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Measurements of urinary adipic acid and suberic acid using high-performance liquid chromatography

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Abstract

A sensitive and specific method was developed for measuring medium-chain dicarboxylic acids (adipic and suberic acid) in urine. These acids were extracted from urine with diethyl ether and converted into fluorescent derivatives with 9-anthryldiazomethane, which can be separated by high-performance liquid chromatography. The reproducibility was high and the recovery from urine was above 90%. Urinary concentrations of adipic acid in streptozotocin-induced diabetic rats were significantly higher than those in control rats. In diabetic patients, both adipic acid and suberic acid tended to be high, but not significantly. This method should be useful for measuring dicarboxylic acids in urine

1. Introduction

Medium-chain dicarboxylic acids such as adipic (C_6), suberic (C_8) and sebacic (C_{10}) acid can be produced from ω-oxidation of long-chain monocarboxylic acids to long-chain dicarboxylic acids, followed by β-oxidation [1]. Adipic acid seems to be the major metabolic end-product and a significant amount of it and its precursor, suberic acid, are excreted in urine. The urinary excretion of these dicarboxylic acids is increased in patients who are ketotic due to diabetes or starvation [2] and in those with congenital defects in fatty acid metabolism [3] or drug intoxication [4].

Urinary dicarboxylic acids have been measured by gas chromatography–mass spectrometry (GC–MS) [5]. However, this method requires specialized equipment and is not commonly used in laboratory medicine. We have developed a method to determine the concentrations of adipic and suberic acid using high-performance liquid chromatography (HPLC). The dicarboxylic acids were converted into fluorescent derivatives and separated by HPLC. We report the results of the application of this method to measure urinary concentrations of dicarboxylic acids in streptozotocin (STZ)-induced diabetic rats and in patients with diabetes mellitus.

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2. Experimental

2.1. Materials

9-Anthryldiazomethane (ADAM) was purchased from Funakoshi Chemical Industry (Tokyo, Japan). Adipic, suberic and azelaic acid and STZ were obtained from Sigma (St. Louis, MO, USA). All solvents were purchased from Wako (Osaka, Japan).

2.2. HPLC apparatus

The HPLC system consisted of an LC-3A pump, an RF-500 LCA spectrofluorimeter, a Chromatopack C-R6A data processor (Shimadzu, Kyoto, Japan) and a sample injector (Rheodyne, Berkeley, CA, USA). Chromatographic separation was performed on a column of 30 × 4 mm I.D. (TSK-Gel, octadecylsilane reversed-phase type, particle size 5 μm; Tosoh, Tokyo, Japan).

2.3. Analytical procedure

Urine specimens were diluted with the same amount of distilled water and 5.3 mmol/l azelaic acid in ethanol was added as an internal standard to a final concentration of 106.3 μmol/l. Diluted urine specimens (0.6 ml) were mixed with 0.6 ml of glycine–HCl buffer (pH 2.3), 1.8 ml of diethyl ether and 150 mg of silicon dioxide. The mixtures were vigorously stirred for 10 min and centrifuged (1800 *g*, 5 min) to separate the organic layer. After three consecutive extractions, the solvent was evaporated. Ethanol (0.2 ml) was added and the solution was diluted with 0.4 ml of ethyl acetate. The sample (0.1 ml), ethanol (0.1 ml) and ADAM solution (0.2 ml) [1 mg/ml in acetone–ethyl acetate (1:9)] were mixed and incubated for 12 h at room temperature. A 20-μl aliquot of the reaction mixture was injected on to the HPLC column and eluted with acetonitrile–water (85:15, v/v) at a flow-rate of 1.5 ml/min at room temperature. The fluorescence intensity was measured at 412 nm with excitation at 365 nm, and the peak area was calculated by a data processor.

Urinary concentrations of adipic and suberic acid were also measured by the GC–MS method as reported previously [5]. Solvent extraction with diethyl ether and ethyl acetate was performed (heptadecanoic acid was added as an internal standard). The eluate was concentrated by lyophilization and the acids were subsequently trimethylsilylated. The derivatives were separated by GC–MS.

To investigate the precision, recovery and linearity and to compare the method with GC–MS, various concentrations of dicarboxylic acids were added to urine.

2.4. Urine samples

Diabetes was induced in rats by intravenous injection of STZ (65 mg/kg body mass) dissolved in 0.01 mmol/l citrate buffer (pH 4.2); control animals received the same amount of the buffer. To evaluate the degree of metabolic disturbance over an extended period, we measured the serum glycohaemoglobin levels [6] for rats or the haemoglobin A1c levels [7] for human. The mean glycohaemoglobin level in STZ-diabetic rats ($n = 18$) was 11.7% and that of control rats ($n = 9$) was 5.2%. Urine was collected from rats in metabolic cages for 24 h. Urinary concentrations of adipic and suberic acid were also measured in eighteen type II diabetic outpatients and 14 normal individuals. The mean haemoglobin A1c of diabetic patients was 9.0% and that of the normal control individuals was 5.4%. The urinary excretion of dicarboxylic acids was expressed as mg/g creatinine.

3. Results

Fig. 1A shows the chromatogram of a standard solution containing adipic, suberic and azelaic acid labelled with ADAM. Adipic acid was eluted at 13.3 min, suberic acid at 18.9 min and azelaic acid at 24.1 min. The medium-chain monocarboxylic acids were separated from adipic, suberic and azelaic acid (caproic acid was eluted at 8.5 min and caprylic acid at 13.7 min). Fig. 1B shows the HPLC profile of urine from a

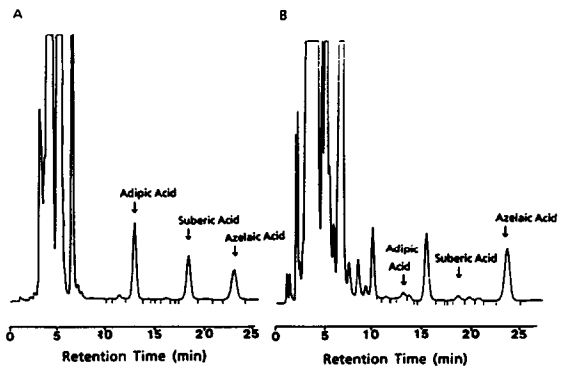

Fig. 1. Chromatograms of (A) standard (200 ng) adipic, suberic and azelaic acid solution derivatized with ADAM and (B) a urinary extract from a diabetic patient.

diabetic pateint (106.3 μmol/l azelaic acid was added as an internal standard). The relationship between the peak area and the concentrations of the dicaboxylic acids was linear over the ranges 0–342 μmol/l (adipic acid), 0–286 μmol/l (suberic acid) and 0–266 μmol/l (azelaic acid). Table 1 shows the intra- and inter-batch precision. The coefficients of variation (C.V.) of adipic acid in two specimens were between 2.0 and 3.7% and those of suberic acid were between 2.5 and 7.1%. Adipic acid (34.2 and 137.0 μmol/l), suberic acid (28.7 and 114.9 μmol/l) or azelaic acid (26.6 and 106.3 μmol/l) was added to the urine specimen and assayed. The recoveries of the added acids ranged from 92 to 106%.

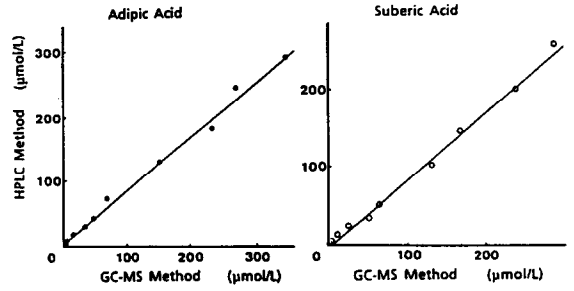

Fig. 2. Comparison of the dicarboxylic acid concentrations measured by HPLC and GC–MS.

Measurement of both asdipic and suberic acid using HPLC correlated well to those obtained by GC–MS ($r = 0.993$, $y = 0.813x + 6.0$ and $r = 0.997$, $y = 0.860x - 2.0$, respectively; x: GC–MS, μmol/l; y: HPLC, μmol/l), but the values measured by HPLC tended to be lower (Fig. 2). Fig. 3 shows the urinary adipic acid in STZ-diabetic and control rats. Urinary adipic acid in STZ-diabetic rats was significantly higher than that in control rats (14.9 ± 8.7 *vs.* 1.7 ± 1.7 mg/g creatinine, mean ± SD, $p < 0.01$). Suberic acid was not detected in either group. Fig. 4 shows urinary adipic and suberic acid levels in diabetic patients and normal controls. The levels of both acids in diabetic patients tended to be higher

Table 1
Precision of the HPLC method

Compound	Concentration (mean ± S.D.) (μmol/l)	C.V. (%)
Intra-batch (n = 10)		
Adipic acid	37.5 ± 1.0	2.67
	65.8 ± 1.3	1.98
Suberic acid	9.7 ± 0.6	6.19
	32.6 ± 0.8	2.45
Inter-batch (n = 10)		
Adipic acid	38.2 ± 1.2	3.14
	59.7 ± 2.2	3.69
Suberic acid	12.7 ± 0.9	7.09
	28.2 ± 1.3	4.61

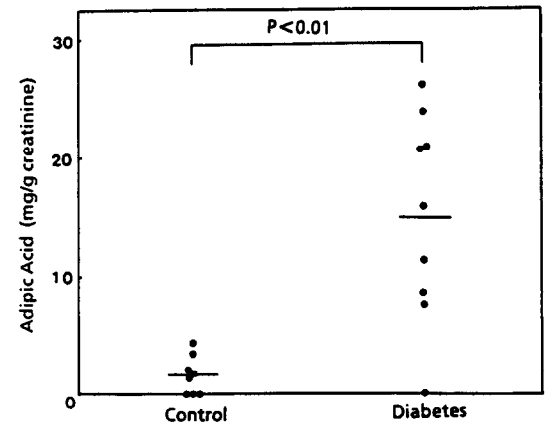

Fig. 3. Urinary adipic acid in STZ-diabetic and control rats.

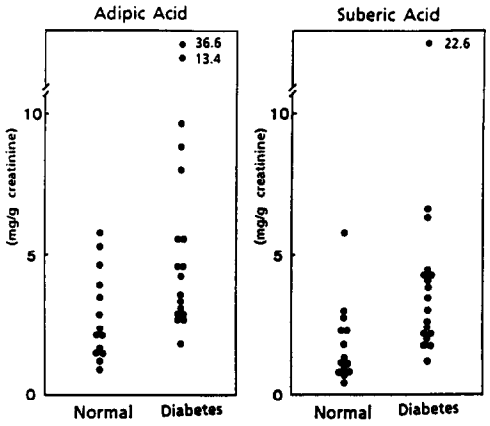

Fig. 4. Urinary adipic acid and suberic acid in normal individuals and diabetic patients.

than those in normal individuals, but not significantly (adipic acid 6.6 ± 7.9 *vs.* 2.8 ± 1.6 mg/g creatinine; suberic acid 4.2 ± 4.8 *vs.* 1.8 ± 1.4 mg/g creatinine).

4. Discussion

The measurement of dicarboxylic acid levels in urine is very important in estimating the ω-oxidation pathway of fatty acids. GC–MS has been used for the measurement of urinary concentrations of dicarboxylic acids, but it is technically complex and requires expensive equipment.

The fluorescence labelling reagent ADAM reacts with carboxyl groups to give an ester derivative which can be separated by the HPLC system and determined by spectrofluorimetry [8]. Fatty acids and oxalic acid have been measured in urine using this procedure [9,10]. We developed a method for the determination of adipic and suberic acid in urine by derivatization with ADAM followed by HPLC. The calibration graphs for adipic and suberic acid were linear over a wide range of concentrations. The reproducibility was good and the recovery was above 90% over a wide range of concentrations of the dicarboxylic acids including an internal standard. When we separated the standard adipic (suberic) acid shortly after admixture with ADAM, two peaks were observed on the chromatogram, but when we separated it every 30 min, the first peak area decreased and the second peak increased with time. After 1 h only the second peak remained and the peak area was stable for about 3 days at room temperature. We therefore believe that both of the carboxyl moieties were tagged with ADAM. To exclude variations in extraction efficiency, we added an internal standard, azelaic acid, which is non-physiological and has a chemical structure similar to those of adipic and suberic acid. The urine pH was adjusted to 2.3 because this gave the highest extraction efficiency (data not shown).

Although the values measured by the HPLC method tended to be lower than those obtained by GC–MS, there was a high correlation between the two assays. To exclude the possibility of the existence of compounds with the same retention time, we checked the retention times of medium-chain monocarboxylic acids (caproic, caprylic and capric acid) and other dicarboxylic acids (azelaic and pimelic acid), but they were different from those of adipic and suberic acid and were completely separated from them. Further, when various concentrations of adipic or suberic acid were added to urine and assayed by HPLC, the retention time for the added adipic or suberic acid coincided exactly with that of the original dicarboxylic acid. We therefore believe that the peaks represent adipic and suberic acids. Hence the proposed method was found to be accurate and specific for measuring adipic and suberic acids in urine.

Diabetic rats had significantly higher concentrations of urinary adipic acid than the controls, but suberic acid was not detected in the urine from either group. In diabetic patients, both adipic and suberic acid levels tended to be high, but not significantly. The discrepancy between diabetic rats and diabetic patients may partly arise from the degree of metabolic disturbance. STZ-induced rats were severely insulin deficient. They also had high blood glucose levels and increased ketone bodies in the blood and urine. These findings suggest that our method of assaying urinary adipic and suberic acid will be useful for studying ω-oxidation in the metabolism of fatty acids.

5. References

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