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Journal of Chromatography B, 783 (2003) 383–389

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of urinary 12(*S*)-hydroxyeicosatetraenoic acid by liquid chromatography–tandem mass spectrometry with column-switching technique: sex difference in healthy volunteers and patients with diabetes mellitus

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Received 25 March 2002; received in revised form 2 September 2002; accepted 5 September 2002

Abstract

We developed a determination method for human urinary 12-hydroxyeicosatetraenoic acid (12-HETE) using LC–MS–MS. This method, which includes simple extraction and detection in the SRM mode, allows precise and accurate determination of 12-HETE. There was a significant sex difference in urinary 12-HETE levels. Chiral analysis of 12-HETE using LC–MS–MS with column-switching technique revealed that the major enantiomer was 12(*S*)-HETE. Furthermore, the urinary level in patients with diabetes mellitus (DM) was analyzed. The present in vivo findings indicate that there could be difference in production of 12(*S*)-HETE between genders and 12(*S*)-HETE may play a role in the pathogenesis of DM.

Keywords: 12(*S*)-Hydroxyeicosatetraenoic acid

1. Introduction

Arachidonic acid metabolites in either the cyclooxygenase or lipoxygenase pathways have been suggested to play a key role in the pathogenesis of vascular disease in diabetes mellitus (DM). For example, thromboxane A_2 (TXA_2) and prostacyclin (PGI_2), a potent platelet activator and platelet antiaggregator, respectively, may be associated with the pathological states of DM. An increased TXA_2/PGI_2 ratio has been reported in diabetic humans [1] and mice [2], and its lack of balance may be connected with progress to complications such as diabetic retinopathy or nephropathy.

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00666-9

12-Hydroxyeicosatetraenoic acid (12-HETE) is mitogenic, proinflammatory and vasoconstrictive [3,4]. Previous studies have suggested that 12-HETE and its labile intermediate 12-hydroperoxyeicosatetraenoic acid (12-HPETE) may also contribute to the pathogenesis of diabetic cardiovascular disease, renal dysfunction and destruction of pancreatic β cells. For instance, activation of the 12-lipoxygenase (12-LO) pathway by high glucose and angiotensin II in porcine aortic vascular smooth muscle cells has been reported [5], suggesting that activation of the 12-LO may be a mechanism for accelerated vascular disease by hyperglycemia and angiotensin II in DM. 12-HETE may play some role in the renal vasoconstriction associated with ischemic renal injury, as dog renal arteries obtained from ischemic-injured kidneys can produce 12-HETE, but those from normal kidneys produce little 12-HETE [4]. A recent study demonstrated that elimination of the 12-LO gene prevented streptozotocin-induced diabetes by decreasing pancreatic β cell damage [6]. Thus, a potential role for enhanced 12-HETE production in the pathogenesis of DM has been suggested, but there are few in vivo studies about activation of the 12-LO pathway in humans. Since it is expected that biological fluids such as urine contain not only extremely small amounts of 12-HETE but also numerous interfering substances, a sensitive and reliable method is needed for 12-HETE determination.

For the purpose of evaluating the role of 12-LO activation in vivo, we first established a simple determination method for urinary 12-HETE using liquid chromatography–tandem mass spectrometry (LC–MS–MS). Then, we determined the urinary levels of 12-HETE in healthy volunteers and patients with DM. There was a significant sex difference in urinary 12-HETE levels in both healthy volunteers and patients with DM. Chiral analysis of 12-HETE using LC–MS–MS with column-switching technique revealed that the major enantiomer was 12(*S*)-HETE, indicating that human urinary 12-HETE was produced by the 12(*S*)-lipoxygenase pathway. Furthermore, increased production of 12(*S*)-HETE in female patients with DM was observed. The present finding suggests that 12(*S*)-HETE may play a role in the pathogenesis of DM.

2. Experimental

2.1. Subjects

Eighteen patients with DM (nine females and nine males) and 17 healthy volunteers (seven females and 10 males) were enrolled in the present study. The mean ages of patients with DM and healthy volunteers were 63 years ranging from 59 to 78 years (female, 68 years; male, 57 years) and 31 years ranging from 22 to 39 years (female, 27 years; male, 34 years), respectively. The treatment was kept unmodified throughout the study. Informed consent was obtained from all subjects.

2.2. Materials

12(*S*)-Hydroxyeicosatetraenoic acid {12(*S*)-HETE}, 12(*R*)-hydroxyeicosatetraenoic acid {12(*R*)-HETE} and [5,6,8,9,11,12,14,15-$^{2}H_{8}$]12(*S*)-hydroxyeicosatetraenoic acid {12(*R*)-HETE-d_8} were purchased from Cayman (Ann Arbor, MI, USA). Empore C_{18} HD disk cartridges (7 mm/3 ml) were purchased from 3M Industrial and Consumer Sector (St Paul, MN, USA). All solvents were HPLC grade obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.3. Determination of urinary 12-HETE

After collection, urine samples were stored at −80 °C until assayed.

12(*S*)-HETE-d_8 (2 ng) as an internal standard (I.S.) was added to urine (2 ml for female or 4 ml for male samples). After centrifugation at 4000×*g* for 10 min, the sample was adjusted to pH 7.5 with 0.5 N sodium hydroxide and was passed through an Empore C_{18} HD disk cartridge, preconditioned with methanol (1 ml) followed by distilled water (1 ml). The cartridge was washed with distilled water (2 ml) and *n*-hexane (2 ml). 12-HETE and I.S. were eluted with *n*-hexane–ethyl acetate (1:2, v/v) (1 ml). After evaporating the solvent, the residue was reconstituted in mobile phase (30 μl) and sonicated. Then, it was transferred to an autosampler vial, and 10 μl was injected.

The LC–MS–MS system was a Quattro II triple-quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface operated in the negative-ion mode. A Nanospace SI-1 HPLC system (Shiseido, Tokyo, Japan) was used.

Chromatography was performed on a C_{18} Capcell Pak UG120 (Shiseido, Tokyo, Japan; 1.5×150 mm, 3 μm) using isocratic elution with acetonitrile–water–acetic acid (60:40:0.02, v/v) with a flow-rate of 100 μl/min. The column was maintained at 40 °C. Column effluent was introduced into the mass spectrometer using a fused-silica capillary between 6.5 and 10.5 min after injection.

12-HETE was detected using LC–MS–MS in the selected reaction monitoring (SRM) mode. SRM was performed by monitoring the transitions between *m/z* 319 and *m/z* 179 for 12-HETE and between *m/z* 327 and *m/z* 184 for I.S. Collision gas (argon) pressure was 2.0×10^{-3} mBar. The capillary voltage was −3000 V and the source temperature was 150 °C. Cone voltage was −25 V with a collision energy of 15 eV. Peak areas and the calibration curve were obtained using the MassLynx program (Micromass, Manchester, UK).

2.4. Chiral analysis

Column-switching technique was used for chiral analysis of urinary 12-HETE. After extraction using Empore C_{18} HD disk cartridge as described above, 12-HETE was separated using a C_{18} Capcell Pak UG120 column (Shiseido, Tokyo, Japan; 1.0×75 mm, 3 μm) using isocratic elution with acetonitrile–water–acetic acid (50:50:0.02, v/v) at a flow-rate of 100 μl/min. The column was maintained at 40 °C. The fraction containing 12-HETE was introduced to Chiral CD-Ph column (Shiseido, Tokyo, Japan; 2.0×250 mm, 5 μm) using isocratic elution with methanol–water–acetic acid (65:35:0.02, v/v) at a flow-rate of 100 μl/min. Chiral CD-Ph column was maintained at 15 °C. Column effluent was introduced into the mass spectrometer using a fused-silica capillary between 40.0 and 65.0 min after injection. 12-HETE was detected using LC–MS–MS in the SRM mode as described above.

2.5. Creatinine contents

Creatinine in human urine was determined using a Creatinine test kit (Wako Pure Chemical Industries, Osaka, Japan). The findings are shown as corrected values.

2.6. Statistical analysis

Values were expressed as mean±standard deviation (SD). Comparison of urinary 12-HETE levels between genders and between patients with DM and healthy volunteers was made using the Mann–Whitney *U*-test.

3. Results

3.1. Mass and product ion mass spectra

Column-switching technique was used for chiral analysis of urinary 12-HETE as reported in other compounds [7–10]. 12-HETE was separated using a C_{18} Capcell Pak UG120 column and introduced to Chiral CD-Ph column. The effluent was introduced into the mass spectrometer between 40.0 and 65.0 min after injection and 12-HETE was detected using LC–MS–MS in the SRM mode.

The mass spectra of 12(*S*)-HETE and I.S. revealed a base peak at *m/z* 319 and 327, respectively, corresponding to deprotonated molecules ($[M-H]^-$). The product ion mass spectra of both the analyte and I.S. were obtained by choosing the molecular anions as the precursor ions (Fig. 1). The fragment ions observed at *m/z* 179 and 184, which are believed to be derived from the cleavage adjacent to the hydroxyl group [11], were most abundant at a collision energy of 15 eV.

3.2. Selected reaction monitoring (SRM)

The selected reaction monitoring (SRM) was performed by monitoring the transitions *m/z* 319 to *m/z* 179 for 12-HETE and *m/z* 327 to *m/z* 184 for I.S. Typical SRM chromatograms of 12-HETE and I.S. extracted from the urine of healthy human are shown in Fig. 2. When the mobile phase of acetoni-

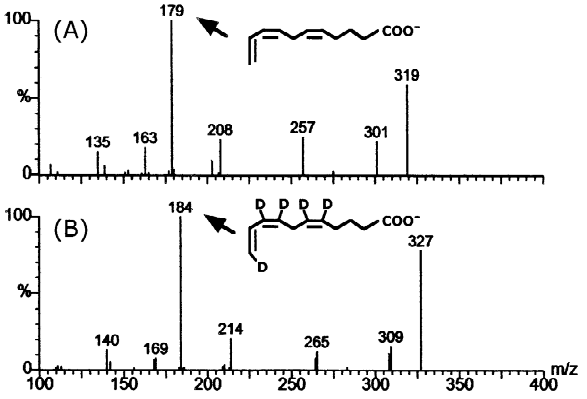

Fig. 1. Product ion mass spectra of 12-HETE (A) and I.S. (B). *Deprotonated molecules (m/z 319 for 12-HETE and m/z 327 for I.S.) were chosen as the precursor ions in the MS–MS experiments.

trile–water–acetic acid (60:40:0.02, v/v) was used at a flow-rate of 100 μl/min, 12-HETE and I.S. were detected in the chromatograms at 8.4–8.7 min. Interfering substances were eliminated during chromatographic separation and selective detection in the SRM mode.

3.3. Calibration graph

The calibration graph for 12-HETE was obtained by plotting the peak area ratio to I.S. against their weight ratio. Duplicate calibration standards were prepared. A good linear response over the range of 10 pg to 10 ng/tube was demonstrated ($r=0.999$).

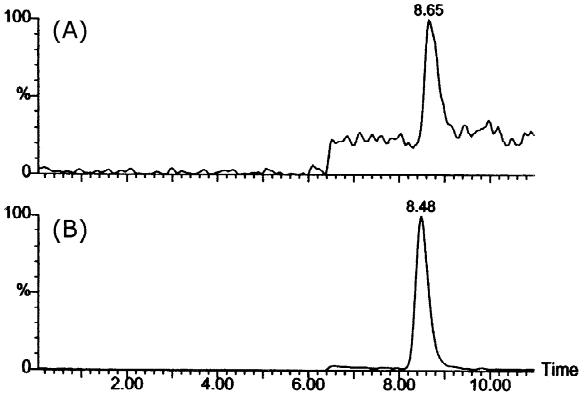

Fig. 2. SRM chromatograms of 12-HETE (A) and I.S. (B) extracted from urine of healthy human. *SRM was performed by monitoring the transition between m/z 319 and m/z 179 for 12-HETE and between m/z 327 and m/z 184 for I.S.

3.4. Validation of the 12-HETE determination method

We examined the accuracy and precision of the present method. Three urine samples containing low levels of 12-HETE (10.8 pg/2 ml; 4.4 pg/mg creatinine) and nine urine samples with 12-HETE added at concentrations of 10, 100 and 1000 pg/2 ml urine (8.4, 44.7 and 406.8 pg/mg creatinine, respectively; three samples each) were prepared. These samples were analyzed by LC–MS–MS in triplicate. The findings are shown in Table 1. The coefficient of variation was less than 9.0% and the accuracy of added 12-HETE ranged from 91.0 to 113.0% with a mean and SD of 99.8±4.3%.

3.5. Application to human urine

We first determined the urinary 12-HETE levels in healthy volunteers. The findings are shown in Fig. 3. The urinary 12-HETE levels in healthy females ($n=7$) and males ($n=10$) were 92.3±38 and 3.4±2 pg/mg creatinine, respectively. There was a significant sex difference ($P<0.005$) in urinary 12-HETE levels.

We also determined the urinary 12-HETE levels in patients with DM (Fig. 3). There was also a sex difference. The urinary level in female patients with DM was 410.8±352 pg/mg creatinine ($n=9$), which was significantly higher ($P<0.005$) than that in male patients (8.0±11 pg/mg creatinine ($n=9$)).

To specify the enzymatic origin of urinary 12-HETE, we analyzed the chirality of urinary 12-HETE in healthy volunteers and patients with DM using LC–MS–MS with column-switching technique. Maintenance of phenylcarbamate-β-cyclodextrin bonded chiral column at 15 °C made it possible to separate each 12-HETE enantiomers. Chiral analysis of 12-HETE revealed that the major enantiomer in human urine was 12(S)-HETE, indicating that human urinary 12-HETE was produced by the 12(S)-lipoxygenase pathway (Fig. 4). In this study, 12(R)-HETE was not detectable in all urine samples.

The urinary level in female patients with DM was significantly higher ($P<0.05$) than that in healthy

Table 1
Accuracy and precision for determination method of 12-HETE

Sample	Urinary levels (pg/2 ml)	C.V. (%)	12 HETE recovered		
			pg/2 ml	Accuracy (%)	Mean (%)
Non-spiked urine					
1	11.9, 10.1, 10.9	7.9			
2	10.1, 11.6, 10.5	6.8			
3	10.4, 10.5, 10.7	1.8			
	10.8	2.2			
Spiked urine					
+10 pg 1	21.9, 21.2, 22.1	5.2	11.1, 10.4, 11.3	111.0, 104.0, 113.0	109.3
2	20.1, 20.7, 21.1	3.9	9.3, 9.9, 10.3	93.0, 99.0, 103.0	98.3
3	19.9, 20.2, 20.0	4.1	9.1, 9.4, 9.2	91.0, 94.0, 92.0	92.3
	20.8	8.9	10.0		100.0
+100 pg 1	109.8, 112.6, 112.8	1.6	99.0, 101.8, 102.0	99.0, 101.8, 102.0	100.9
2	11.5, 108.8, 110.1	1.4	100.7, 98.0, 99.3	100.7, 98.0, 99.3	99.3
3	110.7, 109.5, 109.7	0.7	99.9, 98.7, 98.9	99.9, 98.7, 98.9	99.2
	110.6	1.0	99.8		99.8
+1000 pg 1	1004.8, 1001.9, 1001.8	0.2	994.0, 991.1, 991.0	99.4, 99.1, 99.1	99.2
2	1009.0, 1007.2, 1003.1	0.3	998.2, 9964, 992.3	99.8, 99.6, 99.2	99.5
3	1015.9, 1004.5, 1010.4	0.6	1005.1, 993.7, 999.6	100.5, 99.4, 100.0	100.0
	1006.5	0.4	995.7		99.6
Total (mean±SD)					99.8±4.3

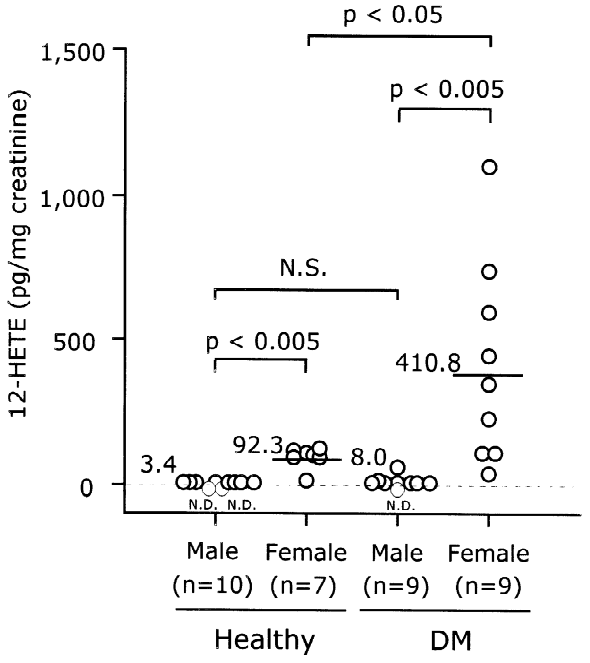

Fig. 3. Urinary 12-HETE levels in healthy volunteers and patients with DM. *N.S., not significant; N.D., not detectable.

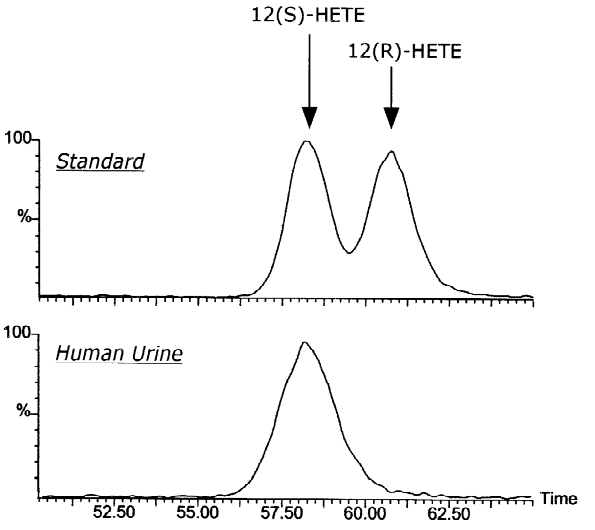

Fig. 4. Chiral analysis of 12-HETE in human urine using LC–MS–MS with column-switching technique. *12-HETE was extracted from urine using solid-phase extraction cartridge and injected to LC–MS–MS.

females, although no significant difference in 12-HETE levels between healthy males and male patients with DM was observed (Fig. 3).

4. Discussion

We developed a new determination method for urinary 12-HETE using LC–MS–MS. The method is characterized by its sensitivity, specificity, accuracy and precision to allow determination of low levels of 12-HETE in a complex biological sample such as plasma and urine. Furthermore, a simple and rapid extraction procedure for urinary 12-HETE makes it possible to treat a number of samples at the same time. We previously reported the similar determination method for urinary leukotriene E_4 using combinations of single extraction and LC–MS–MS [12].

12-HETE is produced by platelets [13], vascular smooth muscle cells [5], pancreatic islets [14], kidney [15] and other tissues or organs [16], but the origins of urinary 12-HETE have not been fully clarified. The in vivo metabolism of 12-HETE has been studied in rats [17] and rabbits [18] but the findings on excretion of 12-HETE into urine were different. Clouet et al. examined the uptake and degradation of tritium-labeled 12-HETE in the rat and no radioactivity was observed in the urine [17]. More recently, however, Westlund and co-workers demonstrated the significant urinary excretion of [^{14}C]12-HETE in the rabbit [18]. As the renal medulla [15], glomeruli and cortical tubules [19] can produce 12-HETE and 12-HETE may be involved in the pathogenesis of renal dysfunction [15], urinary 12-HETE levels determined in the present study appear to mainly reflect renal production.

The present findings indicate that there could be differences in production of 12-HETE between genders, but we have no information to explain the differences. However, arachidonic acid is mainly converted to 12-HETE during mensis and increased metabolism of lipoxygenase as well as cyclooxygenase products is apparently associated with menstrual pain [20]. Furthermore, a previous study suggested that 17β-estradiol, an estrogen, could directly stimulate 12-lipoxygenase activity in rat platelets [21]. In addition to these studies, chiral analysis in this study revealed that the major enantiomer was 12(*S*)-HETE. As formation of the 12(*S*)-enantiomer can be accounted for by the 12(*S*)-lipoxygenase while it is believed that 12(*R*)-HETE is produced by cytochrome P450 [22] or 12(*R*)-lipoxygenase pathways [23], our present data indicate that human urinary 12-HETE was produced by the 12(*S*)-lipoxygenase pathway. These findings may be of some help to explain our current findings about difference between genders. The present study presents the first findings showing a sex difference in 12-HETE production. Further studies are necessary to clarify the mechanisms responsible for the differences.

The urinary 12-HETE level in normal subjects and diabetics with normal renal function and those with microalbuminuria has been determined previously [24]. Increased urinary 12-HETE in patients with non-insulin-dependent diabetes mellitus (NIDDM) with or without microalbuminuria compared with those in normal subjects was observed. Those findings were partly consistent with the present findings of significantly higher 12-HETE levels only in urine of female patients with DM than those of female healthy volunteers, although this difference may be caused by the difference of age between two groups in part. Since increased 12-HETE production has been observed during very early states in DM without complications, vasculotoxic and vasoconstrictive production of 12-HETE may be involved in the progress of diabetic renal disease. However, since we could not get the samples of old healthy volunteers, the mean age of diabetic patients was higher than that of healthy volunteers in this study. Further investigation should be necessary to clarify the relationship between age and 12-HETE level.

5. Conclusion

We developed a determination method for urinary 12-HETE using LC–MS–MS and determined the urinary 12-HETE levels in healthy volunteers and patients with DM. In both healthy volunteers and patients with DM, female urinary 12-HETE levels were significantly higher than the levels in male urine. Urinary 12-HETE in human urine was the 12(*S*)-enantiomer, suggesting that its enzymatic

origin is 12(*S*)-lipoxygenase. Although no significant difference in 12-HETE levels between healthy males and male patients with DM was observed, the urinary level in female patients with DM was significantly higher than that in healthy females. The present findings are the first to show a sex difference in 12-HETE production and support previous findings that suggested activation of the 12-lipoxygenase pathway may play some role in the pathogenesis of DM.

Acknowledgements

This study was supported in part by grants from Uehara Memorial Foundation in Japan, the Health Sciences Research Grants for the Research on Pharmaceutical and Medical Safety from the Ministry of Health and Welfare of Japan, and Grants-in-Aid for the Research on Health Sciences Focusing on Drug Innovation from Japan Health Sciences Foundation.

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