Metabolite Profiling of Urine from Healthy Filipino Women Using High Performance Liquid Chromatography

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Abstract: Breast cancer is currently the leading cause of cancer deaths in the Philippines and there are a lot of instances when it is not detected early enough to be treated. Metabolomics, which is the analysis of metabolites by separation and detection in biological systems, can be used to detect breast cancer. In this study, metabolites in the urine of healthy Filipino women were studied using high performance liquid chromatography (HPLC). Conditions were optimized for the collection, storage and chromatographic separation of urine metabolites. The shelf life of the urine samples was prolonged by the addition of toluene which prevented volatile compounds from evaporating and resulted in minimal loss of compounds in the sample. The conditions for the HPLC separation were successfully optimized. It was found that a linear gradient run is more appropriate than an isocratic run for this study. The solvent system used was a linear gradient (0-10 minutes = 5% A: 95% B, 10-50 minutes = 20% A: 80% B, 50-60 minutes 5% A: 95% B) where the solvent A is 0.1% formic acid in acetonitrile and solvent B is 0.1% formic acid in water. Comparison of the different metabolites found in the urine sample was done. A urinary metabolomic profile for healthy Filipino women was established which shows the minimum, maximum and average concentrations of specific metabolites.

Key Words: urine profiling; metabolomics; biomarkers; high performance liquid chromatography

1. INTRODUCTION

Currently, the World Health Organization ranked cancer as the third cause of deaths in the Philippines ("The Top 10 Causes of Death", 2011). Among all the cancers, breast cancer is the most common type (Valmero, 2010). As of now, there are several tests in which breast cancer can be detected such as mammogram, breast MRI, breast ultrasound, etc ("Breast Cancer: Diagnosis and Tests", n.d.). Some of these tests offered now are not very reliable in detecting breast cancers like mammography.

Mammography cannot detect little tumors if there is a high density of tissues in the breast. It often leads to false positives (Asiago et al., 2010). On the other hand, metabolomic profiling of breast cancer biomarkers using high-performance liquid chromatography (HPLC) offers a non-invasive and cheaper alternative for the early detection of breast cancer.

Metabolomics is the analysis of metabolites in biological systems by separation and detection. It is a rapidly evolving discipline which contributes greatly in

drug discovery and biomedical research. It is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind." It is specifically the study of small-molecule metabolite profiles ("Metabolomics", 2010). It came from the word "metabolome" which mirrors the events in the past which includes the mechanism and the environment interaction ("The Human Metabolome Project", n.d.). Metabolome forms a large network of metabolic reactions which has a system known as the hypercycles where the outputs from one enzymatic chemical reaction are inputs to other chemical reactions. It is like the transcriptome and proteome which is dynamic and changing from second to second. The molecules that constitute the metabolome are called metabolites which are the end products of its gene expression. Metabolites are the intermediates and products of the metabolism (Roux, Lison, Junot, and Heilier, 2011).

Each individual have homeostasis. An interaction with the environment, such as drug and chemical exposure or even the beginning of diseases disorganizes the homeostasis including the metabolites. From this, biomarkers arise (Roux, Lison, Junot, and Heilier, 2011). Biomarkers are quantifiable signals of cellular or molecular changes which appears in the organism during or after exposures to possible diseases or toxicants. It is important in cancer control research because they are sometimes related to the risk of the disease ("Risk Factor Monitoring & Methods", 2011). These biomarkers can be detected in biofluids found in our body such as urine.

In metabolomic testing, urine samples will be obtained from individuals and it will be tested in HPLC to be able to detect the concentration of certain metabolites.

2. METHODOLOGY

1.1. Materials, Reagents, and Apparatus

The compounds were purchased from Chemline. The compounds are all analytical grade. High-performance liquid chromatography gradient grade acetonitrile and formic acid were used. For the analysis, an Agilent Technologies 1200 Series HPLC system with a UV detector was used. The reversed phase column used was a Pinnacle II C18, 250 x 4.6 mm and 5µL particle size.

1.2. Collection of Urine Samples

Urine samples were obtained from 25 healthy Filipino female volunteers. The subjects were of age 35-60

and currently do not have a breast cancer. The subjects were asked to sign an informed consent and questionnaire to participate in the research.

The subjects were asked to obtain the midstream of the urine. The midstream is done to eliminate possible contamination. Right after collection, the sample was immediately brought to the laboratory and toluene was added on top to coat the urine. This will prevent volatile components present in the urine from evaporating. Then the sample was centrifuged for 1 hour at 1500 rpm and 4°C. The centrifuge can only accommodate 4 samples at a time, so the other samples were stored in the refrigerator with a toluene coat on top for the preservation of metabolites.

1.3. Preparation of Internal Standards

Two internal standards were used in the study. These two were 100 ppm tyrosine and 1000 ppm phenol. The phenol was chosen because it contains a ring system and from our references, most of the biomarkers for breast cancer contain an aromatic ring. The tyrosine was also used since it was used in another study which profiles the metabolites found in saliva.

To make the concentrations of the internal standards which are 100 ppm tyrosine and 1000 ppm phenol in the solution in the HPLC vial, 1000 ppm of tyrosine and 10 000 ppm of phenol were prepared.

1.4. Sample Preparation

Aliquots of 5 mL of the urine samples were transferred into centrifuge tubes. Toluene was added on top of the urine sample to preserve the metabolites. These were centrifuged for 60 minutes at 1500 rpm and 4°C. The supernatants that were obtained after centrifugation were used in the analysis. In an HPLC vial, a solution containing 1320 µL of urine sample, 190 µL of 1000 ppm tyrosine, and 190 µL of 10 000 ppm phenol were added. After mixing the other compounds, 200 µL of toluene was added in the vial.

1.5. HPLC Analysis

Separations were performed using a reversed-phase high performance liquid chromatography where Pinnacle II C18, 250 x 4.6 mm and 5 µL particle size and Agilent Technologies 1200 Series HPLC system with UV detector were used. HPLC separation was performed using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) as the mobile phases. The flow rate of the mobile phase used was 0.5mL per minute at

40˚C. The wavelength for the UV absorption used was 254 nm. This was determine by doing the wavelength scan using the UV-Vis Spectrophotometer. The injection volume of the sample was 5μL, and an isocratic run for 0-10 minutes at 5% solvent B then a gradient to 20% solvent B for 40 minutes (10-50 minutes), and lastly a gradient to 5% solvent B for 10 minutes (50-60 minutes) was done.

1.6. Data Processing

The concentrations of the metabolites were calculated through ratio and proportion. The peak area and the concentration of the internal standard 1000 ppm phenol were used in the calculation. The different concentrations of the metabolites calculated from each of the 25 urine samples were compared and the mean, median, maximum values, minimum values, and percent of occurrence were obtained. These values were used to establish the metabolomic profile of healthy Filipino subjects.

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3. RESULTS AND DISCUSSION

The internal standard was injected in the HPLC to determine the retention time of the internal standards, 1000 ppm phenol and 100 ppm tyrosine. The internal standard was used in the study to determine the concentrations of the components eluting in the chromatogram. The retention time of 1000 ppm phenol and 100 ppm tyrosine were found to be at 25.860 minutes and 7.277 minutes, respectively.

Table 1. *Table of Comparison of the Minimum and Maximum Values, Mean, and Frequency of Occurrence of the Metabolites*

Metabolite #	Average Retention Time (min)	Minimum Peak Area (mAU)	Maximum Peak Area (mAU)	Median Peak Area (mAU)	Average Peak Area (mAU)	Conc. Relative to Internal Standard (ppm)	Frequency of Occurrence (%)
1	4.2024	18.12802	60.70305	21.4230	28.7617	22.71061	24
2	4.428	55.03976	74.40704	64.7234	64.7234	47.12485	8
3	4.59875	9.71887	94.37059	41.5794	42.3587	32.68347	40
4	4.85675	39.35015	71.25291	49.1329	53.2453	38.47349	12
5	5.0020833	17.37346	875.10718	43.8319	103.847	83.87601	60
6	5.2245	11.72688	20.39409	16.0605	16.0605	11.72812	8
7	5.6524285	35.75842	6055.1641	183.959	727.499	544.0734	64
8	6.0958888	33.4758	1022.5984	128.011	342.251	288.1738	36
9	6.3993181	987.43866	9168.9141	3848.64	3911.16	3118.833	100
10	6.51975	97.24826	397.32513	146.702	196.994	140.1487	16
11	6.7549545	79.88397	650.77997	272.914	307.740	243.6582	92
12	7.028579	40.67312	699.56488	244.569	275.105	221.5135	84
13	7.269	51.40049	163.53401	100.115	99.3287	82.08228	20
14	7.4295455	18.11467	183.46291	69.3231	91.6789	70.05458	44
15	7.6080526	66.18557	594.61646	150.141	192.152	147.7528	80
16	7.9418636	65.31311	978.84125	371.093	434.808	348.385	96
17	8.211	198.23991	198.23991	198.24	198.24	163.7537	4
18	8.4437333	31.93575	778.76178	181.797	237.697	189.0541	68
19	8.6472	41.25295	244.73167	90.3789	114.847	94.69582	24
20	8.8646	19.12767	240.79298	117.82	137.408	105.7642	44
21	9.1863529	28.52301	499.44208	108.074	145.153	119.2723	68
22	9.504	31.17968	546.49774	118.761	182.015	139.6273	52
23	9.7567	17.7834	976.50726	122.239	218.505	187.2002	40

The samples were then injected in the HPLC after the determination of the retention time of the internal standards. The peaks from the chromatograms of the 25 urine samples were compared and the retention times

were grouped according to the peak shape. The data obtained from the chromatograms of the samples were summarized in Table 1.

The percent of occurrence shown in the table indicates the commonly occurring metabolites in the healthy subjects. These metabolites which are commonly occurring are the ones needed to monitor for the comparison of the breast cancer patients and normal subjects.

In Figure 1, it can be seen that some of the values in the samples are on the extremes. The differences in these values are due to different variables which are not controlled. These are the following: time of collection, time from collection of the sample to addition of toluene, and diet and lifestyle.

The concentration of the urine varies from the different time of the day. The best time to collect a urine sample is during morning; usually the first urine should be collected because this is the most concentrated urine ("Urine Specimens", 2001). This was not strictly followed in the study due to limited time and to the subjects. Not all of the subjects followed the protocols given to them. Since the urine concentration varies depending on the time of the day, the observed data in the Figure 1 are sort of scattered.

The time from collection to the addition of toluene is also a possible cause in the differences in concentration of the metabolites. After collection, the travel time of the sample from the place of collection to the laboratory varies from person to person. The others were able to provide us with the sample faster than the others and this resulted in higher concentrations of metabolites than the ones that gave the urine sample at a longer period of time.

And lastly, according to Star, Kimmel, and Bonventre (n.d.), the diet and lifestyle of the person affects the concentration and components of the urine sample. Due to limited time, it wasn't possible to strictly control this.

The chromatograms of the healthy subjects showed different retention time for the internal standard. This suggests that the other metabolites also elutes this way wherein their retention times differ from one chromatogram to another. According to Waybright et al. (2006), this is due to the different chemical properties present in different urine samples. Since the instrument used was only HPLC and there is no means to gather the mass of the components eluted. A comparison of the chromatograms was done to determine the retention times of the metabolites. The retention times were then grouped according to similarities in the peaks and were recorded in a table.
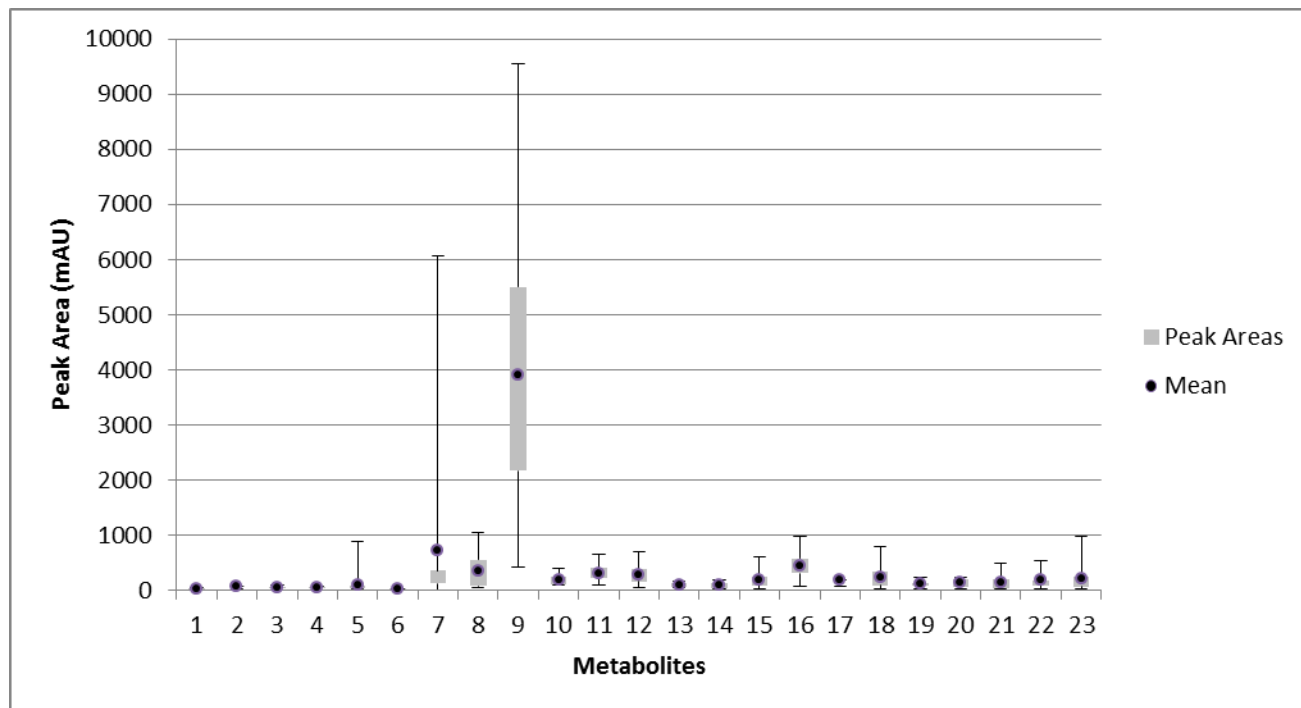
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Figure 1: Comparison of the Minimum and Maximum Value and Mean of Metabolites 1-23

4. CONCLUSIONS

This study aims to profile the different urinary metabolites of healthy Filipino women. The study was able to successfully optimize the conditions for the storage of the sample and the HPLC conditions for separation of urinary metabolites.

The urinary metabolite profiles of healthy subjects were successfully compared. Approximate values of the minimum, maximum, median, average values and frequency of occurrence were determined based on internal standard. The range of concentrations of the metabolites commonly occurring among healthy Filipino female subjects were established.

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