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Qualitative and Relative Quantitative Analysis of Urinary Components with Linear Ion Trap and FT ICR Mass Spectrometer to Search for Biomarkers

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The purpose of the present study was to construct a method to identify varying amounts of urinary components between two groups. We measured urine acquired from 4 healthy men and 4 healthy women with a high performance liquid chromatograph-linear ion trap and Fourier transform ion cyclotron resonance mass spectrometer (HPLC-LIT FT ICR mass spectrometer). We also rigorously compared the different amounts of compounds in men and women by multiple reaction monitoring (object and creatinine). We identified 12 compounds present in equal amounts in men and women as well as pregnanediol glucuronide, a compound found in 5.4-fold larger quantities in women than men. Urinary compounds can be identified by separating them with HPLC, and their exact mass can be measured with an FT ICR mass spectrometer, which has high resolution and high mass accuracy, and by performing multistage mass spectrometry (MS^n) with LIT. In comparing the quantities of a compound from two groups, it is essential to correct the urine volume by measuring creatinine. The method presented here can be used to search for and identify biomarkers of disease.

Metabolomics, following from genomics, transcriptomics, and proteomics, is a remarkable new research field, and involves the analysis of all metabolites (the metabolome) produced by various proteins in cells. Because the purpose of "Omics" is to obtain a comprehensive understanding of phenomena in the cell, metabolomics is as important as genomics, transcriptomics, or proteomics. Nuclear magnetic resonance spectroscopy (NMR), gas chromatography/mass spectrometry (GC/MS), capillary electrophoresis/mass spectrometry (CE/MS), and liquid chromatography/mass spectrometry (LC/MS) are generally utilized in the study of metabolites.

NMR has often been applied to metabolite profiling in plant metabolomics (1,2,3). Although the advantage of NMR is its ability to measure analytes in biofluids quickly and accurately without initial processing or separation, compounds of low concentration are not detected because its dynamic range is poor.

GC/MS has often been applied to metabolite research and to the diagnosis of some diseases, especially disorders of organic acids, which are difficult to measure with LC/MS (4). GC/MS has high resolution separation and high sensitivity, and is available for the

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identification of compounds, because the database of electron impact mass spectrometry is rich. However, GC/MS has several disadvantages, such as the chemical modifications required in the preparation of samples, difficulty in measuring nonvolatile and polar macromolecules, and the use of hard ionization.

CE/MS can measure ionic metabolites at very high resolution and is recognized as a new technique for metabolome analysis (5,6).

LC/MS is available for many fields, including proteomics (7,8,9), measuring drug concentrations in blood and urine (10,11), analyzing the structures of drug metabolites (12), and, recently, searching for biomarkers of disease.

Although a range of equipment is utilized in metabolomics, we used a high performance liquid chromatograph-linear ion trap and Fourier transform ion cyclotron resonance mass spectrometer (HPLC-LIT FT ICR mass spectrometer) in the present study, because it has some advantages in the identification of low molecular weight compounds. FT ICR mass spectrometry has been successfully utilized in metabolomics since 2002 (13,14). The LIT FT ICR mass spectrometer used in this study is a hybrid mass spectrometer, which has an ion trap and Fourier transform ion cyclotron resonance mass spectrometer. The ion trap allows the acquisition of rich structural information about compounds, because it can perform multistage mass spectrometry (MS^n). Fourier transform ion cyclotron resonance mass spectrometry is invaluable for measuring the exact mass since it has high resolution (maximum resolution 500,000 using LTQ FT (Thermo Fisher Scientific, Waltham, MA, USA)) and high mass accuracy. These features enable identification of the compounds in urine.

A major obstacle in performing metabolomics is the presence of compounds with a nominal mass indistinguishable from others. It is difficult for a low resolution mass spectrometer to solve this problem; however, LIT FT ICR mass spectrometer has the potential.

The purpose of the present study was to construct a method to identify varying amounts of urinary components between two groups. We analyzed urine acquired from healthy men and women, and believe that the method can be used to search for biomarkers of disease.

MATERIALS AND METHODS

Materials

The ammonium acetate, acetic acid, and acetonitrile used in preparing for the mobile phases were purchased from Wako Pure Chemical Industries, Osaka, Japan. The standards used in the identification of urinary compounds were as follows. L-phenylalanine, uric acid, aconitic acid, and L-tryptophan were purchased from ChromaDex, Irvine, CA, USA. Indoleacetic acid, hippuric acid, citric acid, 4-amino hippuric aicd, sebacic acid, creatinine, and riboflavin were purchased from Wako Pure Chemical Industries, Ltd. Kynurenic acid was purchased from MP Biochemicals, Solon, OH, USA.

Samples

Urine samples were collected from eight healthy Japanese volunteers: four men, ages 26, 30, 26, and 28, and four women, ages 28, 27, 22, and 25, none of whom were taking regular medication.

Pretreatment

Each urine sample was filtrated using Ultrafree-MC Durapore PVDF 0.22 μm (Millipore Corporation Billerica, MA, USA).

Measurement (First)

Urine analysis was carried out on an HPLC alliance system (Waters, Milford, MA, USA) coupled to a LTQ FT, linear ion trap, and Fourier transform ion cyclotron resonance mass spectrometer with an electrospray ionization source (Thermo Fisher Scientific, Waltham, MA, USA). An Inertsil ODS-3 column (100 mm × 2.1 mm i.d., particle size 3 μm, GL Sciences, Osaka, Japan) was utilized for the separation process. The column temperature was set at 40 °C. The mobile phase, consisting of 10 mmol/L ammonium acetate aqueous solution adjusted to pH 3.75 by acetic acid (solution A) and acetonitrile (solution B), was pumped at a flow rate of 0.2 mL/min. HPLC separation was performed using a linear gradient program of 10 - 10% solution B for 5 min, 10 - 95% solution B for 20 min, 95 - 95% solution B for 5 min, 95 - 10% solution B for 1 min, and 10 - 10% solution B for 9 min. A 10-μL aliquot was injected onto the column. Analysis was conducted in both the positive and negative ion modes. The heated capillary temperature was set at 275 °C. The spray voltage was 4.0 kV. The capillary voltage and the tube lens voltage were 10 V and 70 V in the positive ion mode, and -48 V and -130 V in the negative ion mode, respectively. Three scan events were used: 1. Full scan measurement in a scan range *m/z* 100-1500 with a resolution 25000 with FT ICR mass spectrometer; 2. Data dependent exclusion MS^2 measurement on the most intense ion from the full scan spectrum with LIT; and 3. Data dependent MS^3 measurement on the most intense ion from the MS^2 spectrum with LIT. A precursor ion that acquired its MS^2 spectrum was then placed on a dynamic exclusion list for a period of 18 sec (12). The collision energy was set at 35%.

Measurement (relative quantitation)

A compound known to exist in different amounts in the urine of men and women was rigorously analyzed by multiple reaction monitoring (MRM). Both the MS^2 fragment ion of the compound and that of creatinine were measured to correct the urine volume (15). The *m/z* 114.06 MS^2 fragment ion of the creatinine dimer (precursor ion, *m/z* 227.12) was measured from 0 - 5 min, and the *m/z* 477.60 MS^2 fragment ion of pregnanediol glucuronide (precursor ion, *m/z* 495.30) was measured from 5 - 40 min. Because creatinine tends to be detected as a positive ion and pregnanediol glucuronide as a negative ion, analysis was conducted in the positive ion mode from 0 - 5 min and in the negative ion mode from 5 - 40 min. HPLC conditions were as for those described in the previous section, “Measurement (First)”.

Analysis (Identification of compound)

We analyzed data measured with HPLC-LIT FT ICR mass spectrometer by two methods. Method 1: A mass chromatogram of the *m/z* of protonated ions of compounds known to be abundant in urine was captured (mass tolerance; 3 mDa) (16). If a peak was confirmed, the acquired MS^n spectrum of the compound was checked for consistency against the structure, and then checked against that of the standard. Method 2: A mass chromatogram of the *m/z* detected at strong intensity (not less than 40,000 in the positive ion mode, not less than 20,000 in the negative ion mode) was captured, before the peak shape was checked. This step was performed to enable noise rejection from the equipment. The exact mass (M is defined as mass of the neutral state) was calculated from the measured *m/z* ($[M+H]^+$ or $[M-H]^-$), and a database search was then performed (mass tolerance; 3 mDa). If the database search

produced a hit, the acquired MS^n spectrum was checked for consistency against the structure of the candidate, and then checked against that of the standard. The Human Metabolome Database (http://www.hmdb.ca/, 17) and the database (accessed September 6th, 2006) acquired from Metabolome.JP (http://www.metabolome.jp/) were used. The Human Metabolome Database can be searched under conditions for each mass spectrometer and biofluid, and has rich information for registered compounds. The database acquired from Metabolome.JP has a huge number of compounds, although it has only exact mass information.

An analysis of hippuric acid is shown in Figs. 1 and 2, as examples of fragmentation analysis. The urinary compound was determined as hippuric acid, because the MS^n spectrum was the same as that of the standard of hippuric acid.

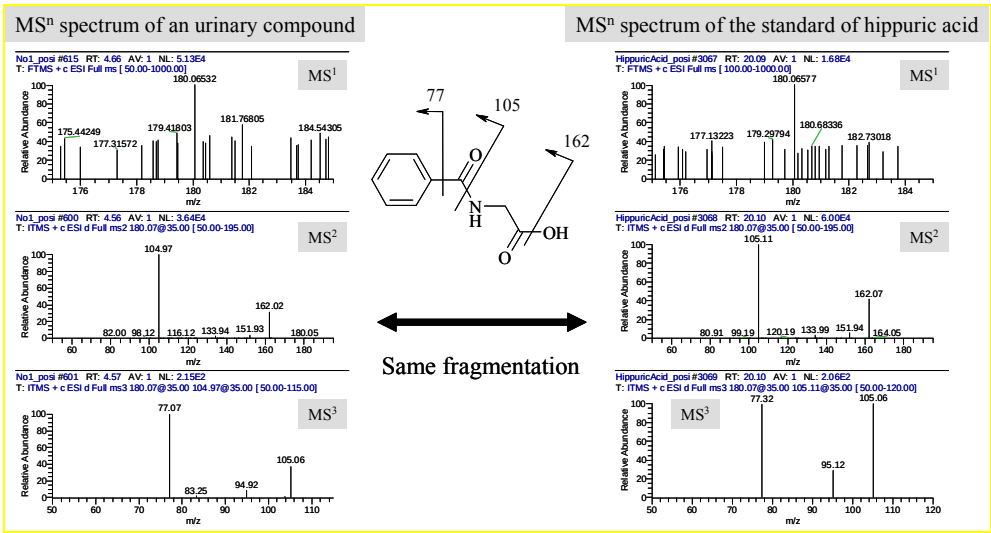

Figure1 Example of structure analysis of hippuric acid in positive ion mode

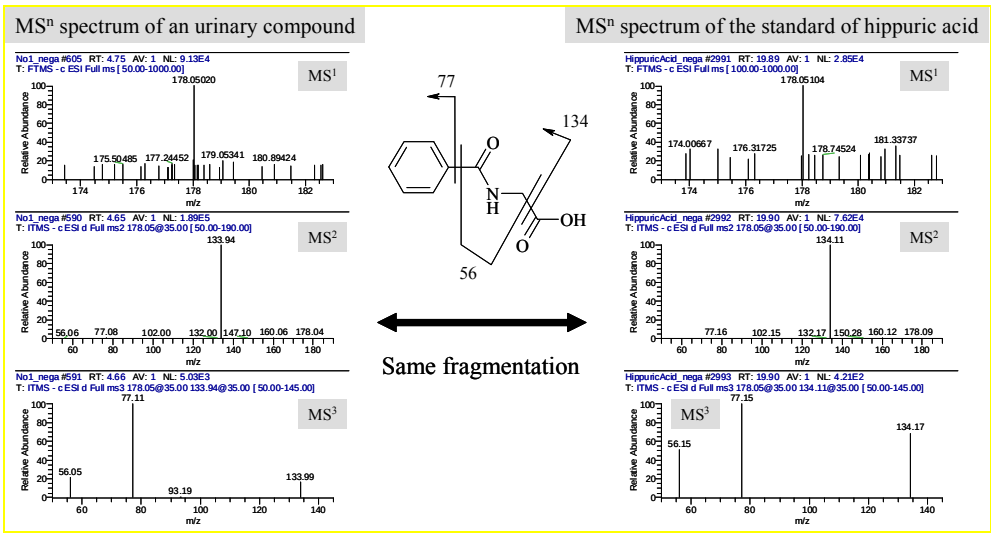

Figure2 Example of structure analysis of hippuric acid in negative ion mode

Analysis (comparison of amounts of compounds in urine)

Conditions for determining equal amounts of a compound in male and female urine were as follows: An *m/z* value was detected in 4 male and 4 female samples and the average of the ion intensity in either males or females was less than 5 times of that of another ion. Conditions that determined different amounts of a compound in the urine of males and females were as follows: An *m/z* value was detected in either 4 male or 4 female samples, or the average of the ion intensity in either male or female samples was not less than 5 times of that of another ion. Furthermore, the amount of a compound determined by the above conditions was precisely compared to that of the compound by MRM. Correction of the volume of each urine sample was performed by dividing the peak area value of the object by that of creatinine.

RESULTS

We identified 13 urinary compounds. Twelve of the compounds were equal in men and women. Of these, 8 compounds were detected in the positive ion mode and 6 in the negative ion mode (Table I). Pregnanediol glucuronide was found in large amounts in female urine. Furthermore, we performed MRM of pregnanediol glucuronide and creatinine and rigorously analyzed the quantities of pregnanediol glucuronide (Figs. 3a and 3b). We ascertained that the peak area value of creatinine is in proportion to the concentration of creatinine in urine under conditions where the peak area value of creatinine is less than 1.36E+08 when using LTQ FT (data not shown). The average area ratios of pregnanediol glucuronide / creatinine were 0.0137 (male) and 0.0734 (female), and we clarified that the amount of female urinary pregnanediol glucuronide was 5.4 times that of male urinary pregnanediol glucuronide (Table II).

Pregnanediol is a metabolite of progesterone, one of the female hormones, and its glucuronate conjugate is excreted in urine (18,19). Therefore, we regard the results of the present study as valid.

Table I. 12 compounds identified in equal amounts in men and women

Compound name	Molecular formula	Positive ion mode				Negative ion mode			
		Detected *m/z*	M *1	Difference *2 (mDa)	RT *3 (min)	Detected *m/z*	M *1	Difference *2 (mDa)	RT *3 (min)
L-Phenylalanine	$C_9H_{11}NO_2$	166.08600	165.07872	-0.26	2.10	-	-	-	-
Uric acid	$C_5H_4N_4O_3$	-	-	-	-	167.01999	168.02727	-1.09	1.46
Aconitic acid	$C_6H_6O_6$	-	-	-	-	173.00838	174.01566	-0.84	1.86
Indoleacetic acid	$C_{10}H_9NO_2$	176.07048	175.06320	-0.18	2.47	-	-	-	-
Hippuric acid	$C_9H_9NO_3$	180.06532	179.05804	-0.20	4.66	178.05020	179.05748	-0.77	4.75
Kynurenic acid	$C_{10}H_7NO_3$	190.04941	189.04213	-0.51	3.27	-	-	-	-
Citric acid	$C_6H_8O_7$	-	-	-	-	191.01891	192.02619	-0.82	1.55
4-Amino hippuric acid	$C_9H_{10}N_2O_3$	195.07626	194.06898	-0.22	3.77	-	-	-	-
Sebacic acid	$C_{10}H_{18}O_4$	203.12761	202.12033	-0.29	11.23	201.11253	202.11981	-0.81	11.25
L-Tryptophan	$C_{11}H_{12}N_2O_2$	205.09695	204.08967	-0.20	2.82	-	-	-	-
Creatinine dimer	$C_8H_7N_3O$ (monomer)	227.12494	226.11766	-0.22	1.42	-	-	-	-
Riboflavin	$C_{17}H_{20}N_4O_6$	-	-	-	-	375.12894	376.13622	-2.18	12.88

*1) Exact mass calculated by "Detected *m/z*"

*2) Difference between "M" and a thoretical mass calculated by "Molecular formula"

*3) RT is the abbreviation of retention time.

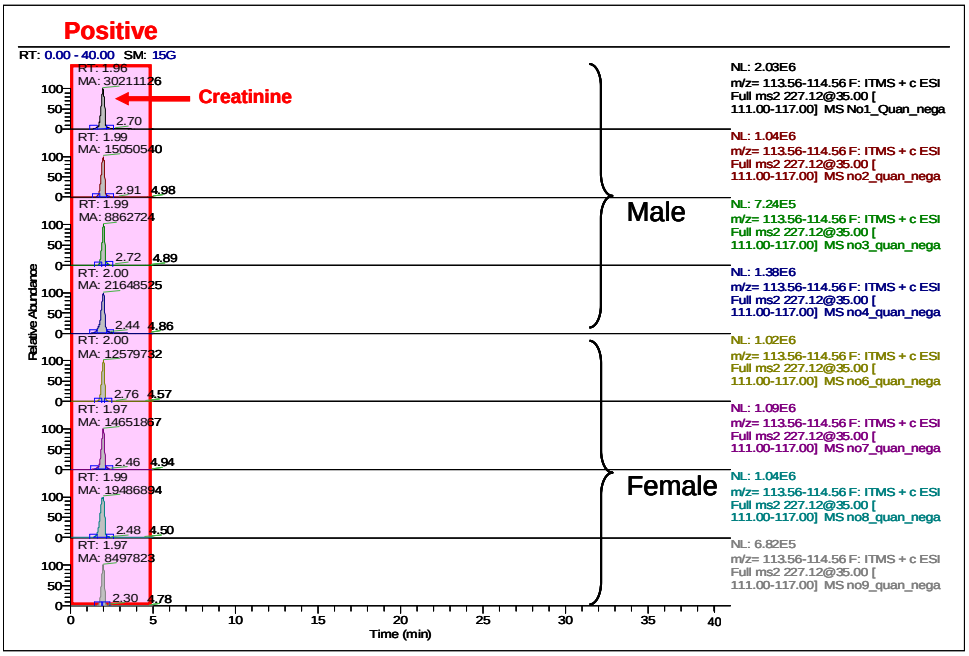

Figure3a Chromatograms of creatinine acquired by MRM of pregnanediol glucuronide and creatinine. RT and MA represent retention time and peak area value, respectively.

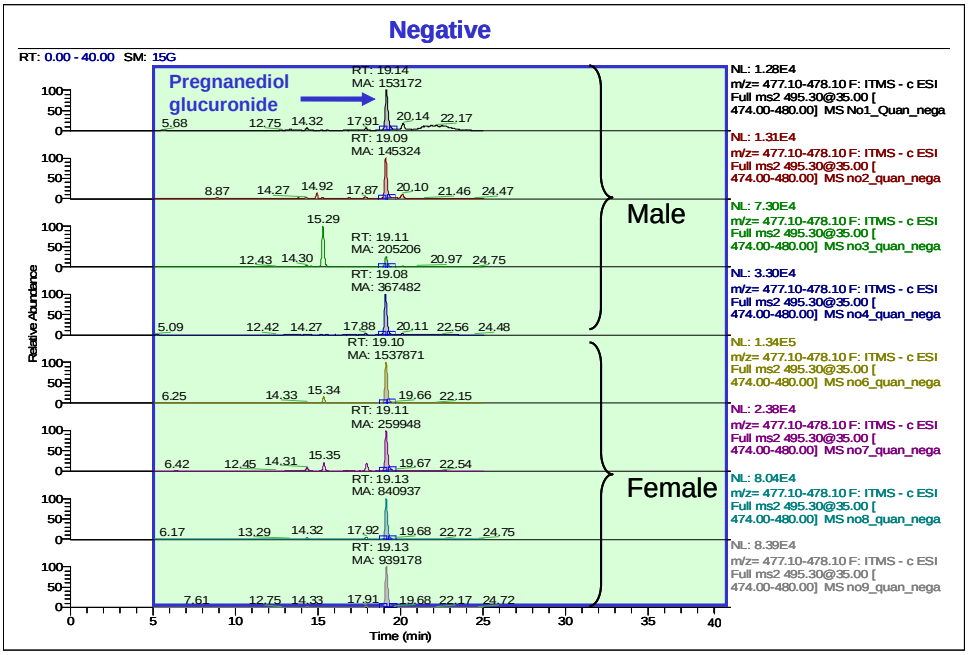

Figure3b Chromatograms of pregnanediol glucuronide acquired by MRM of pregnanediol glucuronide and creatinine. RT and MA represent retention time and peak area value, respectively.

Table II. Area ratio (pregnanediol glucuronide/creatinine) in 4 male and 4 female samples

Sample	Peak area value		Area ratio	Average of area ratio
	Creatinine	Pregnanediol glucuronide	(Pregnanediol glucuronide/Creatinine)	
Male1	30211126	153172	0.00507	0.0137
Male2	15050540	145324	0.00966	
Male3	8862724	205206	0.02315	
Male4	21648525	367482	0.01697	
Female1	12579732	1537871	0.12225	0.0734
Female2	14651867	259948	0.01774	
Female3	19486894	840937	0.04315	
Female4	8497823	939178	0.11052	

DISCUSSION

Compounds with a nominal mass of 258 were searched for using the Human Metabolome Database. Six compounds came back as hits (Fig. 4). Although the nominal mass of 3-Methyluridin, 2-Keto-3-deoxy-6-phosphogluconic acid, and Tetradecanedioic acid are all 258, their exact masses are 258.08521, 258.01407, and 258.18311, respectively. A low resolution mass spectrometer cannot distinguish between them, but the FT ICR mass spectrometer can. Because the exact masses of 3-Methyluridin, Imidazoleacetic acid riboside, and Ribothymidine are the same, it is not possible to use the FT ICR mass spectrometer alone to distinguish between them. Structures and predicted fragmentation sites of 3-Methyluridin, Imidazoleacetic acid riboside, Ribothymidine are shown in Fig. 5. Acquiring MS^n spectra with LIT solves the problem in this case. In a compound having many hydroxy groups, only an *m/z* produced by dehydration can be detected in the MS^2 spectrum. LIT, with the ability to acquire not only MS^2 spectra but also MS^3 spectra, is superior to other mass spectrometers in this case.

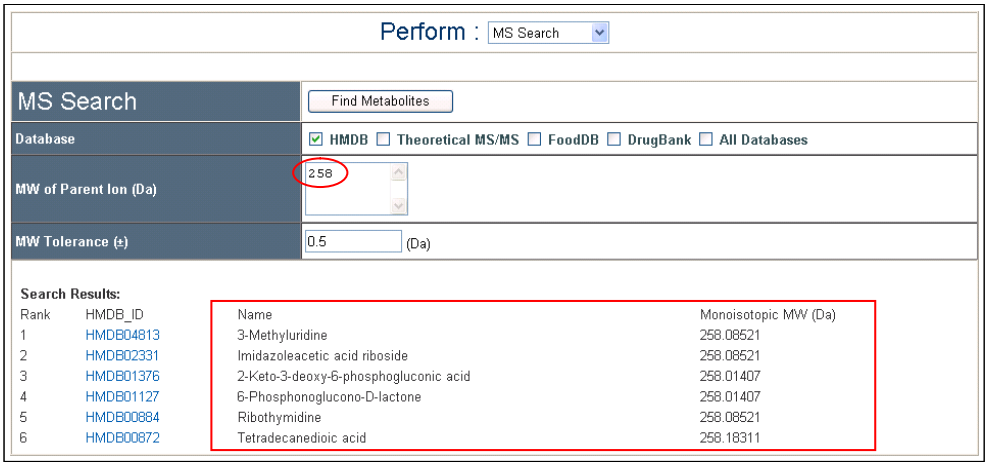

Figure4 Search result for compounds with a nominal mass of 258

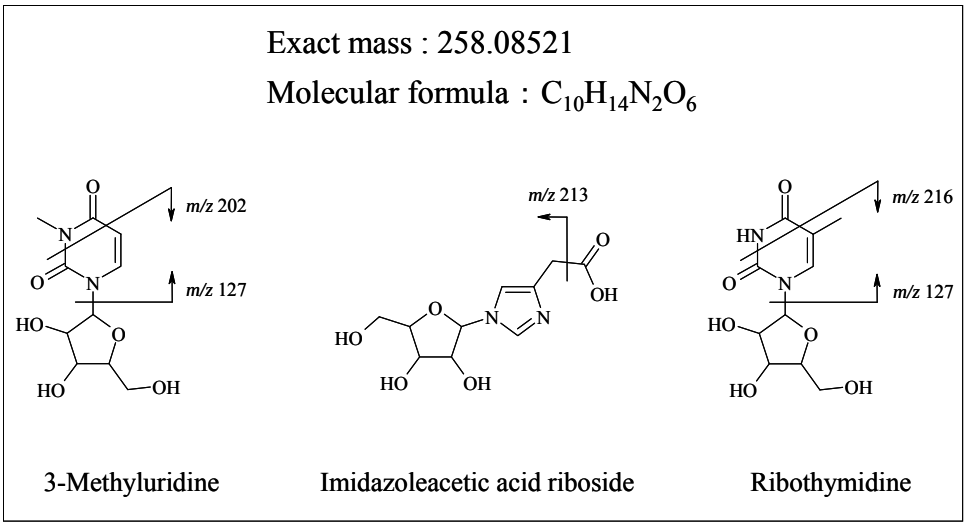

Figure5 Structures and predicted fragmentation sites of 3-Methyluridine, Imidazoleacetic acid riboside, and Ribothymidine. Arrows represent fragmentation sites predicted by MassFrotier 5.0 with *m/z* values expected to be detected.

Although an enormous number of compounds exist in urine, only a few were identified in the present study. One of the reasons is probably the various physical properties of urinary compounds. The column used in the present study was a C18 column and the mobile phase was acidic. These conditions are suitable for the analysis of acidic compounds and for the detection of positive ions, but not for basic compounds or negative ions. In fact, a smaller number of negative ions were detected than positive ions. Therefore, the detection of all compounds by a single method is difficult. By focusing on the objects of measurement, a comprehensive analysis of compounds such as organic acids or lipids seems to be better.

An additional reason for the small number of compounds identified was likely the much lower number of compounds identified in the database search compared to the number of compounds detected. In fact, the number of compounds detected in the present study was more than 100 in the positive ion mode and about 80 in the negative ion mode. It is believed that both nonconjugated and conjugated metabolites are abundant in urine. We subsequently performed a search of the Human Metabolome Database using the keywords glucuronide, sulfate, glutathione, and conjugate (Table III). Few conjugates have been registered, and although the metabolome database is unfinished, its completion and future development is anticipated. Thus, if MS^n spectra are enriched, the work required to identify a compound by acquiring the MS^n spectrum of the reference standard can be omitted. Because every spectrum of compounds acquired by the LIT FT ICR mass spectrometer is a unique fingerprint of that compound, the search for biomarkers of diseases will be advanced dramatically by the completion of the metabolome database.

Table III. Results of a search of the Human Metabolome Database using the keywords glucuronide, sulfate, glutathione, and conjugate. (The search was performed on July 19, 2008. The total number of compounds enrolled was 3001.)

Key word	Number of hit compounds
Glucuronide	23 compounds
Sulfate	65 compounds (Some compounds are not conjugate)
Glutathione	9 compounds (no conjugates)
Conjugate	10 compounds

In conclusion, the identification of urinary compounds is possible through separation using HPLC, measurement of the exact mass using an FT ICR mass spectrometer, which has high resolution and high mass accuracy, and acquiring MS^n with LIT. It is essential to correct the urine volume by measuring creatinine when comparing the amounts of compounds from two groups. When aliquots of tissues, cells, or culture media are measured, the acquired intact data are available for comparative analysis. However, in the case of urine, acquired intact data are not available for rigorous comparative analysis because urinary compounds are concentrated or diluted under the influence of water intake or sweating. Therefore, the urine volume must be corrected. Measuring creatinine is a suitable method for the correction of urine volume in a clinical examination because creatinine is scarcely reabsorbed in the renal tubules, exists abundantly in urine, and is not affected by changes in physiological factors. Thus, a change in creatinine concentration reflects urine volume error. In addition to urine metabolomics, it is essential that an object and creatinine are measured by MRM, the urine volume is corrected, and an intergroup comparison is performed for the precise examination of intergroup differences. The method presented in this article can be applied to the search and identification of biomarkers of disease.

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