further purification.

2.2. Instrumentation

Schematic representations of the column-switching HPLC systems with heart-cut procedure are shown in Fig. 1. The system consisted of a solvent delivery system of two pumps (A and B) (LC-9A, Shimadzu, Kyoto, Japan), an automated gradient controller (SCL-9A, Shimadzu), a column oven (CTO-10A, Shimadzu), a manual four-port switching valve (HPV-4, GL Sciences, Tokyo, Japan), a Shimadzu SPD-10AV UV–Vis detector, a fluorescence detector (FP-920S, Nippon Bunko, Tokyo, Japan), and a computing integrator (Chromatopac CR6A, Shimadzu).

2.3. Columns and column-switching conditions

Table 1 summarises the present column switching conditions. The clean-up column (C1) was Cosmosil

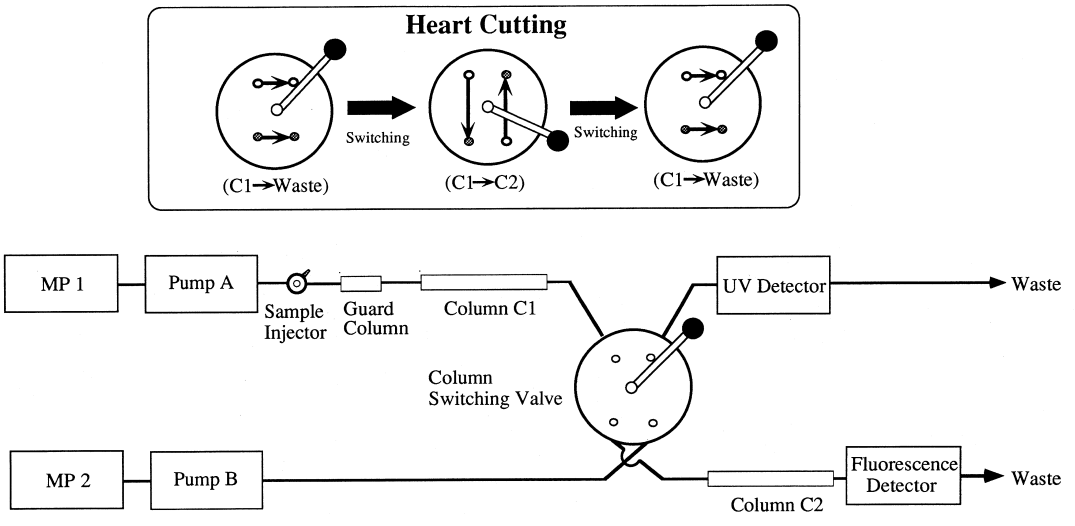

Fig. 1. Schematic representation of the column-switching HPLC system for the determination of endogenous angiotensins in plasma.

Table 1
HPLC conditions for determination of angiotensins by the column-switching technique

Angiotensins	Column (∅ 4.6×250 mm)		Solvent system (%CH_3CN/0.1% TFA)		Heart-cut (min)
	C1	C2	MP1	MP2	
ANG I	5C_{18}-ARII	5C_{18}-ARII	20–40 (40 min)	30–40 (25 min)	54.5–57.5
ANG II	5C_{18}-ARII	5C_{18}-ARII	20–40 (40 min)	30–40 (25 min)	53.5–56.5
ANG (5–8)	5Ph	5C_{18}-ARII	40–60 (60 min)	55	62.0–65.0
ANG (3–4)	5Ph	5C_{18}-ARII	40–60 (60 min)	60	65.5–68.5

5Ph for ANG (3–4) and ANG (5–8) assays or Cosmosil 5C_{18}-ARII for ANG I and ANG II assays (each column: 5 μm particle size, 250×4.6 mm I.D.; Nacalai Tesque, Kyoto, Japan). In this study, 250 mm column length was necessary to achieve complete separation for the ANG analyte on the analytical column (C2). In our preliminary study using the analytical scale (100×4.6 mm I.D.) column for C1, poor separation of the analyte on C2 column was obtained due to the overlap of many contaminated NDA-derivatives. Cosmosil 5Ph or Cosmosil 5C_{18}-ARII guard column (5 μm, 10×4.6 mm I.D.) was connected in front of the C1 column. The C2 column was Cosmosil 5C_{18}-ARII (5 μm, 250×4.6 mm I.D.). Both the columns (C1 and C2) were operated at 30°C. The mobile phase for C1 (MP1) was acetonitrile (CH_3CN)–water mixture containing 0.1% trifluoroacetic acid (TFA). The following linear gradient system of MP1 was used: 40–60% CH_3CN within 60 min for ANG (3–4) and ANG (5–8) assays, 20–40% CH_3CN within 40 min for ANG I and ANG II assays. The mobile phase for C2 (MP2) was the CH_3CN–water–0.1% TFA containing 5 m*M* SOS. The following system of MP2 was used for C2 to achieve complete separation of each ANG: 60% CH_3CN for ANG (3–4) assay, 55% CH_3CN for ANG (5–8) assay, 30–40% CH_3CN within 25 min for ANG I and ANG II assays. The flow-rates of MP1 and MP2 were 0.4 ml/min for both columns.

The sample (50 μl) was injected on C1. The retention times of derivatized ANGs with NDA on the C1 were checked by a Shimadzu SPD-10AV UV detector at 220 nm prior to the column switching. Each ANG solution for determining their retention time on C1 was prepared at a concentration of 0.5 μ*M* individually, and then subjected to the NDA-derivatization described in Section 2.5. The retention times of NDA-ANG I, II, (5–8), and (3–4) on C1 were 55.5±0.51, 54.5±0.42, 63.0±0.41 and 66.5±0.62 min, respectively. As the retention time slightly varied over the repeated runs of the column, the heart-cut of the eluate was selected from 1 min before to 2 min after the retention time of each ANG (Table 1). The heart-cut of the eluate was then transferred to C2 by manual valve operation. The analyte separated on C2 was determined by the fluorescence detector with excitation at 420 nm and emission at 490 nm.

2.4. Human plasma pre-treatment

Subjects of the study were five healthy male volunteers, 24–34 years of age (mean, 28.6 years). Venous blood samples were taken after 30-min in a supine posture [9]. Each blood sample was drawn into chilled vacutainer tubes containing EDTA-2Na (Terumo, Tokyo, Japan). After immediate centrifugation at 1500 *g* for 15 min (4°C), the plasma was ultrafiltrated through Millipore Molucut L (M.W. <5000). Specific adsorption of the ANGs on this filter during ultrafiltration would be negligible because this membrane is a cellulose having little absorptivity, and its exclusion molecular weight of 5000 is much higher than ANGs (e.g. ANG I, 1296.5). In order to achieve complete and reproducible NDA derivatization of analyte ANG, removal of a large number of other endogenous compounds present in the ultrafiltrated plasma sample (250 μl) was performed on the reversed HPLC system (Shimadzu LC-9A instrument; column, Cosmosil 5C_{18}-AR II, 250×4.6 mm I.D., 5 μm, Nacalai Tesque), eluted with a linear CH_3CN gradient (10–25%, 150 min) in 0.1% TFA at a flow-rate of 0.4 ml/min. Other rapid preliminary clean-up methods by using disposable ODS cartridge or ultrafiltration with M.W. <1000 was not sufficient for the removal

of interfering endogenous compounds to react with NDA. The zone of the analyte retention under this HPLC condition was collected to dryness, and was then subjected to the NDA derivatization. The retention time of each ANG on this HPLC system was 104.5 min for ANG I, 99.0 min for ANG II, 91.0 min for ANG (5–8), and 40.0 min for ANG (3–4).

2.5. Derivatization of ANG with NDA

The derivatization of ANG with NDA was performed according to our previous report [5]. Briefly, the plasma sample after clean-up treatment or ANG standard for calibration was prepared in 50 ml of 20 m*M* borate buffer (pH 9.5). A 10-μl aliquot of 10 m*M* sodium cyanide solution in the borate buffer and 50 μl of 0.1 m*M* NDA solution in methanol were added to 50 μl of sample or standard solution. After reaction for 60 min at ambient temperature, an aliquate (50 μl) of each NDA derivatized ANG sample was directly injected into the column-switching HPLC system with fluorescence detection described above.

2.6. Calibration

For calculating the calibration curve, four separate replicates of each five different concentrations of ANG in 20 m*M* borate buffer (pH 9.5) were performed in the concentration range of 0–1000 fmol/ml for ANG I, 0–250 fmol/ml for ANG II, 0–2000 fmol/ml for ANG (5–8) and ANG (3–4). The concentration of ANGs in human plasma was calculated from the equation for the linear regression ($y=a+bx$, where y is the concentration in fmol/ml in plasma and x is the peak height [μV s] of the analyte ANG).

3. Results and discussion

3.1. Chromatography

As many kinds of ANGs are metabolized in plasma [10,11], selective chromatography of a desired ANG from them and/or other endogenous compounds was required. Prior to the fluorimetric column-switching HPLC, the pre-extraction of a desired ANG fraction by the conventional reversed HPLC was primarily employed to avoid the lowering of fluorescence intensity by contaminations in plasma. Nevertheless, large interfering peaks were observed at the retention time of NDA-derivatized ANG by the only C1 column analysis as shown in Fig. 2A. As Montigny et al. [12] suggested, NDA, which reacts with primary amino group, were liable to produce a lot of fluorescence derivatives, though it had a high sensitivity, e.g. for NDA-amino acid, the detection limit was >200 fmol/injection volume. Thus, the application of column-switching HPLC method for determining a trace of desired ANG in plasma would be effective in achieving a selective detection and good separation. The column-switching technique has been recently carried out by combining HPLC, many of which have been established to measure exogenous compounds such as aspirin [8] and dimiracetam [6] spiked in plasma or serum. However, there has been no application to determine endogenous ANGs in human plasma by using this technique. Most conventional determination of the typical endogenous ANGs, in particular ANG I, II or III is the RIA or ELISA method [4], but there has been no expansion of the method to determine the smaller ANG metabolites. The first attempt to develop the column-switching HPLC conditions in this study was done for ANG (3–4). After the heart-cutting of the NDA-derivatized ANG (3–4) eluate on the C1 column, the zone was separated by the ion-pair chromatography with 5 m*M* SOS on the analytical C2 (Cosmosil $5C_{18}$-AR II) column. As shown in Fig. 2B, complete separation was achieved for the smallest ANG (3–4). Although data were not shown, when the MP2 without SOS was used, poor separation of the analyte was observed on the fluorescence detection. Yamaguchi et al. [13] reported that the column-switching technique combined with ion-pair chromatography was sufficient for the determination of amines due to the ion-pair formation. Thus, we applied ion-pair chromatography to the determination of all of the analyte ANGs (ANG I, II, (5–8), and (3–4)). The elution conditions of MP2 varied with ANGs as summarized in Table 1. The heart-cutting time of each ANG for 3 min was decided on the basis of the retention time of 500 nmol/ml standard ANG on the C1 column at 30°C with the UV-detection at 220 nm: NDA-ANG I,

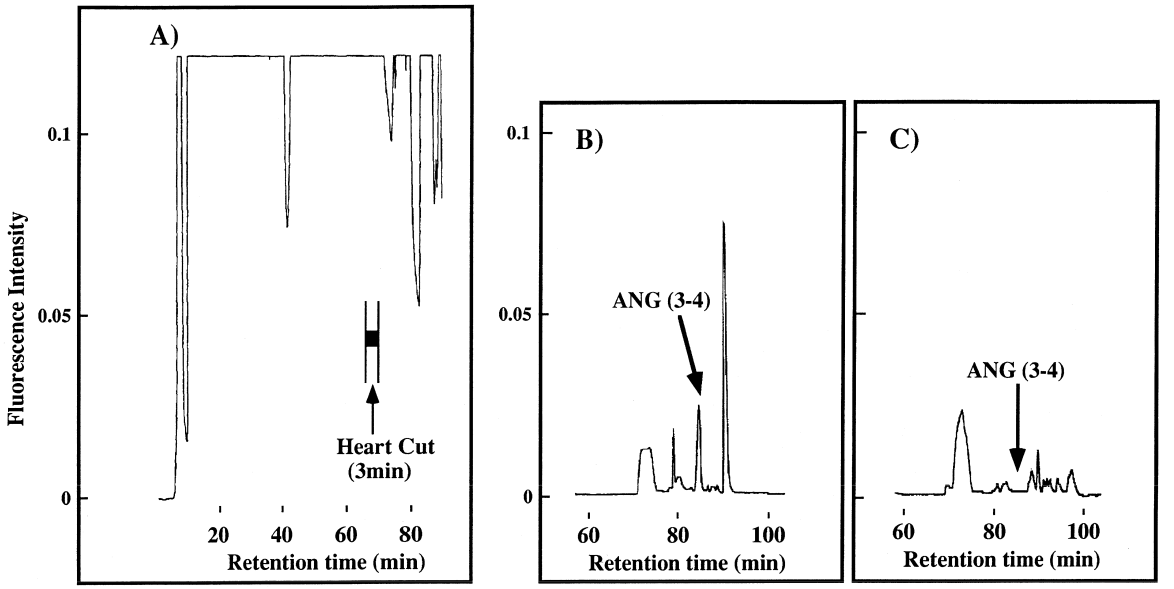

Fig. 2. Application of the column-switching method to detect endogenous ANG (3–4). (A) Before column-switching; (B) after column-switching of plasma sample; (C) after column-switching of blank plasma sample.

55.5±0.51 min; NDA-ANG II, 54.5±0.42 min; NDA-ANG (5–8), 63.0±0.41 min; NDA-ANG (3–4), 66.5±0.62 min. The longer time up to 6 or 9 min brought about poor separation due to the appearance of interfering peaks. Fig. 3 shows the chromatograms of other ANGs in human plasma after column-switching. The difference in the profiles for ANG I and ANG II would be caused by their different zone of heart-cutting fraction on the C1 column; namely the retention range of 54.5–57.5 min of NDA-ANG I

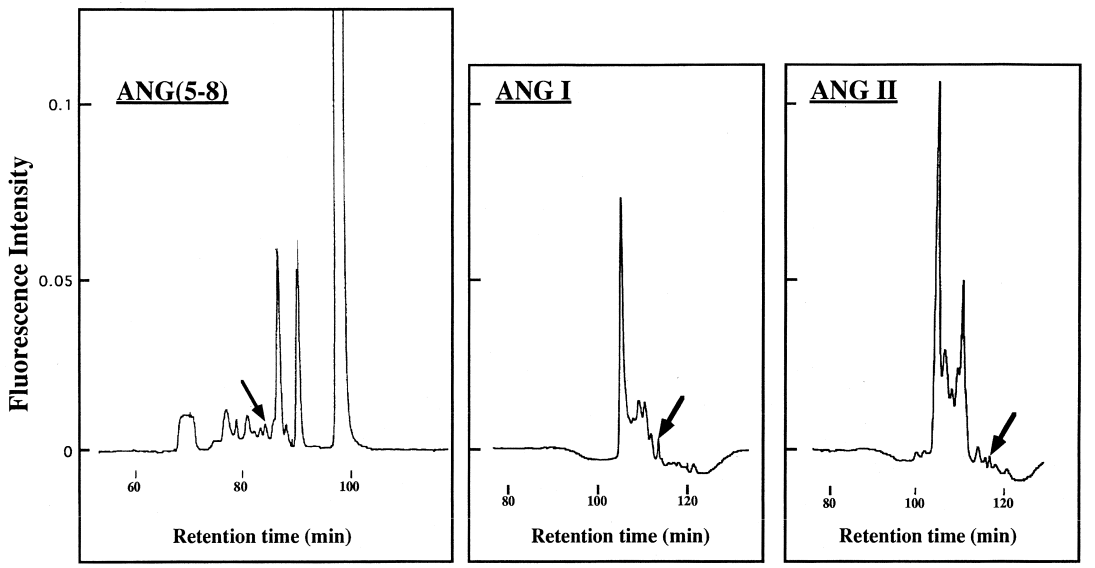

Fig. 3. Typical chromatograms of ANG I, II and (5–8) in human plasma.

on C1 column was subjected to C2 column separation, whereas the retention range of 53.5–56.5 min of NDA-ANG I on C1 was subjected to C2 separation. As shown in Fig. 2C, no interfering peak in blank plasma was observed at the retention time of ANG (3–4). Blank plasma was prepared by mixing 1 ml of ultrafiltrated human plasma with 5 mg of Cosmosil $5C_{18}$-AR II ODS resin at 4°C for 30 min. Although chromatograms were not shown, the high selectivity of each ANG analyte from other endogenous compounds was also observed at their retention time under proposed MP2 conditions. Also, shorter analysis time, within 120 min for all analytes, was achieved by using this column-switching system than that (<400 min) by our proposed conventional HPLC method [5]. Further attempts to shorten the analysis time, e.g. extraction of one sample on C1 during simultaneous separation of another sample on C2 would be impossible for the present HPLC method due to the restriction of the MP2 system in linear-gradient mode.

3.2. Linearity

Under the conditions described, linear calibration graphs were obtained for each ANG analyte over their fixed concentration range. The equation for the regression line was as follows: ANG I, $y=15.28+1.71x$ (correlation coefficient, $r=0.9995$); ANG II, $y=-0.19+0.92x$ ($r=0.9995$); ANG (5–8), $y=-423.6+8.47x$ ($r=0.9951$); ANG (3–4), $y=-54.17+0.97x$ ($r=0.9928$).

3.3. Validation performance

The accuracy and reproducibility for ANG (3–4) in human plasma were determined by the analysis of plasma standard spiked with two different concentrations (50 and 100 fmol/ml) of ANG (3–4) on one assay of each three separate days. As shown in Table 2, the present method for determining ANG (3–4) was consistent with low within-run C.V. of less than 4.0% ($n=3$). The other ANGs also had a high reproducibility with C.V. values of 3.6, 3.4 and 2.6% from three runs for ANG I, II, and (5–8), respectively. The recoveries of ANGs from spiked (100 fmol/ml) plasma samples were 93.0±3.3, 95.0±5.6, 97.5±4.9 and 92.4±2.4% for ANG I, ANG II, ANG (5–8) and ANG (3–4), respectively. The quantitative limit of each analyte was 18, 6, 17 and 21 fmol/ml for ANG I, II, (5–8) and (3–4), respectively, while their detection limits were estimated to be 1.9, 1.5, 1.6 and 2.8 fmol/ml for ANG I, II, (5–8) and (3–4), respectively, (signal-to-noise ratio, >2).

3.4. Application for plasma sample

The present column-switching HPLC method was applied to the determination of endogenous ANGs in human plasma from five normotensive subjects (Table 3). The reliability of this method was supported by the agreement of the range of ANG II plasma level by the present method (25–47 fmol/ml in plasma) with that by the RIA method (28–52 fmol/ml in plasma).

4. Conclusion

A new fluorimetric column-switching HPLC method has been developed for the determination of endogenous ANGs (ANG I, II, (5–8) and (3–4)) in human plasma. Only 250 μl of plasma is required for the assay, and even the smallest ANG (3–4) could be determined within 120 min by derivatizing it with NDA. The quantitative limit of ANG (3–4) was 21 fmol/ml in plasma. In addition, the method employed only one pre-extraction of the analyte ANG

Table 2
Between-day variability for the assay of ANG (3–4)

Added concentration (fmol/ml)	Measured concentration (fmol/ml)			C.V. (%)	Accuracy (%)
	Day 1	Day 2	Day 3		
0	350	368	379	4.00	–
50	406	425	438	3.80	103
100	479	486	477	0.98	105

Table 3
Concentration of angiotensins in normotensive human plasma obtained by the column-switching HPLC method

Subject (age)	Systolic/diastolic blood pressure (mmHg)	Concentration (fmol/ml in plasma)				
		Column switching method				RIA method
		ANG I	ANG II	ANG (5–8)	ANG (3–4)	ANG II
A (34)	117/73	238	25	145	350	28
B (29)	121/76	208	44	169	244	29
C (24)	124/64	218	35	189	330	52
D (32)	123/71	213	44	203	328	47
E (24)	114/66	276	47	175	239	44

by the conventional reversed HPLC from plasma to remove a large number of other endogenous compounds capable of interfering NDA fluorescence derivatization. The high reproducibility and good agreement of the range of ANG II plasma level between the present and the RIA methods indicate that this method may be applicable for investigating the metabolic behavior of ANGs as well as for clinical assay.

Acknowledgements

The authors thank Mr Yoshiaki Kasubuchi for his technical assistance.

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