Journal of Chromatography, 490 (1989) 43–51
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4673

DETERMINATION OF RENIN ACTIVITY IN HUMAN PLASMA BY COLUMN-SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

TAKASHI MIYAZAKI

Department of Quality Control, Yoshitomi Pharmaceutical Industries Ltd , Yoshitomi-chou, Chikujou-gun, Fukuoka 871 (Japan)

and

MASAAKI KAI and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

(First received October 19th, 1988; revised manuscript received January 4th, 1989)

SUMMARY

A column-switching high-performance liquid chromatographic (HPLC) method with fluorescence detection is described for the determination of the renin activity in human plasma. The method is based on the quantification of the enzymatically produced angiotensin I. Angiotensin I liberated from a synthetic substrate (tridecapeptide of human angiotensinogen) and [Val^5]-angiotensin I as an internal standard are converted into fluorescent derivatives by reaction with benzoin. The derivatives are separated from various interfering substances by column-switching HPLC using three reversed-phase columns. The limit of detection (signal-to-noise ratio = 3) of the renin activity is 2.7 pmol of angiotensin I formed per h per ml of plasma, which corresponds to approximately 820 fmol of angiotensin I injected. The column-switching method in combination with pre-column derivatization for the fluorimetric detection permits the sensitive and selective determination of the enzymatically formed angiotensin I. Hence low activities of renin in normal human plasma are readily measured.

INTRODUCTION

The renin–angiotensin (ANG) system plays an important role in the regulation of blood pressure [1]. Renin (EC 3.4.99.19) is the rate-limiting enzyme in this system and it mediates the liberation of ANG I (Asp-Arg-Val-Tyr-Ile-

0378-4347/89/$03.50 © 1989 Elsevier Science Publishers B.V.

His-Pro-Phe-His-Leu) from the substrate of endogenous angiotensinogen [1]. The enzyme activity of renin in normal human plasma is generally very low.

Current methods for the determination of plasma renin activity are based on radioimmunoassay (RIA) [2,3] or enzyme immunoassay [3] of ANG I generated from the endogenous angiotensinogen. However, the concentration of the angiotensinogen in human plasma is insufficient for zero-order kinetic reaction with the endogenous renin [4]. Thus, the renin activity is affected by the angiotensinogen concentration in the sample. Therefore, the activity values obtained by these methods do not always reveal the concentration of the endogenous renin [4,5].

Synthetic renin substrates have also been used for renin determination. The synthetic substrates use a tridecapeptide (TRDP; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His) [6] and a tetradecapeptide (TEDP; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) [7], which correspond to the thirtheen amino terminal residues of human angiotensinogen and the fourteen amino terminal residues of horse angiotensinogen, respectively. The methods using these substrates are based on spectrofluorimetry [8,9] and high-performance liquid chromatography (HPLC) coupled with ultraviolet detection [10]. However, these methods are insufficiently sensitive for the determination of the renin activity in normal human plasma.

We previously developed a pre-column fluorescence derivatization method for the sensitive HPLC determination of synthetic ANGs at the femtomole level, using benzoin as a fluorogenic reagent [11]. This reagent reacts selectively with the guanidino moiety of compounds and yields highly fluorescent derivatives, viz., 2-substituted amino-4,5-diphenylimidazoles [12,13]. Therefore, ANGs that contain an arginyl residue can be converted into fluorescent compounds by the reaction [11,14].

Our present objective was to develop a column-switching HPLC method involving the pre-column fluorescence derivatization of ANGs with benzoin for the sensitive determination of the renin activity in human plasma. TRDP as a renin substrate and [Val^5]-ANG I as an internal standard were employed.

EXPERIMENTAL

Chemicals and samples

The following synthetic peptides were obtained from the Protein Research Foundation (Osaka, Japan): ANG I, ANG II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), ANG III (Arg-Val-Tyr-Ile-His-Pro-Phe), [Val^5]-ANG 1 and TRDP. Synthetic TEDP was purchased from Peninsula Labs. (Belmont, CA, U.S.A.). Water was deionized and then distilled before use. Tris(hydroxymethyl)aminomethane (Tris; Wako, Osaka, Japan) was recrystallized from methanol–water (3:2, v/v) to remove fluorescent impurities. Other chemicals were of the highest purity available and were used as received.

The synthetic peptides were dissolved in water and stored at −80°C. McIlvaine buffer (pH 7.4) was prepared by mixing 0.4 *M* citric acid and 0.8 *M* disodium hydrogenphosphate. The reagent solutions used for the fluorescence derivatization were prepared as described previously [13].

A renin assay kit composed of antibody-coated tubes, ^{125}I-labelled ANG I (2.0 μCi) and phenylmethylsulphonyl fluoride, an angiotensinase inhibitor, was purchased from Green Cross (Osaka, Japan).

Blood specimens were from healthy volunteers. Plasma of the blood contained disodium EDTA (3 mg/ml).

Apparatus

The column-switching HPLC system (Fig. 1) consisted of a Hitachi 655 high-pressure pump, a Tosoh CCPM dual pump, a Tosoh PT-8000 autovalve, a Tosoh AS-48 autosampler (500-μl injection volume) and a Shimadzu RF-530 spectrofluorimeter equipped with a 12-μl flow cell. Two short columns of Nucleosil $7C_8$ (column I; 35 mm×4.6 mm I.D.; particle size 7 μm) (Macherey-Nagel, Düren, F.R.G.) and TSKgel ODS-80 TM (column II; 10 mm×4 mm I.D.; particle size 5 μm) (Tosoh, Tokyo, Japan) and an analytical column of TSKgel ODS-80 TM (column III; 250 mm×4.6 mm I.D.; particle size 5 μm) were used.

An Aloka PS-9 well-type scintillation counter and an Aloka TDC-601 scaler were used for the measurement of radioactivity.

HPLC assay

Enzyme reaction and deproteinization. A mixture of 50 μl of plasma, 50 μl of the McIlvaine buffer (pH 7.4), 20 μl of ethanol–water (1:1, v/v) containing 28.9 m*M* 8-hydroxyquinoline and 13.6 m*M* dimercaprol and 50 μl of 100 m*M* TRDP was incubated at 37°C for 16 h. The mixture was then cooled in ice–

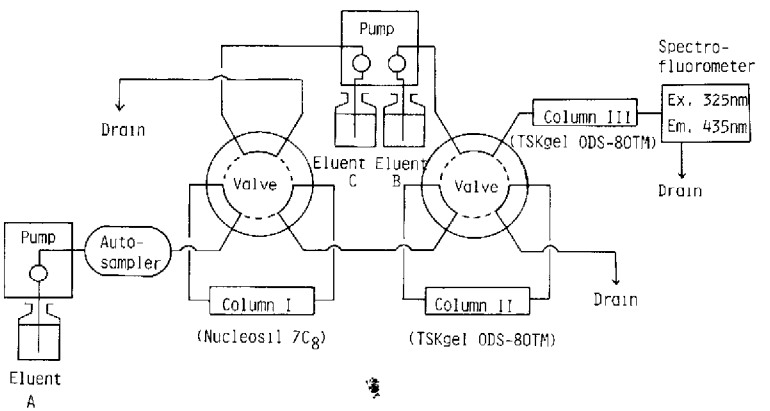

Fig. 1. Flow diagram of the column-switching HPLC system.

water, to which 50 μl of 200 n*M* [Val^5]-ANG I and 100 μl of water (or 10–500 n*M* ANG I) were added. The mixture was quickly ultrafiltered through a Tosoh Air Press 30 membrane, which excludes substances of molecular mass greater than ca. 30 000. For the enzyme blank, the same procedure was carried out but without incubation.

Fluorescence derivatization. A 200-μl volume of the ultrafiltrate was placed in a test-tube, to which were added 100 μl of 5 m*M* benzoin (in methylcellosolve), 100 μl of an aqueous solution containing 0.1 *M* 2-mercaptoethanol and 0.2 *M* sodium sulphite and 200 μl of 0.8 *M* potassium hydroxide. The mixture was then heated in a boiling water-bath for 90 s. After the reaction, a 200-μl aliquot of an acidic solution containing 0.5 *M* Tris–hydrochloric acid buffer (pH 8.0) and 0.8 *M* hydrochloric acid was added to adjust the pH of the final reaction mixture to ca. 8.0.

HPLC. A 500-μl aliquot of the derivatization mixture was automatically injected into the chromatograph. The fluorescent derivatives of ANG I and [Val^5]-ANG I were first passed through column I (Nucleosil 7C$_8$) and then retained on column II (TSKgel ODS-80 TM) between 0 and 9.0 min after the sample injection while a mobile phase (eluent A) of acetonitrile–0.2 *M* phosphate buffer (pH 8.1)–water (25:15:60, v/v) was pumped through both columns. At 12.1 min after the injection, the effluent from column II was switched to column III (TSKgel ODS-80 TM). The derivatives of ANG I and [Val^5]-ANG I were then separated on column III by eluting with a mobile phase (eluent B) of acetonitrile–0.2 *M* phosphate buffer (pH 8.1)–water (35:15:50, v/v). During the separation, column I was washed with 50% aqueous acetonitrile (eluent C) to remove hydrophobic substances retained on the column. The column was then equilibrated with eluent A until the start of the next analysis. The above column-switching operation was performed automatically according to the time programme depicted in Table I. The flow-rates of the three eluents were all 0.6 ml/min. The column temperature was ambient (24 ± 4°C).

The fluorescence intensity in the effluent from column III was monitored at

TABLE I

TIME PROGRAMME FOR COLUMN-SWITCHING HPLC

Time after injection (min)	Eluent (A, B or C)		
	Column I	Column II	Column III
0– 9.0	A	A	B
9.1–12.0	C	A	B
12.1–14.0	C	B	B
14.1–start	A	A	B

435 nm (emission) and 325 nm (excitation). The peak height was used for the quantification of ANG I.

RIA assay

For the comparison of the present HPLC method with a general RIA method, the RIA assay using a renin assay kit was carried out. The renin assay mixture (pH 6.0, 560 μl) containing 500 μl of plasma sample was incubated at 37°C for 1.5 h. A 50-μl aliquot of the incubation mixture was placed in an antibody-coated tube, to which were added 400 μl of 40 m*M* phosphate buffer (pH 7.4) and 100 μl of [^{125}I]ANG I. The mixture was then incubated at 25°C for 24 h. The radioactivity of [^{125}I]ANG I bound to the tube was measured after the assay mixture in the tube had been removed by suction,

RESULTS AND DISCUSSION

Column-switching HPLC

Fig. 2A shows a chromatogram of a renin assay mixture of the enzyme blank spiked with [Val^5]-ANG I, ANG I, II and III and TRDP, obtained by column-switching HPLC with fluorescence detection. A single fluorescent derivative for each ANG was formed by the derivatization with benzoin, as reported previously [11]. The derivatives of ANG I and [Val^5]-ANG I were separated and

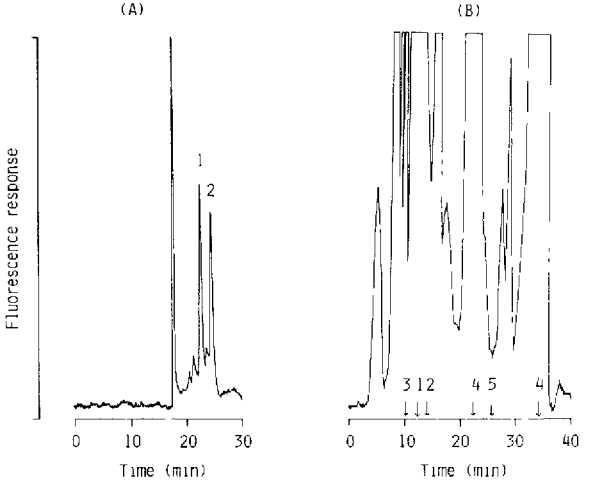

Fig. 2. Chromatograms of a renin assay mixture of the enzyme blank spiked with ANGs I, II and III, [Val^5]-ANG I (10 pmol each) and TRDP (5 nmol), obtained by (A) the proposed column-switching HPLC method and (B) common HPLC using only column III. Chromatogram B was obtained as follows: the same sample was injected directly into column III and separated by isocratic elution with eluent B. Peaks: 1 = [Val^5]-ANG I; 2 = ANG I. Arrows 1–5 indicate the retention times of [Val^5]-ANG I, ANG I, ANG II, TRDP and ANG III, respectively.

detected fluorimetrically within 27 min after the sample injection. The detection conditions are suitable for the derivatized ANGs, as previously described [11,14]. The derivatives of TRDP, ANG II and ANG III were not introduced to column III.

When the same sample as that for Fig. 2A was separated only with column III, the peaks of ANG I and [Val^5]-ANG I could not be separated from large peaks of the other substances in the assay mixture (Fig. 2B). Most of these interfering peaks were due to the plasma constituents. The derivatized TRDP gave two fluorescent peaks (No. 4 in Fig. 2B), probably because of the decomposition of TRDP during the bezoin reaction. In addition, a fluorescent compound, 8-hydroxyquinoline, added to the assay mixture as an inhibitor of angiotensinase and AMG-converting enzyme, was detected at a retention time of 29 min under the HPLC conditions in Fig. 2B. However, these fluorescent compounds were almost removed by switching the two short columns in the HPLC system, as shown in Fig. 2A.

The recoveries of ANG I and [Val^5]-ANG I (10 pmol each) added to the renin assay mixture were 75.8 ± 4.5 and $78.0 \pm 3.4\%$, respectively (mean $\pm$ S.D., $n = 5$). The limit of detection at a signal-to-noise ratio of 3 for ANG I was 820 fmol per injection volume (500 μl), corresponding to a renin activity of 2.7 pmol ANG I per h per ml of plasma.

Renin activity in plasma

For the assay of the renin activity in human plasma, the enzyme reaction was carried out at pH 7.4 in the presence of 8-hydroxyquinoline and dimercaprol as inhibitors of both angiotensinase and ANG-converting enzyme. The combination of the inhibitors was required to prevent the enzymatic degradation of ANG I formed during the incubation, as demonstrated by other workers [15,16].

Fig. 3 shows the chromatograms obtained with the renin assay mixture and its enzyme blank. In the blank chromatogram, a small peak was observed at the same retention time as that of ANG I. This peak may be ascribed to ANG I produced by non-enzymatic degradation of the substrate during the fluorescence derivatization, because the peak was formed even when only the substrate was derivatized with benzoin. The height of the peak was constant and did not vary with the individual plasma samples. For the calculation of the amount of ANG I formed enzymatically, therefore, the blank peak was subtracted from the peak of ANG I for test.

The calibration graph for ANG I, constructed by plotting the ratio of the peak height of the spiked ANG I to that of the internal standard versus the amount (0–50 pmol) of ANG I added to the assay tube, was linear. The correlation coefficient (r) of the graph ($n = 3$, each plot) was 0.998. The peak-height ratio of ANG I to the internal standard (10 pmol each per assay tube) was 0.77 ± 0.018 (mean $\pm$ S.D., $n = 5$).

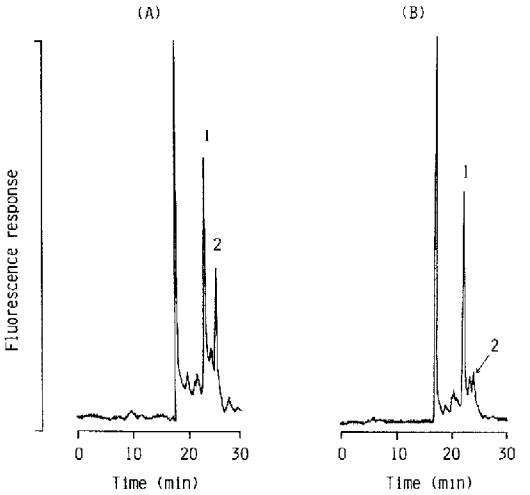

Fig. 3. Chromatograms showing (A) the renin reaction and (B) the enzyme blank. Peaks: 1 = [Val^5]-ANG I; 2 = ANG I.

TABLE II

EFFECT OF pH OF THE ASSAY MIXTURE ON THE FORMATION OF ANG I FROM SYNTHETIC RENIN SUBSTRATES

Substrate[a]	ANG I formed (pmol per assay tube)		
	pH 7.4	pH 6.0	pH 4.5
TRDP	8.4	11.8	58.6
TEDP	5.4	179.8	> 2500

[a]The concentrations of these substrates were the same as in the recommended procedure.

The endogenous ANG I present in the plasma sample could not be detected by the present HPLC method because its concentration in human plasma was ca. 40 p*M* or less [17].

The proposed HPLC method can use either TRDP (human angiotensinogen type) or TEDP (horse angiotensinogen type) as the renin substrate. Table II shows the amount of ANG I formed enzymatically from these substrates by the renin reaction at neutral and acidic pH. TEDP was more readily cleaved than TRDP at acidic pH. This result agrees with other reports [18,19] in which TEDP was degraded by acid proteases such as cathepsin D. The acid proteases generally show low activity at a neutral pH. At pH 7.4, the formation of ANG I from TRDP was greater than that from TEDP. In the recommended assay procedure, TRDP was therefore selected as the substrate.

The amount of ANG I formed enzymatically was proportional to the plasma

TABLE III

COMPARISON OF THE RENIN ACTIVITIES IN NORMAL HUMAN PLASMA, MEASURED BY THE PROPOSED HPLC METHOD AND BY RIA

Sample	Renin activity (pmol ANG I per h per ml plasma)			
	A group[a]		B group[a]	
	Present method	RIA method	Present method	RIA method
1	3.1	0.2	7.3	0.4
2	3.2	0.8	7.7	1.9
3	3.3	0.9	7.3	1.0
4	3.6	1.4	8.1	3.4
5	3.6	1.6	5.0	3.2
6	7.6	4.3	12.7	8.7
Mean	4.1	1.5	8.0	3.1
S.D.	1.6	1.3	2.3	2.7

[a]Blood was drawn in a sitting state for group A and an upright state after exercise (5 min jogging) for group B.

volume up to at least 80 μl, and also to the incubation time up to at least 20 h. The Michaelis constant (K_M) of the substrate was 15 μM for the plasma renin.

Table III shows a comparison of renin activities determined by the proposed HPLC method with those determined by a RIA method. The HPLC method gave higher values than the RIA method. The regression equation for the HPLC method (y) against the RIA method (x) is $y=1.78x+2.26$ ($r=0.972$) for the group A samples and $y=0.67x+5.95$ ($r=0.786$) for the group B samples. The values of the renin activity obtained by the HPLC method do not correlate satisfactorily with those obtained by the RIA method. In the HPLC method, a sufficient concentration of the substrate was used for the catalytic formation of ANG I with renin. Therefore, the renin activity measured by the HPLC method may reveal only the endogenous renin. Conversely, the RIA method using a renin assay kit is based on the immunochemical determination of ANG I generated from the endogenous angiotensinogen present in the plasma sample. Hence the renin activity measured by the RIA method may reflect the concentrations of both the endogenous renin and angiotensinogen. The synthetic substrate of TRDP could not be used for the RIA method because cross-reaction of the antibody with the substrate occurred.

The proposed method of pre-column fluorescence derivatization followed by column-switching HPLC is less sensitive (approximately 1/60) than the RIA method. However, this method can automatically and readily determine ANG I formed enzymatically. The method using a sufficient concentration of the synthetic renin substrate can determine the low activity of renin in normal

human plasma. The method should be useful for clinical and biological investigations of the renin–ANG system.

ACKNOWLEDGEMENT

The authors express their gratitude to Mr. Kozo Hikida of the Department of Quality Control, Yoshitomi Pharmaceutical Industries, for valuable discussions.

REFERENCES

1 M.R. Lee, Br. J. Clin. Pharmacol., 12 (1981) 605.
2 E. Haber, T. Koerner, L.B. Page, B. Kliman and A. Purnode, J. Clin. Endocrinol., 29 (1969) 1349.
3 W. Hubl, K. Haussig, F. Hofmann, M. Buchner, W. Rohde and G. Dorner, Endokrinologie, 77 (1981) 333.
4 S. Oparil, Clin. Chem., 22 (1976) 583.
5 T. Kokubu, K. Hiwada, H. Takahasi, K. Kimura and N. Yoshida, Jpn. Circ. J., 43 (1979) 15.
6 D.A. Tewksbury, R.A. Dart and J. Travis, Biochem. Biophys. Res. Commun., 99 (1981) 1311.
7 L.T. Skeggs, J.R. Kahn, K.E. Lentz and N.P. Shumway, J. Exp. Med., 106 (1957) 439.
8 A. Reinharz and M. Roth, Eur. J. Biochem., 7 (1969) 334.
9 K. Murakami, T. Ohsawa, S. Hirose, K. Takada and S. Sakakibara, Anal. Biochem., 110 (1981) 232.
10 L.B. Klickstein and B.U. Wintroub, Anal. Biochem., 120 (1982) 146.
11 M. Kai, T. Miyazaki, Y. Sakamoto and Y. Ohkura, J. Chromatogr., 322 (1985) 473.
12 M. Kai, M. Yamaguchi and Y. Ohkura, Anal. Chim. Acta, 120 (1980) 411.
13 M. Kai, T. Miura, K. Kohashi and Y. Ohkura, Chem. Pharm. Bull., 29 (1981) 1115.
14 Y. Sakamoto, T. Miyazaki, M. Kai and Y. Ohkura, J. Chromatogr., 380 (1986) 313.
15 S. Oparil, T.J. Koerner and E. Haber, J. Clin. Endocrinol. Metab., 39 (1974) 965.
16 A. Delorme, P.T. Guyene, P. Carvol and J. Menard, Am. J. Med., 61 (1976) 725.
17 M.D. Cain, K.J. Catt and J.P. Coghlan, Biochim. Biophys. Acta, 199 (1969) 322.
18 E. Hackenthal, R. Hackenthal and U. Hilgenfeldt, Biochim. Biophys. Acta, 522 (1978) 574.
19 F.E. Dorer, K.E. Lentz, J.R. Kahn, M. Levine and L.T. Skeggs, J. Biol. Chem., 253 (1978) 3140.