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Gas chromatographic profiling and pattern recognition analysis of urinary organic acids from uterine myoma patients and cervical cancer patients

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Abstract

An efficient organic acid profiling and pattern recognition method is described for the correlation between urinary organic acid profiles and uterine cervical cancer. After methoximation of keto acids in alkalinized urine samples, all free organic acids were recovered by a dual solid-phase extraction procedure, followed by conversion to *tert*.-butyldimethylsilyl derivatives for the profiling analysis by dual-capillary column gas chromatography (GC) with subsequent screening for acids by retention index (*I*) library matching. A total of 50 organic acids were positively identified in urine samples (0.25 ml) from 12 uterine myoma (benign tumor group) and 14 uterine cervical cancer (malignant tumor group) patients studied. When the GC profiles were simplified to their corresponding organic acid *I* spectra in bar graphical form, characteristic patterns were obtained for each average of benign and malignant tumor groups. Stepwise discriminant analysis performed on the GC data selected 16 acids as the variables discriminating between the two groups. Canonical discriminant analysis applied to these 16 variables correctly classified 26 urine samples into two separate clusters according to tumor types in the canonical plot.

Keywords: Organic acids

1. Introduction

Carcinogenesis is known to affect a complex network of metabolic interrelationships, leading to significant changes in concentration of a large number of constituents of body fluids. In the literature, the assay of urinary polyamines [1–5] and modified nucleosides [6–9] as the chemical markers for cancers among the various constituents, has been exclusively investigated.

As the major metabolic end products, urinary organic acids are well established as important biochemical indicators of abnormal metabolism created by various diseases [10–14]. But, attempts were rarely made to correlate the urinary organic acid profiles to carcinogenic diseases. Among the various cancers, uterine cervical cancer is known to be one of the most prevalent to women and its early

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detection is thus desirable [15,16]. The measurements of urinary organic acids could provide a noninvasive diagnostic tool for the cervical cancer screening. It seems, therefore, of interest to investigate the effects of uterine cervical cancer on urinary organic acid profiles.

The accurate discrimination of normal states from abnormal states requires the application of a proper computer-aided pattern recognition method to the complex metabolic profiles such as gas chromatographic (GC) data obtained. In the literature, a nonparametric pattern recognition method was used for the distinction of diabetic patients [17]. SIMCA multivariate data analysis was employed for the classification of brain and pituitary tumor cells [18]. The differentiation of leprosy patients from normal controls was achieved by principal component analysis (PCA) and partial least-square models [14].

In a previous work [19], simpler pattern recognition methods [20] combined with our routine organic acid profiling analysis [21,22] were found to be potentially useful for the comparative analysis of urinary organic acid profiles among the groups of nonsmokers and smokers. The procedure involves solid-phase extraction (SPE) of organic acids using Chromosorb P in normal-phase partition mode, with subsequent single-step conversion to *tert*.-butyldimethylsilyl (TBDMS) derivatives, followed by direct GC analysis on dual-capillary columns of different polarity. Each organic acid was then identified through computer library matching based on the two retention index (*I*) sets and area ratio comparison. The GC profiles were simplified to their corresponding organic acid *I* spectra for the visual comparison between sample groups. Stepwise discriminant analysis on the GC data was performed to produce characteristic star symbol plots for the easier visual pattern recognition. The canonical discriminant analysis performed on the same GC data produced a canonical plot depicting correct group classification.

This work discusses our recent investigation on the discrimination of changes in urinary organic acid levels associated with uterine cervical cancer by applying the organic acid profiling analysis combined with *I* spectral and canonical plotting methods [19]. In this study, direct methoximation of keto acids in alkalinized urine with subsequent SPE of organic acids in strong anion-exchange mode was performed prior to the SPE in normal-phase partition mode for more accurate profiling analysis.

2. Experimental

2.1. Urine specimens

Mid-portions of the first morning urine specimens were collected from 12 uterine myoma patients and 14 uterine cervical cancer patients (ages from 33 to 69). All patients were hospitalized at Department of Obstetrics and Gynecology, Ajou University Hospital (Suwon, South Korea). The samples were immediately alkalinized (pH about 10) and stored at −20°C until analyzed. The concentration of creatinine in each urine was measured according to the Jaffe reaction [23] in triplicate.

2.2. Chemicals

Silylating reagent, *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was purchased from Pierce (Rockford, IL, USA) and triethylamine (TEA) from Aldrich (Milwaukee, WI, USA), *trans*-cinnamic acid and methoxyamine hydrochloride from Sigma (St. Louis, MO, USA) and *n*-hydrocarbon standards (C_{12}–C_{34}, even numbers only) from Polyscience (Niles, IL, USA). All other chemicals were of analytical grade and used as received. Chromosorb P (acid-washed, 80–100 mesh) and Superclean LC SAX column (Cl^- form) were purchased from Supelco (Bellefonte, PA, USA). A luer-tipped glass tube (5 mm I.D.) packed with Chromosorb P (600 mg) was washed successively with 0.1 *M* sulfuric acid, methanol, acetone, dichloromethane and diethyl ether, followed by activation under vacuum (150°C, 3 h) prior to being used as a tube for SPE in normal-phase partition mode. A polyethylene tube (1 ml) packed with SAX (200 mg) was conditioned by washing sequentially with methanol, 0.1 *M* sodium fluoride and distilled water before being used as a tube for SPE in anion-exchange mode.

2.3. Sample preparation

All urine samples of each patient were individually processed in triplicate for organic acid analysis as

follows. After addition of *trans*-cinnamic acid as internal standard (I.S.) at 10 ppm to urine, an aliquot (1.0 ml) was adjusted to pH about 13 with 0.1 *M* sodium hydroxide and then methoximated with methoxyamine hydrochloride (10 mg) at 60°C for 1 h. The resulting mixture was subjected to SPE in two stages. First, an aliquot (0.25 ml) of the mixture was loaded to a preconditioned SAX tube and rinsed with diethyl ether (0.25 ml). The rinsed SAX tube was eluted with 0.1 *M* sodium sulfate (saturated with sodium chloride; 0.45 ml) and diethyl ether (0.25 ml) in sequence. After the removal of diethyl ether (under a stream of nitrogen) from the combined eluates, the aqueous eluate was acidified with conc. sulfuric acid and saturated with sodium chloride. It was then loaded onto an activated Chromosorb P tube and eluted with diethyl ether (2 ml). The ethereal eluate was collected into the mixture of TEA (20 μl) and isooctane (40 μl) and excess ether was removed (under a steam of nitrogen), followed by silylation with MTBSTFA (20 μl) at 60°C for 2 h for the direct analysis GC or GC–mass spectrometry (MS).

2.4. Gas chromatography and gas chromatography–mass spectrometry

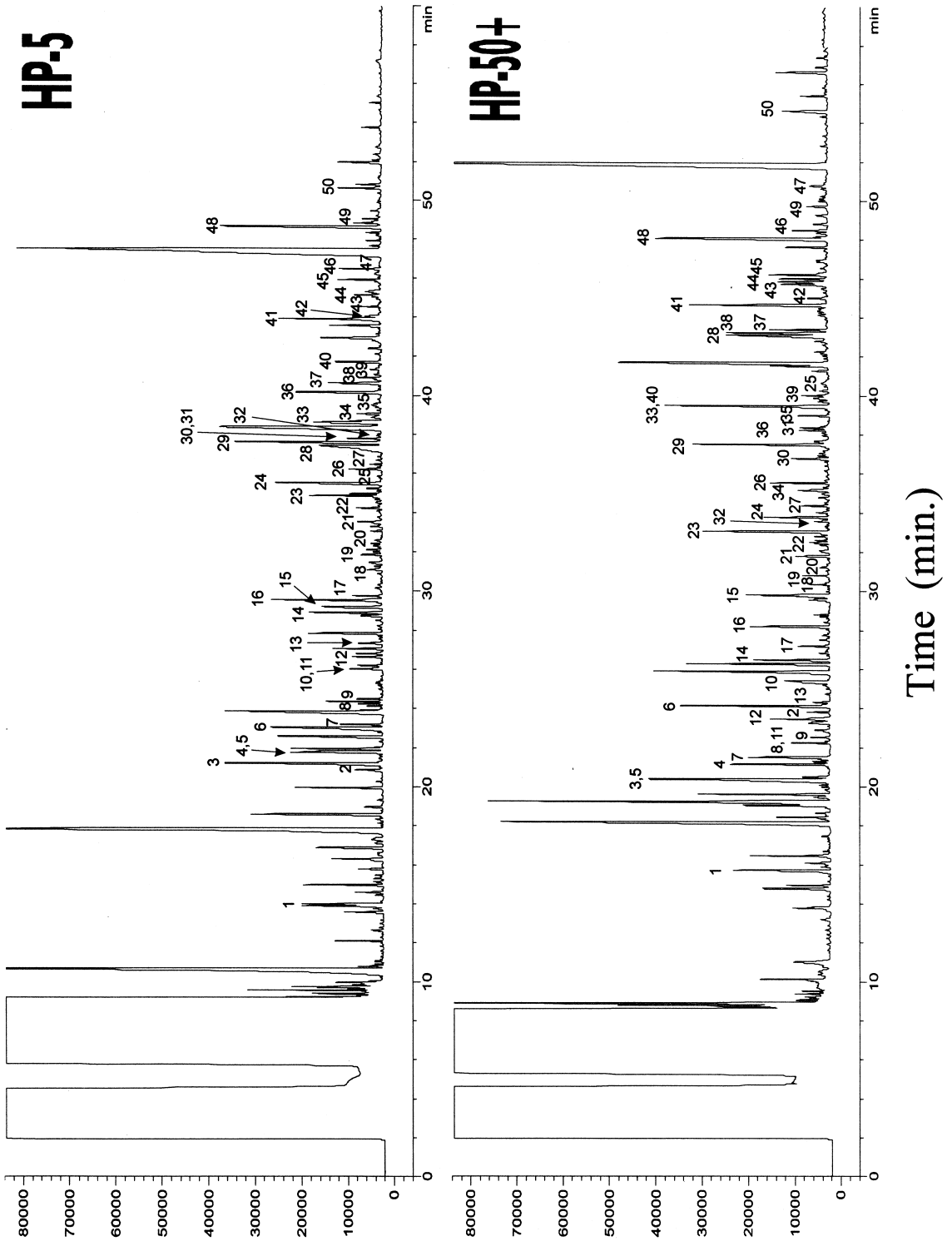
GC analyses were performed with a Hewlett-Packard HP Model 5890A gas chromatograph series II, equipped with a split/splitless capillary inlet system and two flame ionization detection (FID) systems and interfaced to an HP 3365A GC Chemstation (Hewlett-Packard, Avondale, PA, USA). Samples (ca. 0.5 μl) were injected in the splitless mode with a purge delay time of 42 s. The oven temperature was held at 60°C for 2 min, then programmed to 280°C at a rate of 4°C/min. The injector and detector temperatures were 260 and 280°C, respectively. The inlet pressure of helium as the carrier gas was set to 137.9 kPa. *I* measurements with subsequent screening for acids were carried out using a dual-capillary column system made of HP-5 (SE-54 bonded phase) and HP-50+ (OV-17 bonded phase) fused-silica capillary columns (Hewlett-Packard; dimensions 30 m×0.25 mm I.D., 0.25 μm film thickness), which were connected to a deactivated fused-silica tubing (1 m×0.25 mm I.D.) as retention gap via Y-splitter. The two FID signals were processed simultaneously in dual-channel mode by the GC Chemstation. A standard solution of *n*-hydrocarbons (C_{12}–C_{34}, even numbers only) in isooctane was injected to be used as external retention standards. Temperature-programmed *I* values were computed via built-in retention index program by linear interpolation between the retention times of adjacent hydrocarbon standards. For peak identification by computer *I* matching [22], the *I* set on the two columns for each peak was compared with those in the previously compiled *I* reference library containing about 180 organic acid standards as their TBDMS derivatives. For the quantitative analysis, an Ultra-2 (SE-54 bonded phase) capillary column (25 m×0.20 mm I.D., 0.33 μm film thickness) was used under the identical operating conditions. The peak area ratio of each identified organic acid with respect to I.S. was calculated using Ultra-2 chromatographic data for the subsequent pattern recognition analysis.

The confirmation of peak identities was done on a HP 5890A series II gas chromatograph, interfaced to an HP 5970B mass spectrometer (70 eV, electron impact mode), which was on-line to an HP 59940A MS Chemstation. An Ultra-2 (SE-54 bonded phase) capillary column (25 m×0.20 mm I.D., 0.11 μm film thickness) was used in the split injection mode (10:1). The oven temperature was initially 60°C for 2 min and then raised to 280°C at 4°C/min. The injector and interface temperatures were 260 and 280°C, respectively. The mass range scanned was 50–650 u at a rate of 0.99 scan/s. Each peak was identified by library match using our MS library file containing organic acid standards as their TBDMS derivatives.

2.5. Pattern recognition analysis

The mean peak area ratios (relative to I.S.) of organic acids identified in urine of each patient were corrected for the creatinine amount in 0.25 ml of urine and then normalized to the largest peak as the base peak. Using MS Excel program, the percentage normalized area ratios were plotted against *I* values in bar graphical form to obtain organic acid *I* spectra of each patient urine as described earlier [19]. For group average *I* spectra, normalized median area ratios were used.

The mean peak area ratios (corrected for creatinine) of all organic acids identified were subjected to stepwise discriminant analysis and canoni-


HP-5
HP-50+
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10
20
30
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50
10000
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30000
40000
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60000
70000
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Time (min.)

cal discriminant analysis by means of the statistical software package SAS. Canonical discriminant analysis was applied to the mean peak area ratios of the variables preselected by stepwise discriminant analysis as the data vectors. And a canonical plot was drawn on the basis of the first canonical discriminant function (CAN1) against the second canonical discriminant function (CAN2) of the preselected variables for each urine specimen.

3. Results and discussion

In this study, after the direct methoximation of keto acids in alkalinized urine, SPE of organic acids was carried out in two stages: first in anion-exchange mode and then in normal-phase partition mode. SAX tubes were preconditioned in the form of weakly bound F^- for the stronger retention of carboxylic anions by the anion-exchange sites during the application of urine specimens. The bound organic acid anions were eluted with sodium sulfate solution (with high ionic strength) containing more strongly bound sulfate ion. The eluted organic acid anions were recovered as unionized free forms by the subsequent SPE in normal-phase partition mode using Chromosorb P [21], followed by single-step conversion to TBDMS derivatives for the direct GC analysis on dual-capillary columns of different polarity. As expected, SPE procedure in anion-exchange mode using SAX tube was more efficient for clean-up of unwanted neutral components from urine matrix than solvent washing. In the preliminary experiments using aqueous samples spiked with known amounts of organic acid standards, the present dual SPE method was found to recover most of the organic acids tested with good precision. The relative standard deviation (R.S.D.) ($n=3$) was lower than 10% on average except for enanthic acid, α-ketovaleric acid, hippuric acid, protocatechuic acid and citric acid which gave R.S.D.s ranging from 11 to 16%.

First morning urine specimens of 12 uterine myoma patients and 14 uterine cervical cancer patients were screened for organic acids. Myoma patients were served as the benign tumor group (B-1 through B-12) and cervical cancer patients as the malignant tumor group (M-1 through M-14). As demonstrated in typical dual chromatograms of urinary organic acids from a myoma patient of the benign group (Fig. 1), the present profiling method was useful for producing good GC profiles with 0.25 ml of urine in comparative studies among the benign and malignant tumor patients. The peaks unresolved on the non-polar HP-5 column were well separated on the HP-50+ column of medium polarity, and vice versa. By computer comparison of *I* sets with the reference values in our *I* library, a total of 50 organic acids were positively identified from 26 urine samples studied. The *I* sets measured with dual-capillary columns were very useful in cross-checking each organic acid, which was further confirmed by GC–MS. Presently, our *I* and GC–MS libraries contain about 180 organic acid standards as TBDMS derivatives, which are being expanded for a more complete organic acid data base.

The concentrations of acids identified from triplicate runs of each urine sample on the Ultra-2 column were expressed as the mean peak area ratios corrected for urine creatinine (Tables 1 and 2). Large variations in the levels of organic acids from patient to patient even within each group were observed. Hippuric acid was most abundant only in two cases for the benign group, while it was in seven cases for

Fig. 1. Dual chromatograms of urinary organic acids from a uterine myoma patient of benign tumor group separated on HP-5 and HP-50+ (both 30 m×0.25 mm I.D., 0.25 μm film thickness) dual-capillary column system. GC conditions are described in Section 2.4. Peaks: 1=pyruvic acid; 2=benzoic acid; 3=lactic acid; 4=glycolic; 5=2-hydroxyisobutyric acid; 6=oxalic acid; 7=2-hydroxybutyric acid; 8=2-hydroxy-2-methylbutyric acid; 9=3-hydroxybutyric acid; 10=α-ketocaproic acid; 11=α-hydroxyisovaleric acid; 12=malonic acid; 13=methylmalonic acid; 14=ethylmalonic acid; 15=maleic acid; 16=succinic acid; 17=methylsuccinic acid; 18=glutaric acid; 19=3-methylglutaric acid; 20=*trans*-3-hexenedioic acid; 21=α-hydroxyphenylacetic acid; 22=adipic acid; 23=3-methyladipic acid; 24=α-ketoglutaric acid; 25=3-phenyllactic acid; 26=*m*-hydroxyphenylacetic acid; 27=pimelic acid; 28=hippuric acid; 29=*p*-hydroxyphenylacetic acid; 30=*p*-hydroxybenzoic acid; 31=malic acid; 32=citramalic acid; 33=phthalic acid; 34=3-indoleacetic acid; 35=suberic acid; 36=2-hydroxyglutaric acid; 37=4-hydroxy-3-methoxyphenylacetic acid; 38=4-hydroxy-3-methoxybenzoic acid; 39=azelaic acid; 40=palmitic acid; 41=*trans*-aconitic acid; 42=*cis*-aconitic acid; 43=*p*-hydroxymandelic acid; 44=stearic acid; 45=4-hydroxy-3-methoxymandelic acid; 46=*p*-hydroxy-phenyllactic acid; 47=ferulic acid; 48=citric acid; 49=isocitric acid; 50=hydroxyhippuric acid.

Table 1
Organic acids found in urine samples from 12 uterine myoma patients (benign tumor group)

No.	Acid	Amount (mean area ratio/mg creatinine)[a]													
		B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8	B-9	B-10	B-11	B-12	Mean[b]	Median[c]
1	Pyruvic	7.87	9.78	45.22	7.29	4.66	4.56	6.19	2.44	1.71	2.02	8.40	2.75	8.57	5.43
2	Benzoic	0.52	0.67	3.22	1.04	1.94	0.54	0.69	0.43	0.13	0.15	0.46	0.24	0.84	0.53
3	Lactic	11.68	14.19	64.91	10.36	7.52	3.92	8.12	7.28	2.50	5.91	8.57	5.16	12.51	7.82
4	Glycolic+2-hydroxyisobutyric[d]	6.86	6.08	25.68	5.26	5.29	2.11	4.41	3.26	1.36	1.58	3.55	3.47	5.74	3.98
5	Oxalic	17.08	15.60	9.12	8.16	15.16	6.59	9.37	8.74	2.81	2.60	9.07	9.42	9.48	9.10
6	2-Hydroxy-butyric	0.40	0.77	4.95	0.52	0.80	0.24	0.68	0.40	0.62	0.67	0.68	0.42	0.93	0.65
7	2-Hydroxy-2-methylbutyric	0.83	0.81	0.88	0.69	0.47	0.46	0.82	0.64	0.61	0.30	1.23	0.66	0.70	0.67
8	3-Hydroxybutyric	0.54	0.41	24.68	0.51	1.79	0.28	0.77	0.34	0.55	0.58	1.52	0.00	2.66	0.55
9	α-Ketocaproic+α-hydroxyisovaleric[d]	0.18	0.52	1.38	0.35	0.43	0.19	0.44	0.28	0.10	0.38	0.27	0.25	0.40	0.32
10	Malonic	1.48	1.61	1.81	0.40	0.58	0.46	0.64	0.35	0.16	0.13	0.39	0.35	0.70	0.43
11	Methylmalonic	0.85	3.53	1.87	1.31	0.87	0.98	4.16	1.44	0.67	0.73	0.88	1.29	1.55	1.14
12	Ethylmalonic	0.87	1.59	2.79	1.66	1.29	2.57	3.16	1.49	0.80	1.49	2.57	1.40	1.81	1.54
13	Maleic	0.27	0.95	2.78	0.71	0.58	0.27	0.31	0.32	0.13	0.12	0.38	0.44	0.61	0.35
14	Succinic	4.62	8.40	14.36	12.92	9.55	6.93	9.68	12.23	4.89	3.67	3.67	6.62	8.13	7.67
15	Methylsuccinic	0.57	0.88	2.37	1.64	1.30	0.78	1.60	0.81	0.62	0.41	1.44	0.78	1.10	0.85
16	Glutaric	0.78	1.67	2.50	1.25	1.18	0.90	0.89	1.07	0.63	0.29	0.88	0.65	1.06	0.90
17	3-Methylglutaric	1.18	0.63	1.56	0.58	0.65	0.54	0.52	0.28	0.45	0.31	1.16	0.39	0.69	0.56
18	*trans*-3-Hexenedioic	2.62	0.83	2.31	1.25	0.78	0.64	1.09	0.52	0.40	0.19	0.77	0.62	1.00	0.77
19	α-Hydroxyphenylacetic	0.74	0.55	2.40	0.42	0.32	0.56	0.33	0.28	0.25	0.10	0.38	0.37	0.56	0.38
20	Adipic	0.87	1.70	3.39	0.86	1.58	1.01	1.16	0.58	0.68	0.38	1.88	0.74	1.24	0.94
21	3-Methyladipic	0.93	0.83	3.78	2.76	1.35	1.49	1.57	1.21	0.68	0.45	0.60	1.19	1.40	1.20
22	α-Ketoglutaric	3.38	3.26	4.96	7.39	2.69	2.87	1.88	1.24	3.41	2.53	9.47	2.40	3.79	3.07
23	3-Phenyllactic	0.59	0.00	1.08	0.17	0.17	0.06	0.21	0.58	0.18	0.11	0.68	0.00	0.32	0.18
24	*m*-Hydroxyphenylacetic	0.39	0.25	1.70	0.83	0.17	0.37	0.34	0.15	0.17	0.15	0.27	0.33	0.43	0.30
25	Pimelic	0.26	0.29	0.98	0.18	0.19	0.14	0.25	0.32	0.11	0.08	0.21	0.32	0.28	0.23

26	Hippuric	8.74	14.86	10.98	26.04	14.90	24.-61	14.81	0.81	1.44	2.49	2.09	2.07	10.32	9.86
27	*p*-Hydroxyphenylacetic	11.84	15.77	39.32	8.07	15.07	3.72	45.84	1.87	8.67	2.79	7.87	6.33	13.93	8.37
28	*p*-Hydroxybenzoic+malic[d]	1.70	0.91	2.03	1.47	0.60	0.74	0.91	0.74	0.72	0.46	1.03	1.61	1.08	0.91
29	Citramalic	0.52	1.31	2.28	0.54	0.35	0.83	0.21	0.43	0.31	0.18	0.80	0.56	0.70	0.53
30	Phthalic	5.40	10.47	9.09	7.90	5.24	1.99	5.66	1.90	1.22	1.03	2.70	2.42	4.58	3.97
31	3-Indoleacetic	0.70	0.31	1.07	0.31	0.62	0.50	4.84	0.22	0.22	0.19	0.61	0.66	0.85	0.55
32	Suberic	0.56	1.00	1.42	0.69	1.02	0.61	0.73	0.14	0.08	0.00	0.12	0.00	0.53	0.59
33	2-Hydroxyglutaric	0.13	0.70	1.00	0.73	0.63	0.42	0.53	0.58	0.33	0.23	0.81	0.65	0.56	0.60
34	4-Hydroxy-3-methoxyphenylacetic	1.12	1.87	2.36	1.19	1.14	1.09	1.79	0.15	0.30	0.30	0.57	0.35	1.02	1.11
35	4-Hydroxy-3-methoxybenzoic	0.29	0.58	2.19	0.23	0.17	0.36	0.30	0.22	0.27	0.15	0.66	0.32	0.48	0.30
36	Azelaic	0.29	0.00	0.46	0.15	0.20	0.22	0.31	0.19	0.14	0.00	0.44	0.18	0.21	0.19
37	Palmitic	2.14	3.65	2.97	3.26	2.09	0.60	2.81	1.55	0.35	0.39	1.21	1.40	1.87	1.82
38	*trans*-Aconitic	7.34	13.01	11.13	9.40	13.81	9.21	12.25	10.22	6.67	3.92	7.01	12.18	9.68	9.81
39	*cis*-Aconitic	0.38	0.55	1.02	0.45	0.62	0.36	0.76	0.37	0.25	0.24	0.29	0.50	0.48	0.41
40	*p*-Hydroxymandelic	0.66	2.16	2.46	0.89	0.79	0.56	1.01	0.64	0.34	0.37	0.72	0.63	0.94	0.69
41	Stearic	3.04	5.79	4.28	4.87	3.26	0.71	4.68	1.85	0.43	0.43	1.39	1.80	2.71	2.44
42	4-Hydroxy-3-methoxymandelic	2.19	2.50	2.95	1.30	1.25	1.44	1.48	0.91	1.00	0.75	1.61	1.27	1.55	1.37
43	*p*-Hydroxyphenyllactic	0.46	0.69	1.64	0.50	0.38	0.40	0.80	0.42	0.24	0.25	0.85	0.44	0.59	0.45
44	Ferulic	0.38	0.76	0.87	0.66	0.37	0.23	0.30	0.24	0.12	0.00	0.40	0.27	0.38	0.34
45	Citric	2.23	6.69	4.50	20.00	5.92	4.20	3.35	5.44	8.29	4.16	5.73	5.55	6.34	5.49
46	Isocitric	1.31	2.63	5.55	0.68	1.42	1.20	1.02	0.90	0.86	0.49	2.51	2.10	1.72	1.26
47	Hydroxyhippuric	5.87	4.97	11.42	5.71	1.70	1.40	1.00	2.14	0.76	1.71	4.26	0.98	3.49	1.93

[a] Mean peak area ratios relative to I.S. (*trans*-cinnamic acid) from triplicate runs of each urine on an Ultra-2 column were corrected for the creatinine amount in 0.25 ml of urine.
[b] Mean peak area ratios of 12 patients.
[c] Median peak area ratios of 12 patients.
[d] Unresolved peaks.

Table 2
Organic acids found in urine samples from 14 uterine cervical cancer patients (malignant tumor group)

No.	Acid	Amount (mean area ratio/mg creatinine)[a]															
		M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	M-9	M-10	M-11	M-12	M-13	M-14	Mean[b]	Median[c]
1	Pyruvic	6.55	6.85	3.49	5.61	5.57	7.34	6.15	2.37	6.36	7.80	5.25	9.46	9.01	6.24	6.29	6.30
2	Benzoic	2.47	1.39	1.25	1.06	0.62	0.76	1.39	14.32	1.11	1.42	1.27	1.52	2.72	1.21	2.32	1.33
3	Lactic	12.32	8.29	9.92	5.54	9.31	13.23	5.67	10.61	12.47	21.00	7.10	17.40	11.48	9.79	11.01	10.26
4	Glycolic+2-hydroxyisobutyric[d]	4.37	4.24	4.50	4.36	4.15	3.57	3.64	3.36	6.09	13.04	3.11	10.24	7.35	3.83	5.42	4.30
5	Oxalic	1.91	11.79	8.87	4.55	9.18	3.23	5.40	2.52	28.92	50.44	20.91	19.63	25.60	10.60	14.54	9.89
6	2-Hydroxy-butyric	0.90	0.44	1.02	0.19	0.48	0.37	1.08	0.87	1.75	1.43	3.05	1.87	0.80	0.51	1.05	0.88
7	2-Hydroxy-2-methylbutyric	0.47	0.86	0.80	0.34	1.47	0.47	0.73	0.42	1.38	1.81	0.84	0.00	2.30	1.17	0.93	0.82
8	3-Hydroxybutyric	0.24	0.40	1.91	0.05	0.53	5.70	0.37	0.74	0.95	0.00	0.55	0.00	0.00	0.60	0.86	0.47
9	α-Ketocaproic+α-hydroxyisovaleric[d]	0.46	0.19	0.44	0.51	0.17	0.22	0.35	0.27	1.00	1.57	0.30	0.00	0.81	0.24	0.47	0.33
10	Malonic	1.19	0.44	0.52	0.68	0.56	0.91	0.74	0.34	1.20	0.78	0.70	1.10	0.98	0.54	0.76	0.72
11	Methylmalonic	1.43	0.89	2.90	2.60	1.07	1.27	1.13	0.63	1.07	1.63	0.83	2.44	2.51	1.63	1.57	1.35
12	Ethylmalonic	1.34	1.42	11.81	1.70	4.03	2.18	1.69	1.00	3.30	18.38	1.34	3.05	2.34	1.32	3.92	1.94
13	Maleic	0.58	0.81	1.09	0.67	0.53	0.32	0.57	0.43	0.84	0.71	0.34	1.28	1.12	0.31	0.69	0.63
14	Succinic	4.55	9.79	10.15	6.72	16.77	4.47	7.76	69.47	17.12	11.27	8.27	24.41	18.59	12.95	15.88	10.71
15	Methylsuccinic	0.57	0.93	0.91	1.49	1.29	2.46	1.65	1.41	0.94	1.38	1.19	1.75	2.18	1.98	1.44	1.40
16	Glutaric	0.88	1.13	1.87	2.16	0.99	0.72	1.40	2.22	2.43	2.06	1.30	3.17	1.66	1.94	1.71	1.76
17	3-Methylglutaric	0.83	0.74	0.98	0.81	1.59	3.61	0.45	0.97	0.93	1.55	1.83	0.50	2.75	0.89	1.32	0.95
18	*trans*-3-Hexenedioic	1.31	0.64	0.92	1.17	2.56	0.84	0.56	1.86	1.25	3.37	3.58	1.06	4.51	2.48	1.86	1.28
19	α-Hydroxyphenylacetic	14.54	0.95	0.63	1.02	0.80	5.49	0.93	0.27	0.67	0.00	0.39	0.00	0.00	0.48	1.87	0.65
20	Adipic	1.85	1.30	2.96	1.16	2.11	3.91	0.98	1.04	1.26	1.35	1.02	1.86	1.88	1.56	1.73	1.45
21	3-Methyladipic	2.18	2.32	3.12	2.65	2.67	2.41	0.81	0.81	2.67	5.80	1.27	0.00	3.84	3.27	2.42	2.53
22	α-Ketoglutaric	7.44	1.90	1.50	4.98	9.15	0.52	1.62	0.38	2.34	9.25	3.92	3.99	8.74	7.96	4.55	3.95
23	3-Phenyllactic	0.39	0.61	0.24	0.55	0.68	0.08	0.42	3.42	0.57	0.84	0.47	0.00	0.00	0.49	0.62	0.48
24	*m*-Hydroxyphenylacetic	0.35	0.31	0.21	0.53	0.33	0.57	0.47	0.40	0.36	0.00	4.24	1.21	1.50	1.20	0.83	0.44
25	Pimelic	0.57	0.52	0.20	0.53	0.27	3.67	0.08	0.39	0.62	0.00	0.68	0.00	0.90	0.40	0.63	0.46

26	Hippuric	93.77	37.25	17.72	47.89	8.48	6.59	30.97	3.63	3.15	15.37	20.18	16.60	73.57	30.94	29.01	18.95
27	*p*-Hydroxyphenylacetic	3.61	8.08	5.14	27.97	13.74	4.51	7.71	10.78	19.40	27.93	5.00	8.92	12.90	5.86	11.54	8.50
28	*p*-Hydroxybenzoic+Malic[d]	3.29	1.47	1.51	0.85	0.63	0.92	1.51	4.78	2.32	1.30	5.23	1.09	3.05	2.22	2.15	1.51
29	Citramalic	4.25	0.59	0.30	1.57	1.11	1.38	0.56	0.62	0.41	2.16	2.39	1.43	1.58	1.27	1.40	1.33
30	Phthalic	6.89	6.90	17.55	5.53	5.95	3.11	3.86	2.98	6.48	17.52	2.94	5.92	3.30	4.95	6.71	5.72
31	3-Indoleacetic	0.92	0.90	0.63	0.90	0.37	1.53	0.19	0.40	1.81	0.00	0.79	0.96	1.15	0.36	0.78	0.84
32	Suberic	0.77	0.12	1.15	1.01	0.71	0.60	0.20	0.46	0.14	0.33	0.00	0.45	0.38	0.21	0.47	0.42
33	2-Hydroxyglutaric	0.20	0.11	3.67	0.48	0.94	7.54	0.47	0.74	1.16	1.36	9.03	1.96	12.67	9.68	3.57	1.26
34	4-Hydroxy-3-methoxyphenylacetic	1.77	1.76	2.66	2.07	1.16	1.60	0.98	1.78	0.62	0.51	0.80	0.64	1.14	5.22	1.62	1.38
35	4-Hydroxy-3-methoxybenzoic	0.45	0.75	0.24	0.98	0.66	0.92	0.83	0.51	0.36	0.41	0.50	0.65	0.47	0.18	0.57	0.51
36	Azelaic	0.39	0.81	0.19	0.32	0.65	0.51	0.26	0.22	0.37	0.00	0.26	0.62	1.09	1.69	0.53	0.38
37	Palmitic	1.33	1.16	5.13	1.01	1.49	0.97	2.21	1.69	3.21	3.38	1.86	3.03	2.19	2.27	2.21	2.03
38	*trans*-Aconitic	8.80	15.81	13.53	12.94	17.93	13.24	9.17	1.58	15.04	15.32	10.70	30.48	19.74	16.03	14.31	14.28
39	*cis*-Aconitic	1.24	0.11	2.07	0.87	0.78	0.44	0.86	0.04	0.76	3.96	0.71	1.17	0.75	0.89	1.05	0.82
40	*p*-Hydroxymandelic	2.58	0.87	0.56	1.42	0.80	1.03	1.04	0.97	0.71	1.61	0.68	0.89	1.28	3.78	1.30	1.00
41	Stearic	1.32	1.22	6.59	1.31	2.18	0.24	3.77	1.77	3.72	3.97	2.21	3.29	2.19	2.34	2.58	2.20
42	4-Hydroxy-3-methoxymandelic	2.28	2.23	1.00	2.03	4.29	2.54	1.00	1.32	0.77	1.64	1.45	1.40	2.52	1.42	1.85	1.54
43	*p*-Hydroxyphenyllactic	1.82	0.55	0.95	0.60	0.34	0.79	0.35	0.88	0.61	2.10	0.70	0.00	0.88	1.10	0.83	0.75
44	Ferulic	0.00	0.84	0.42	2.12	0.98	0.66	0.73	0.59	0.57	0.00	0.55	0.89	1.77	0.50	0.76	0.63
45	Citric	3.96	4.86	6.88	3.74	22.57	8.52	4.69	3.67	9.58	9.78	7.45	15.23	33.70	17.59	10.87	7.99
46	Isocitric	2.22	1.99	1.33	1.54	2.02	1.99	13.01	0.08	1.58	2.16	7.58	2.82	3.69	0.91	3.07	2.00
47	Hydroxyhippuric	4.28	6.25	1.86	4.10	3.21	3.32	2.74	4.46	6.41	1.45	8.60	2.86	3.22	9.46	4.45	3.71

[a] Mean peak area ratios relative to I.S. (*trans*-cinnamic acid) from triplicate runs of each urine on an Ultra-2 column were corrected for the creatinine amount in 0.25 ml of urine.
[b] Mean peak area ratios of 14 patients.
[c] Median peak area ratios of 14 patients.
[d] Unresolved peaks.

the malignant group. In median area ratios, the most abundant acid of the benign group was hippuric acid, followed by *trans*-aconitic acid, oxalic acid, *p*-hydroxyphenylacetic acid, lactic acid, succinic acid, citric acid, while the orders of oxalic acid and succinic acid were switched, and lactic acid and *p*-hydroxyphenylacetic acid were switched in the malignant group. Overall organic acid levels of the malignant group appeared to be higher compared to those of the benign group.

When the complex GC data in tabular form were transformed to their corresponding organic acid *I* spectra in bar graphical form [19], visual comparison between samples was much easier as exemplified by two group average *I* spectra (Fig. 2). Overall patterns of the average *I* spectra look similar qualitatively, but the differences between them in quantities of several prominent and minor acid peaks are readily detected by visual inspection.

It was desired to classify organic acid profiles of 26 urine samples into two groups according to tumor types. However, the jumbles of numbers in Tables 1

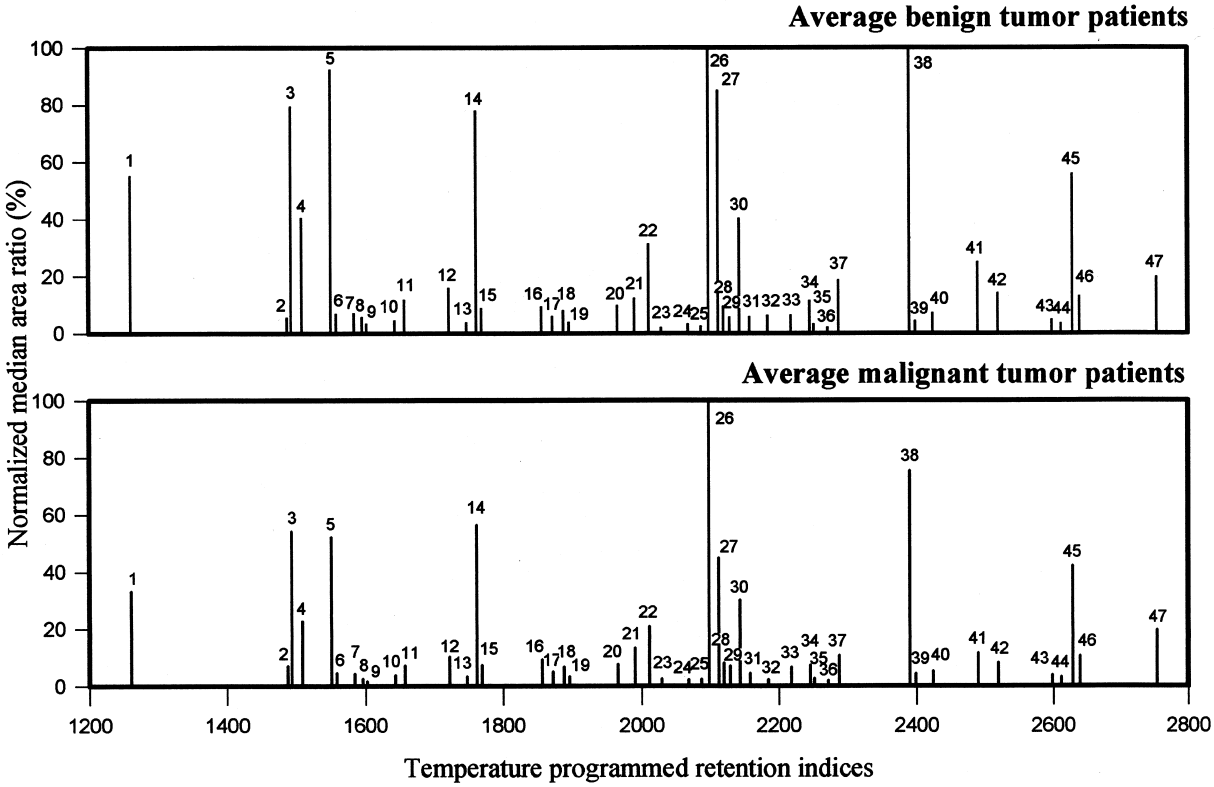

Fig. 2. Average retention index spectra of urinary organic acids from benign tumor group (uterine myoma patients) and malignant tumor group (uterine cervical cancer patients) separated on an Ultra-2 (25 m×0.20 mm I.D., 0.33 μm film thickness) capillary column. GC conditions are described in Section 2.4. Peaks: 1=pyruvic acid; 2=benzoic acid; 3=lactic acid; 4=glycolic+2-hydroxyisobutyric acid; 5=oxalic acid; 6=2-hydroxybutyric acid; 7=2-hydroxy-2-methylbutyric acid; 8=3-hydroxybutyric acid; 9=α-ketocaproic acid+α-hydroxyisovaleric acid; 10=malonic acid; 11=methylmalonic acid; 12=ethylmalonic acid; 13=maleic acid; 14=succinic acid; 15=methylsuccinic acid; 16=glutaric acid; 17=3-methylglutaric acid; 18=*trans*-3-hexenedioic acid; 19=α-hydroxyphenylacetic acid; 20=adipic acid; 21=3-methyladipic acid; 22=α-ketoglutaric acid; 23=3-phenyllactic acid; 24=*m*-hydroxyphenylacetic acid; 25=pimelic acid; 26=hippuric acid; 27=*p*-hydroxyphenylacetic acid; 28=*p*-hydroxybenzoic acid+malic acid; 29=citramalic acid; 30=phthalic acid; 31=3-indoleacetic acid; 32=suberic acid; 33=2-hydroxyglutaric acid; 34=4-hydroxy-3-methoxyphenylacetic acid; 35=4-hydroxy-3-methoxybenzoic acid; 36=azelaic acid; 37=palmitic acid; 38=*trans*-aconitic acid; 39=*cis*-aconitic acid; 40=*p*-hydroxymandelic acid; 41=stearic acid; 42=4-hydroxy-3-methoxy-mandelic acid; 43=*p*-hydroxyphenyllactic acid; 44=ferulic acid; 45=citric acid; 46=isocitric acid; 47=hydroxyhippuric acid.

and 2 showed no readily apparent patterns. Therefore, the GC quantitative data were subjected to stepwise discriminant analysis. Of the 50 organic acids, only 16 were chosen as the variables which discriminate 14 cancer patients from 12 myoma patients. Glutaric acid was selected as the most discriminant variable, followed by 3-methylglutaric acid, glycolic acid+2-hydroxyisobutyric acid, hippuric acid, *cis*-aconitic acid, 4-hydroxy-3-methoxybenzoic acid, methylmalonic acid, azelaic acid, α-ketoglutaric acid, 4-hydroxy-3-methoxymandelic acid, suberic acid, palmitic acid, 3-indoleacetic acid, oxalic acid, ferulic acid and lactic acid. Unlike the previous result on the smoking effect [19], star symbol plots drawn based on the area ratios of the 16 variables were not useful for visual pattern recognition between individual as well as groups.

However, when canonical discriminant analysis was applied to these 16 acids as data vectors, 26 urine specimens were well separated into two distinct clusters in the two-dimensional canonical function space (Fig. 3). All of the urine samples were correctly classified into their respective tumor types. Each of the cervical cancer patients was represented by M and myoma patients by B.

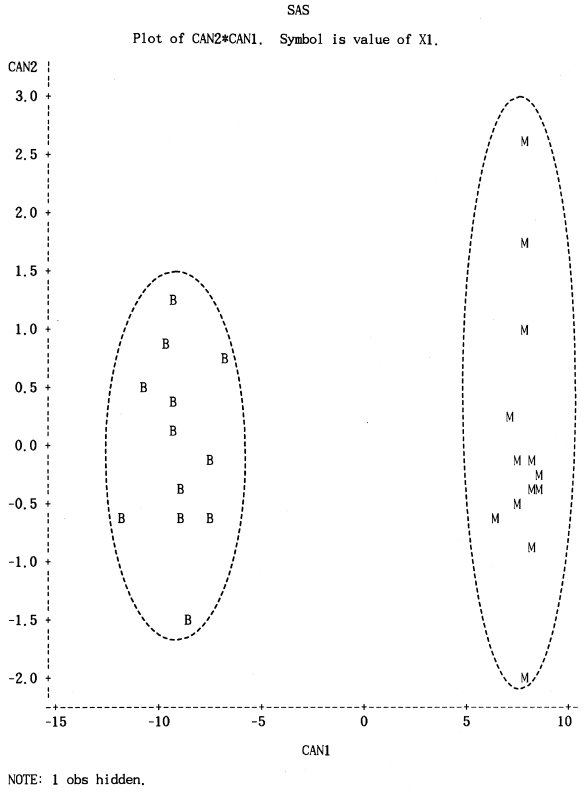

Fig. 3. Plot of the first and second canonical functions of the 16 acid variables for the 12 uterine myoma patients (each represented by B) and 14 uterine cervical cancer patients (each represented by M). Variables: glutaric acid, 3-methylglutaric acid, glycolic acid+2-hydroxyisobutyric acid, hippuric acid, *cis*-aconitic acid, 4-hydroxy-3-methoxybenzoic acid, methylmalonic acid, azelaic acid, α-ketoglutaric acid, 4-hydroxy-3-methoxymandelic acid, suberic acid, palmitic acid, 3-indoleacetic acid, oxalic acid, ferulic acid and lactic acid.

4. Conclusions

The present dual SPEs and silylation with subsequent dual-capillary column GC analysis were suitable for the routine profiling and screening for organic acids in urine. Simplification of GC profiles to their corresponding organic acid *I* spectra of bar graphs enabled one to readily detect quantitative differences among samples. When stepwise discriminant analysis was performed to select variables discriminating GC profiles from 14 uterine cancer patients and 12 uterine myoma patients, glutaric acid was selected as the most discriminant variable, followed by 3-methylglutaric acid, glycolic acid+2-hydroxyisobutyric acid, hippuric acid, *cis*-aconitic acid, 4-hydroxy-3-methoxybenzoic acid, methylmalonic acid, azelaic acid, α-ketoglutaric acid, 4-hydroxy-3-methoxymandelic acid, suberic acid, palmitic acid, 3-indoleacetic acid, oxalic acid, ferulic acid and lactic acid. Canonical discriminant analysis applied to these 16 organic acid variables correctly classified 26 urine samples into two separate clusters according to cervical cancer or myoma. From our results, it can be stated that there might be some correlation between urinary organic acid levels and uterine cervical cancer for the 26 urine specimens studied.

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