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First observation of *N*-acetyl leucine and *N*-acetyl isoleucine in diabetic patient hair and quantitative analysis by UPLC–ESI–MS/MS

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ABSTRACT

Background: Type 2 diabetes patients (DP) have significantly higher plasma levels of valine, leucine, isoleucine and alanine than the controls. Specific amino acids may acutely and chronically regulate insulin secretion from the pancreatic β-cells. We recently identified a metabolic signature of *N*-acetyl leucine (Ac-Leu) that strongly predicts diabetes development in mice hair. The Ac-Leu appears to be a potential biomarker candidate related to diabetes. However, the determination of Ac-Leu in human hair has not been reported. We measured the Ac-Leu, and its structure is similar to *N*-acetyl isoleucine (Ac-Ile) in human hair by ultra-performance liquid chromatography (UPLC) with electrospray ionization tandem mass spectrometry (ESI–MS/MS). The developed method was applied to the determination of Ac-Leu and Ac-Ile in the hair of healthy volunteers (HV) and DP.
Methods: Ac-Leu, Ac-Ile and *N*-acetyl norleucine (Ac-Nle, IS) were extracted from human hair samples by a micropulverized extraction procedure, then separated on a C18 column by isocratic elution of acetonitrile–0.1% formic acid in water:0.1% formic acid (14:86, vol./vol.). MRM using the fragmentation transitions of *m/z* 174.1 → 86.1 in the positive ESI mode was performed to quantify the *N*-acetyl leucine, *N*-acetyl isoleucine and IS.
Results: Ac-Leu, Ac-Ile and Ac-Nle in the human hair samples were completely separated by isocratic elution of a 5.0 min duration wash program using a reversed-phase column, and sensitively detected by LC–MS/MS in the ESI^+ MRM mode. The amounts of Ac-Leu and Ac-Ile in the hairs of HV and DP were determined. When comparing the concentrations between DP and those from HV, a statistically significant correlation was observed for the Ac-Leu ($p < 0.001$) and Ac-Ile ($p < 0.01$).
Conclusions: The proposed method is useful for the determination of Ac-Leu and Ac-Ile in the hairs of DP and HV. Human hair may serve as a noninvasive biosample for the diagnosis of diabetes.

1. Introduction

Type 2 diabetes is the most common form of diabetes, and its prevalence is dramatically increasing in both developed and developing countries [1,2]. As an endocrine metabolic disease, diabetes would lead to the metabolic disorder of several kinds of endogenetic metabolites [3]. Therefore, early identification of individuals at risk is particularly important for delaying or preventing the onset of type 2 diabetes and decreasing the burden of the condition worldwide [4,5].

Recent studies have shown that the amino acid metabolism plays a potential key role in the pathogenesis of diabetes, and amino acid profiling is very helpful in diabetic risk assessment [3,6]. Higher concentrations of branched chain amino acids (Leu, Ile, and Val) and aromatic amino acids (Trp, Tyr and Phe) have emerged as predictors of the future development of diabetes [7–10]. In previous studies, we recently identified the *N*-acetyl leucine (Ac-Leu) metabolite to be a potential biomarker candidate related to diabetes. Metabolic signature of *N*-acetyl leucine (Ac-Leu) strongly predicts diabetes development in the mice hair [11]. These results proved that amino acids play essential roles in the energy metabolism as a cluster metabolite.

Urine and serum samples have been extensively investigated for the amino acid assay in biological specimens [3,8,12,13]. However, the inherent problems of serum and urine specimens, such as the fluctuation in its composition during the day and the hygienic practice during its collection and handling, prompted us to search for other types of noninvasive samples. Recently, the value of hair as a noninvasive biosample has been recognized in the biomedical field [14,15]. In contrast, the human hair is relatively clean and the sampling is easy and noninvasively collected and easily stored. Hair analysis provides one important means for

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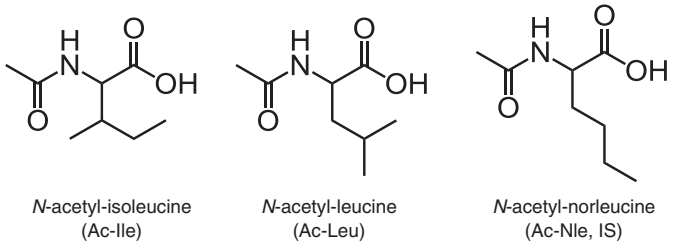

Fig. 1. Structures of *N*-acetyl isoleucine, *N*-acetyl leucine and *N*-acetyl norleucine.

determining the individual past history of long-term chemical exposures, because many substances have been detected in the hair [16–21]. Many studies concerning hair analysis have dealt with drugs of abuse, such as heroin, cocaine and amphetamines [22]. In recent years, however, interest in hair analysis has gradually shifted to other drug species, e.g., doping agents and therapeutic drugs [23,24]. Hair is a biological sample that is not considered to be important in the field of clinical testing, except in limited cases such as the detection of drug abuse [25–27]. Furthermore, hair samples contain long-term histories of diseases and medicines used for treatment as compared to other biological samples; it is a notable biological sample in that it compensates for the limitations of blood or urine samples. However, for analyzing hair samples, an appropriate extraction method is required for sample preparation and the method is usually time-consuming. Also, the concentrations of the target compounds obtained from the hair extracts are expected to be lower compared to those obtained from blood or urine samples; therefore, highly sensitive detection methods are required for hair analyses. According to recent reports, human hair may be used to obtain physiologic information, and may serve as the noninvasive biosample for the diagnosis of chronic disease. Basic compounds are efficiently incorporated into hair. Certain kinds of endogenous biogenic amino acids have been detected in human hair [28–30]. However, a method for the determination of Ac-Leu and Ac-Ile in human hair has not been reported.

2. Methods

2.1. Materials and reagents

The Ac-Leu and Ac-Ile were from Watanabe Chemical Industries, Co. Ac-Nle (Watanabe Chemical Industries) was used as the internal standard (IS). Formic acid (FA), hydrochloric acid (HCl), trifluoroacetic acid (TFA), sodium dodecylsulfate (SDS), methanol (CH_3OH), and acetonitrile (CH_3CN) were of special reagent grade (Wako Pure Chemicals). All other chemicals were of analytical reagent grade and were used without further purification. Deionized water and distilled water (H_2O) were used throughout the study (Aquarius pwu-200 automatic water distillation apparatus, Advantec).

2.2. UPLC–ESI–MS/MS conditions

The UPLC–ESI–MS/MS analysis was performed using a Xevo™ TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA) connected to an Acquity ultra-performance liquid chromatograph (UPLC I-class, Waters). An Acquity UPLC BEH C18 column (1.7 μm, 100 mm × 2.1 mm i.d.; Waters) was used at the flow rate of 0.35 ml/min and 40 °C. The mobile phase A consisted of 0.1% FA in water. Mobile phase B was 0.1% FA in acetonitrile and the total run time was 5.0 min. The isocratic steps were as follows: 0–5.0 min from 14% solvent B. The injection volume was 2 μl. The Ac-Leu, Ac-Ile and Ac-Nle were analyzed by UPLC–ESI–MS/MS in the positive-ion mode unless otherwise stated, and the multiple reaction monitoring mode (MRM) using a switching ionization mode. The detection conditions were a capillary voltage of 3.00 kV; sample cone voltage of 10 V; source temperature of 120 °C; desolvation gas flow of 1000 l/h; cone gas flow of 150 l/h; nebulizer gas flow of 7.0 l/h; collision gas flow of 0.15 ml/min; collision energy of 15 eV; collision cell exit potential of 5 V; and desolvation temperature of 500 °C. The analytical software (MassLynx, ver 4.1) was used for the system control and data processing.

2.3. Human hair samples

We obtained human hair sample 10.0 mg of the posterior part of the head from 12 healthy volunteers (age: 21–56 years; 3 men and 9 women, black hair) and 9 diabetic patients (age: 57–82 years; 2 men and 7 women, black hair) treated at the Fengxian Branch of Shanghai Sixth People's Hospital from January 2013 to September 2013. All patients provided written informed consent before entry into the study. The human hair samples were rinsed with 1 ml of 0.1% SDS for 1 min by ultrasonication. The procedure was repeated another two times. After rinsing, the SDS on the hair samples was removed by three washings with distilled water. The hairs were then dried in a desiccator

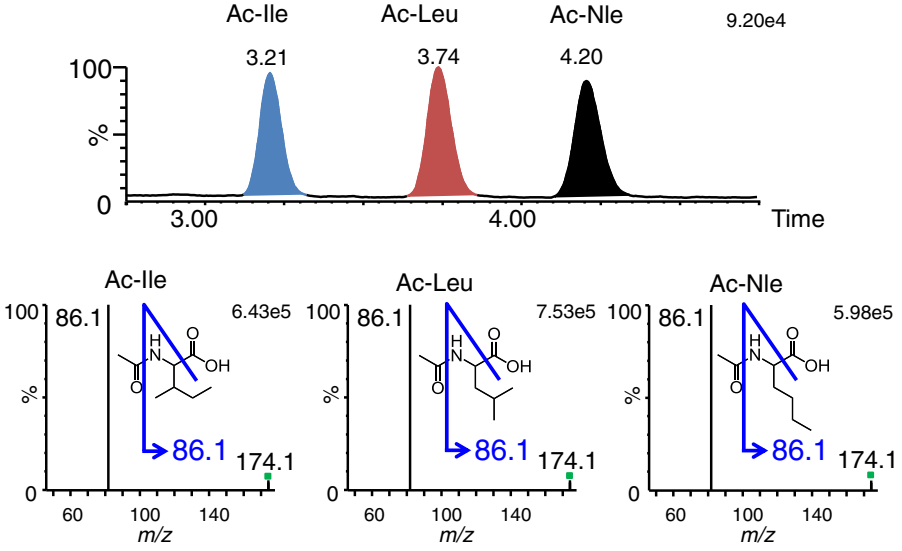

Fig. 2. MRM chromatograms and MS/MS spectrum of ion produced from Ac-Ile, Ac-Leu and Ac-Nle by UPLC–ESI–MS/MS. The MRM chromatograms were obtained from the monitoring at *m/z* 174.1 → *m/z* 86.1. The MS/MS spectra were recorded by the collisional activation of *m/z* 174.1 $[M + H]^+$.