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# Determination of acetone in saliva by reversed-phase liquid chromatography with fluorescence detection and the monitoring of diabetes mellitus patients with ketoacidosis

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A B S T R A C T

*Background:* In diabetes mellitus (DM) patients with ketoacidosis, ketone bodies, i.e., acetone, acetoacetic acid (AA) and β-hydroxybutyric acid (HA), are increased in the blood and urine. Acetone is also excreted by breathing due to the spontaneous decomposition of AA. Thus, the increase in acetone has been considered as one of the biomarkers for the diagnosis of DM. However, the determination of acetone in one's breath is not recommended because of the sample handling difficulty. We measured acetone in saliva by reversed-phase liquid chromatography (LC) with fluorescence (FL) detection. The proposed method was applied to the determination of acetone in the saliva of healthy volunteers and DM patients with and without ketoacidosis.
*Methods:* 3-Pentanone (I.S.) and DBD-H in acetonitrile were added to freshly collected saliva and reacted at room temperature for 20 min in the presence of trifluoroacetic acid. After the reaction, the solution was centrifuged at 10,000 ×*g* and 4 °C for 5 min. The supernatant was separated by reversed-phase LC and the FL detected at 550 nm (excitation at 460 nm).
*Results:* The concentrations of acetone in the DM patients with ketoacidosis were significantly higher than those of the normal subjects and DM patients without ketoacidosis. Furthermore, the total contents of the ketone bodies in the blood correlated with acetone in the saliva of the DM patients. The concentrations of acetone in the saliva of an emergency patient also correlated with the ketone bodies in the blood at each sampling time.
*Conclusion:* The proposed method using LC–FL seems to be useful for the determination of acetone in the saliva of DM patients with ketoacidosis. The method offers a new option for the diagnosis and monitoring of DM patients with ketoacidosis.

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## 1. Introduction

Diabetes mellitus (DM) is one of the diseases of interest due to its increasing worldwide prevalence, not only in European–American, but also in Asian countries [1,2]. The DM in humans is a multifactorial disorder based on environmental factors and genetic background. The main symptom of DM, one of the most common endocrine diseases, is a high blood sugar concentration because DM patients cannot fully use glucose, and relative or absolute insulin deficiency causes fat to metabolize instead of glucose for energy [3]. When a carbohydrate is very scarce, energy must be obtained from the β-oxidation of fatty acids in the liver, which can be converted into ketone bodies. The three endogenous ketone bodies are acetone, acetoacetic acid (AA) and β-hydroxybutyric acid (HA). The ketone bodies produced by the liver are peripherally used as an energy source when glucose is not readily available. The two main ketone bodies are AA and HA, while acetone is the third, and the least abundant ketone body. In the DM patients with ketoacidosis, a significant increase in the ketone bodies, i.e., acetone, AA and HA, is observed, and AA and HA affect the blood bicarbonate concentration and lower the pH [4].

Measurement of one or all of the ketone bodies in the blood is used as an indication of ketoacidosis, and the enzymatic oxidation of HA is performed in most cases [5,6]. Following the oxidation of HA, the resulting AA is then decarboxylated into acetone. By determining the sample with and without the addition of the enzymatic reactants, the combined concentration of acetone and AA can be subtracted from the total ketone concentration [7,8].

Acetone is a direct byproduct of the spontaneous decomposition of AA and is excretes during breathing, especially in ketoacidosis patients. Thus, an increase in acetone has gradually been considered as one of the biomarkers for the diagnosis of DM [9–12]. The analysis of acetone in the DM patients has been widely reported in blood and urine. The

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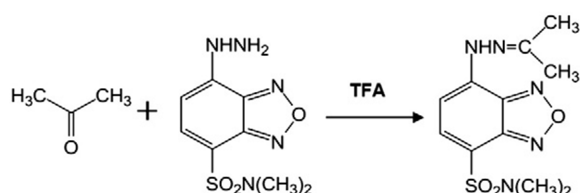


**Fig. 1.** Derivatization reaction of acetone with DBD-H.

breath analysis of acetone in the DM patients is a noninvasive technique and has been used as a tool for the diagnosis of diabetes [13]. The determination of acetone is essentially performed by various GC techniques such as head-space GC-MS [14,15] and GC-FID [16–21]. Although the GC technique is superior for the determination of volatile compounds such as acetone and alcohols [22,23], the head-space sample handling seems to be cumbersome. Furthermore, the determination of acetone in one's breath is inconvenient because acetone is easily lost during sample pretreatment before the GC analysis. Based on these considerations, we attempted to determine the acetone in saliva by reversed-phase liquid chromatography (LC) with fluorescence (FL) detection. Saliva has recently become the focus as a non-invasive sample because of the easy sampling and storage [24–28]. The proposed method was applied to the determination of acetone in the saliva of healthy volunteers and DM patients. The concentrations of acetone in the DM patients with and without ketoacidosis were also compared to those in healthy persons. Although the determination of acetone in saliva of isopropanol exposed persons was also carried out by Rose et al. [29], the method is head-space GC-FID. Thus, the present LC–FL method is essentially different from the GC-FID.

## 2. Experimental

### 2.1. Materials and chemicals

4-(*N*,*N*-Dimethylaminosulfonyl)-7-hydrazinobenzofurazan (DBD-H) was from Tokyo Kasei (Fig. 1). Acetone and trifluoroacetic acid (TFA) were from Kanto Chemicals. 3-Pentanone (Tokyo Kasei) was used as an internal standard (I.S.). Acetonitrile and formic acid (HCOOH) of HPLC grade were from Kanto Chemicals. Deionized and distilled water ($H_2O$) was used throughout the study (Aquarius PWU-200 automatic water distillation apparatus, Advantec). All other reagents and solvents were of analytical grade.

### 2.2. UPLC–FL analysis

The UPLC–FL analysis was performed using an Acquity ultra-performance liquid chromatograph (UPLC H-class: Waters) connected to an Acquity UPLC FLR detector (Waters). An Acquity UPLC BEH C18 column (1.7 μm, 100 × 2.1 mm i.d.; Waters) was used at the flow rate of 0.3 ml/min and 40 °C. The separation and detection conditions

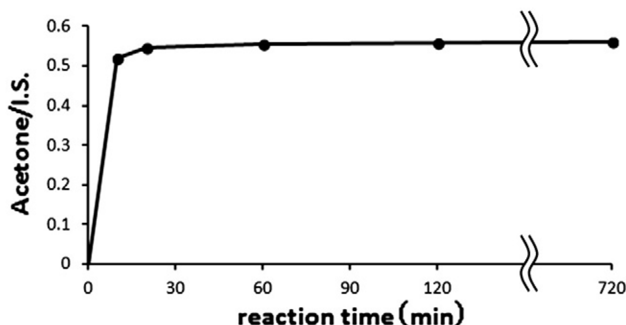


**Fig. 2.** Time course of the reaction of acetone with DBD-H at room temperature.

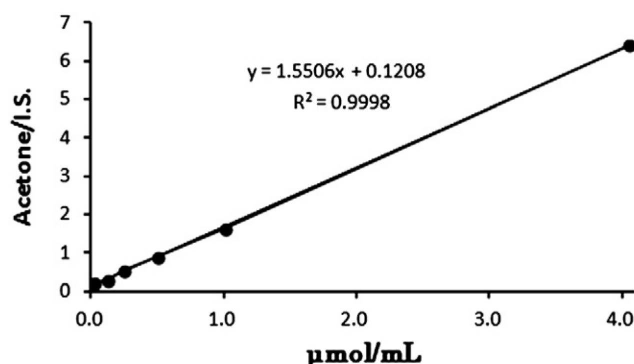


**Fig. 3.** Calibration curve of acetone. Concentration at 0.032–4.0 μmol/ml.

were as follows: Mobile phase, water–acetonitrile mixture (65:35) containing 0.1% (v/v) HCOOH; FLR detector, excitation wavelength, 460 nm; emission wavelength, 550 nm. Analytical software (MassLynx, version 4.1) was used for the system control and data processing.

### 2.3. Optimization of reaction time

The working solution of acetone (0.50 mmol/l) in water (100 μl) was added to 3-pentanone (I.S., 0.47 mmol/l) in acetonitrile (10 μl) and DBD-H (10 mmol/l) in acetonitrile (90 μl). The solution was vigorously mixed with TFA (2 μl) and reacted at room temperature over 12 h to determine the suitable reaction time. After fixed time intervals, the reaction vials were removed and the peak areas of the derivatives were determined by UPLC–FL.

### 2.4. Recommended reaction conditions of acetone with DBD-H

The freshly prepared samples (100 μl) containing acetone were added to 3-pentanone (0.47 mmol/l I.S.) in acetonitrile (10 μl) and DBD-H (10 mmol/l) in acetonitrile (90 μl). The solution was vigorously mixed with TFA (2 l and reacted at room temperature for 20 min). After the reaction, the derivative solution was centrifuged at 10,000 ×*g* and 4 °C for 5 min. An aliquot (2 μl) of the supernatant was subjected to the UPLC–FL system.

### 2.5. Analytical validation

#### 2.5.1. Calibration curve

The fixed concentrations of acetone (0.032–4.0 μmol/ml) in water were prepared by sequential dilutions. The working solutions (100 μl) were pretreated and derivatized. Each 2 μl of the derivatization solutions was then subjected to the UPLC–FL system. The calibration curve was constructed by plotting the peak area ratio of the derivatives of acetone to I.S. (y) vs the concentration of acetone (x, μmol/ml) by linear regression ($n = 5$). The limit of detection (LOD, $S/N = 3$) and the limit of quantitation (LOQ, $S/N = 10$) were calculated from the comparison of the peak height ratios of the injected amounts of the acetone and the baseline noise.

#### 2.5.2. Precision

The pooled saliva from DM patients with ketoacidosis was used for the determination of the precision of the proposed method. Each 100 μl of the saliva was pretreated and derivatized. Each 2 μl of the derivatization solutions was then subjected to the UPLC–FL system. The concentration of acetone in the saliva was determined from the calibration curve. The CV was calculated from 5 determinations.

#### 2.5.3. Recovery

The acetone (0.32, 5.0 or 40 mmol/l) in water (10 μl) was spiked into 90 μl of a saliva samples collected from a healthy volunteer ($n = 5$). The spiked- and unspiked samples were then pretreated and determined by

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