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DETERMINATION OF HIPPURIC ACID BY RP-HPLC USING TWO DIFFERENT ANALYTICAL COLUMNS – A SHORT REPORT

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Key words: hippuric acid, RP-HPLC, angiotensin I-converting enzyme (ACE), hypertension, bioactive peptides

Reversed-phase HPLC of liberated hippuric acid (HA) from an ACE assay in the presence of ACE inhibitory peptides derived from a crackling hydrolysate was conducted. The efficacies of two different analytical HPLC columns using identical mobile phases with an isocratic system were tested. Chromatograms revealed that the shorter C8 column (4.6 × 150 mm, 5 μm) was just as efficient as the longer C18 column (4.6 × 250 mm, 5 μm) in resolving liberated HA, but achieved this in a much shorter time (*i.e.*, 3.67 *cf* 12.52 min). The presence of the crackling hydrolysate exhibited ACE-inhibiting activity by retarding the liberation of HA from the substrate hippuryl-L-histydyl-L-leucine (HHL) in a dose-dependent manner, and did not interfere with the chromatography. Hence, a quick reliable analytical methodology is at hand for the *in vitro* examination of various "bioactive peptide concoctions" for possible use in the development of functional foods.

INTRODUCTION

The rennin-angiotensin system in the human body is central to the pathophysiology of a number of cardiovascular disorders including hypertension [Nicholls *et al.*, 1998]. In fact, hypertension is considered as one of the major risk factors associated with cardiovascular diseases (*e.g.* heart failure, stroke, coronary heart disease, myocardial infarction) [Kannel, 1996]. Although pharmaceutical options are available (*e.g.*, Captropril, Enalapril, Lisinopril), the search for diet-related preventive measures for hypertension is of interest within the scope of functional foods [Li *et al.,* 2004]. Some peptides obtained from the hydrolysis of food proteins have exhibited angiontensin I-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1 or kininase II)-inhibiting properties, thereby reducing blood pressure. ACE converts the inactive decapeptide (angiotensin I: Asp-Arg-Val-Tyr--Ile-His-Pro-Phe-His-Leu) to the active salt-retaining octapeptide (angiotensin II: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; a potent vasoconstrictor) by cleavage of the dipeptide (L-histidyl-L-leucine) from the carboxy terminus of angiotensin I [Ondetti & Cushman, 1982]. ACE also cleaves a dipeptide moiety from bradykinin, thereby inactivating this potent nonapeptide vasodilator [Wu *et al.*, 2006]. Inactivation or inhibition of ACE is quite effective in lowering blood pressure or preventing the pathogenesis of hypertension. Consequently, "bioactive peptides" arising from food proteins with antihypertensive efficacy show great promise as a therapeutic treatment for arterial hypertension.

Many ACE inhibitory peptides have been obtained from enzymatic hydrolysates of dairy proteins such as casein [Kim & Chung, 1999; Tauzin *et al*., 2002], whey protein [Pihlanto--Leppälä *et al.,* 2000; Hernández-Ledesma *et al.,* 2002], muscle protein [Arihara *et al.,* 2001; Nakashima *et al.,* 2002], fish protein [Curtis *et al*., 2002], chicken egg [Yoshii *et al.,* 2001], gelatin [Kim *et al.,* 2001], and blood plasma proteins [Wanasundara *et al.,* 2002]. As an example of one such product, short peptides like VPP and IPP from sour milk were administered in a human trial. Results showed a reduction in the blood pressure of hypertensive patients; a product containing VPP and IPP has since been commercialized [Hata *et al*., 1996; Seppo *et al*., 2003; FitzGerald *et al*., 2004]. Therefore, examining the efficacy of bioactive peptides using an *in vitro* ACE assay is of clinical, commercial and scientific interest. A number of assays have been developed using the tripeptide substrate hippuryl-L-histydyl-L-leucine (HHL) (Figure 1), whose composition matches the terminal two amino acids of angiotensin I [Cushman & Cheung, 1971; Cheung *et al.*, 1980; Meng & Oparil, 1996]. The procedure is based on the quantity of HA or L-histydyl-L-leucine liberated from the substrate at various enzyme concentrations (Figure 1). Thus, reliable and quick HPLC techniques to detect and quantify the substrate breakdown products are the cornerstone of ACE assays.

The aim of this study was to examine the efficacy of two different analytical HPLC columns by reversed-phase HPLC at measuring liberated HA from an ACE assay in the presence of ACE inhibitory peptides derived from a crackling hydrolysate.

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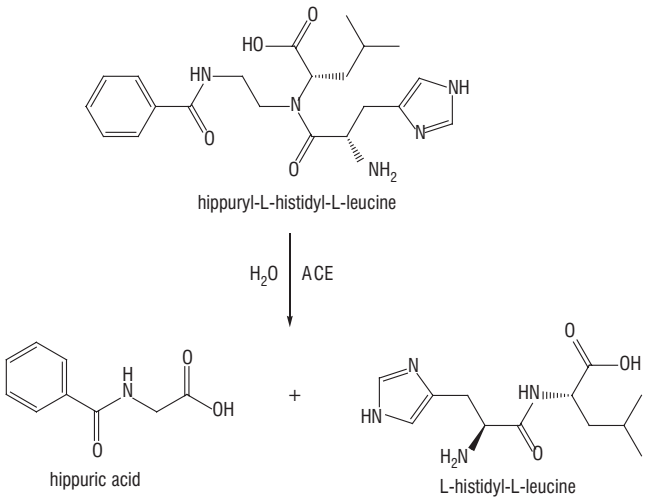

FIGURE 1. Hydrolysis of the substrate hippuryl-L-histydyl-L-leucine by the angiotensin I-converting enzyme (ACE) to hippuric acid and L-histydyl-L-leucine.

MATERIAL AND METHODS

Material. Hippuric acid (HA), hippuryl-L-histydyl-L-leucine (HHL), and angiotensin I-converting enzyme (ACE) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). The enzymatic hydrolysate from cracklings (degree of hydrolysis = 14.0% [Flaczyk *et al*., 2003]) was purchased from the Department of Human Nutrition and Food Technology, Agricultural University in Poznań (Poland). All other chemicals and solvents were acquired from VWR International (Suwanee, GA) and were of analytical reagent grade, unless specified otherwise.

Analytical procedure. Hippuric acid was determined from the test conditions described by Wanasundara *et al.* [2002]. Briefly, the assay was conducted in a Tris buffer (50 mmol/L, pH 8.3) containing 300 mmol/L NaCl. The same buffer was used to dilute the crackling hydrolysate, enzyme, and substrate. The initial assay volume consisted of 50 μL of the substrate (3.0 mmol/L), 50 μL of the enzyme (*i.e*., ACE from porcine kidney; Sigma) solution containing 1.25 mU of declared enzyme activity, and 50 μL of a hydrolysate sample added to successive tests in a dose-dependent fashion. All solutions were incubated for 30 min at 37°C in a water bath without mixing and then for an additional 30 min after mixing. Glacial acetic acid (150 μL) was added to stop the ACE activity. The reaction mixture so obtained was used as is in the determination of liberated HA resulting from ACE activity on the substrate.

An HPLC method was developed to separate and quantify free HA in the reaction mixture liberated due to the action of ACE on HHL. A reversed-phase C18 column (LUNA, 4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA) and a C8 column (ZORBAX Eclipse XDB-C8, 4.6 × 150 mm, 5 μm; Agilent Technologies, Wilmington, DE) were employed. The mobile phase comprised an isocratic system consisting of 12.5% (v/v) HPLC-grade acetonitrile in HPLC-grade water, and its pH was adjusted to 3.0 by adding glacial acetic acid. The injection volume used was 10 μL; flow rate of 1 mL/min. Elution of HA was detected at 228 nm. An Agilent 1200 Series HPLC system consisting of a quaternary pump with degasser, autosampler, UV-vis diode array detector with standard flow cell, and 2D ChemStation software (Agilent Technologies, Wilmington, DE) was used for the chromatography.

Preparation of standard hippuric acid (HA) solutions. An HA stock solution at a concentration of 2 mmol/L (*i.e.* 358.4 mg/L) in HPLC-grade water was prepared and then further diluted to give working solution concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/L. The standard solutions were injected in triplicate and the detector response was measured as peak area.

RESULTS AND DISCUSSION

Figure 2 shows representative chromatograms obtained using a reversed-phase C18 column after injection of the HA standard solution onto the HPLC column (A); an actu-

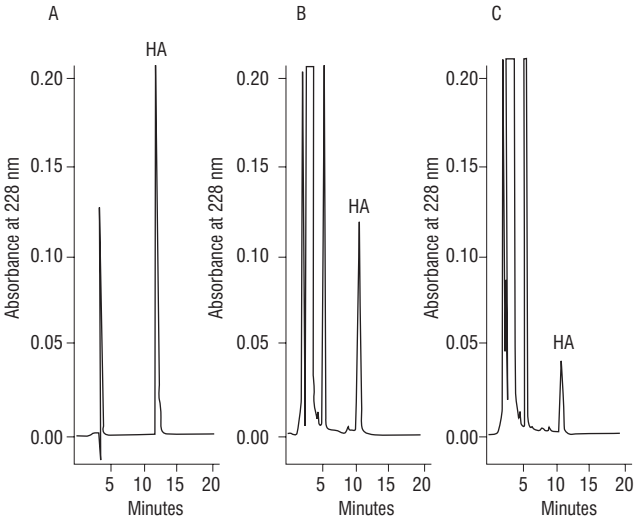

FIGURE 2. Representative chromatograms using a reversed-phase C18 column for a hippuric acid standard (A); a mixture containing the substrate hippuryl-L-histydyl-L-leucine and ACE (B); and mixture of hippuryl-L-histydyl-L-leucine, ACE, and crackling hydrolysate (C).

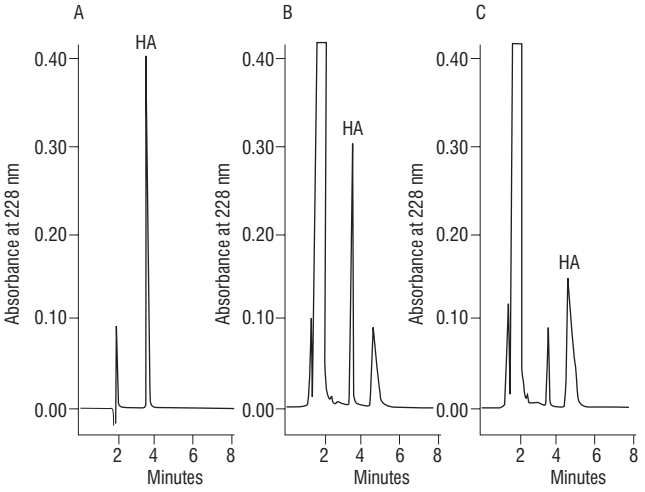

FIGURE 3. Representative chromatograms using a reversed-phase C8 column for a hippuric acid standard (A); a mixture containing the substrate hippuryl-L-histydyl-L-leucine and ACE (B); and mixture of hippuryl-L-histydyl-L-leucine, ACE, and crackling hydrolysate (C).

al assay mixture containing the HHL substrate and ACE (B); and a mixture of HLL, ACE, and crackling hydrolysate. Whereas, Figure 3 shows representative chromatograms for the same treatment depicted in Figure 2, but using the shorter reversed-phase C8 column. The conditions of HPLC analysis employed in this study afforded complete resolution (*i.e.* baseline separation) of HA with both columns. The retention time for the HA peak was 12.52 min with the LUNA column and 3.67 min with the ZORBAX column. The unreacted HHL substrate (*i.e.* no proteolytic cleavage by the ACE) eluted from the column together with added hydrolysate at retention times of 6.67 min (C18 column) and 4.93 min (C8 column). The presence of the crackling hydrolysate exhibited ACE-inhibiting activity by retarding the liberation of HA from the HHL substrate in a dose-dependent manner, and did not interfere with the chromatography. On the chromatograms there were also recorded peaks with retention times of 3.75 min (column C18) and 2.08 min (column C8), which most likely originated from L-histydyl-L-leucine. Noteworthy is the well-resolved efficient separations of HA and L-histydyl-L-leucine by both the reversed-phase C18 and C8 analytical columns using an isocratic system comprising a mobile phase of Tris buffer (50 mmol/L, pH 8.3) containing 300 mmol/L NaCl.

The retention times obtained in this study using two different columns were slightly longer than that reported by Mehanna & Dowling [1999], but gave a slightly better peak shape. Mehanna & Dowling [1999] reported a HA retention time of 2.77 min; they employed a Bondoclone 10 μ C18 column (150 × 3.9 mm) protected by Bondoclone 10 μ C18 guard column (30 × 3.9 mm). Furthermore, their flow rate of analysis was 2 mL/min compared with a flow rate of 1 mL/min used in this study.

The standard curves for both columns used in this work showed a linear response with a slope of 10,000,000 (column C18) and 6,000,000 (column C8) and correlation coefficients (R^2) of 0.9984 and 0.9998, respectively (Figure 4). The RSD values for 10 injections at a HA concentration of 0.3 mol/L were calculated to be 2.12% (C18 column) and 2.33% (column C8). Mehanna & Dowling [1999] reported a correlation coefficient of 0.999 for their HA standard curve, and RSD values for standard solutions ranged from 2.14% to 2.87%.

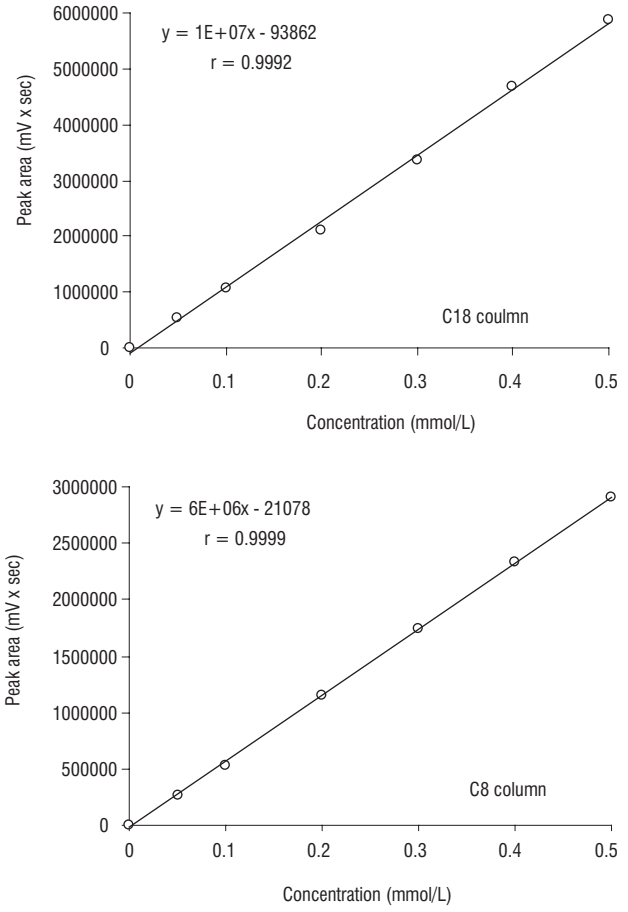

FIGURE 4. Calibration curves for hippuric acid determined using RP-HPLC.

CONCLUSIONS

Chromatograms showed that the shorter C8 column (4.6 × 150 mm, 5 μm) was just as efficient as the longer C18 column (4.6 × 250 mm, 5 μm) in resolving liberated HA from an ACE assay, but achieved this in a much shorter time (*i.e.* 3.67 *cf* 12.52 min). This analytical methodology can quickly determine HA concentrations for *in vitro* ACE assays, but also has applicability to measure the activity of bioactive peptides in the development of functional food formulations.

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OZNACZANIE KWASU HIPUROWEGO METODĄ RP-HPLC PRZY UŻYCIU DWÓCH RÓŻNYCH KOLUMN ANALITYCZNYCH – KRÓTKI KOMUNIKAT

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W pracy oznaczano kwas hipurowy (HA) metodą HPLC w układzie odwróconych faz. Posłużono się metodą stosowaną przy pomiarze aktywności peptydowych inhibitorów ACE, których źródłem w tym przypadku był hydrolizat białek skwarek wieprzowych. Do dwóch kolumn analitycznych zastosowano identyczną fazę ruchomą w układzie izokratycznym. Skuteczność rozdziału kwasu hipurowego przy użyciu krótszej kolumny C8 (4,6 × 150 mm, 5 μm) była podobna do tej, którą otrzymano stosując dłuższą kolumnę C18 (4,6 × 250 mm, 5 μm). Czas retencji dla HA w przypadku kolumny C8 był krótszy (3,67 min) niż przy zastosowaniu kolumny C18 (12,52 min). Użyty w badaniach hydrolizat wykazywał aktywność hamowania ACE poprzez hamowanie uwalniania HA z substratu HHL (hipurylo-L-histydylo-L-leucyna). Zawarte w hydrolizacie peptydy nie interferowały z HA. Zaprezentowana w pracy szybka metoda chromatograficzna może być stosowana w analizie *in vitro* biologicznie aktywnych peptydów w badaniach z obszaru żywności funkcjonalnej.