Development of Quality Control Method for Glucofarmaka Antidiabetic Jamu by HPLC Fingerprint Analysis

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Received October 31, 2016; Accepted January 6, 2017

ABSTRACT

Herbal medicines become increasingly popular all over the world for preventive and therapeutic purposes. Quality control of herbal medicines is important to make sure their safety and efficacy. Chromatographic fingerprinting has been accepted by the World Health Organization as one reliable strategy for quality control method in herbal medicines. In this study, high-performance liquid chromatography fingerprint analysis was developed as a quality control method for glucofarmaka antidiabetic jamu. The optimum fingerprint chromatogram was obtained using C18 as the stationary phase and linear gradient elution using 10–95% acetonitrile:water as the mobile phase within 60 min of elution and detection at 210 nm. About 20 peaks were detected and could be used as a fingerprint of glucofarmaka jamu. To evaluate the analytical performance of the method, we determined the precision, reproducibility, and stability. The result of the analytical performance showed reliable results. The proposed method could be used as a quality control method for glucofarmaka antidiabetic jamu and also for its raw materials.

Keywords: fingerprint analysis; glucofarmaka; antidiabetic jamu; HPLC; quality control

ABSTRAK


Kata Kunci: analisis sidik jari; KCKT; jamu antidiabetes; glucofarmaka; kendali mutu

INTRODUCTION

Nowadays, diabetes is one of the most worrisome health issues. Its prevalence has increased significantly in recent years. In 2015, a patient with diabetic reached about 415 million people all around the world. Diabetes contributes up to 14.5% to global death [1]. Treatment for this disease usually involves synthetic drugs to decrease the blood sugar level. However, the use of the synthetic drugs sometimes causes negative side effects [2]. This situation makes some people change their choice of drugs to herbal medicines which are considered as relatively safer.

Centre-Bogor Agricultural University has developed a new formula of antidiabetic jamu called glucofarmaka. This antidiabetic jamu consists of four plants (Tinospora crispa, Zingiber officinale, Blumea balsamifera, and Momordica charantia). In vivo assay for glucofarmaka using streptozotocin-induced mice showed a fairly good result in decreasing blood glucose level after 2 weeks of treatment [3].

The use of different sources of raw material in a large scale production will result in inconsistency of efficacy and quality of herbal medicine. Raw materials from different sources will give the different composition of chemical components that in the end, it

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DOI: 10.22146/ijc.23616
will affect the final product's efficacy. Thus, a quality control method is required to guarantee the quality of the raw materials used in the production of the herbal medicines in order to provide consistency in efficacy. In general, there are two approaches to the development of the quality control method for herbal medicines, namely compound-based and pattern-based approaches [4].

The compound-based approach uses one or some chemical compounds in the herbal medicines related to their quality. However, the use of marker compound sometimes is not relevant because generally, the efficacy of jamu comes from many compounds work in synergy [5]. The pattern-based approach sometimes is also called fingerprint analysis, will evaluate all detectable compounds in herbal medicines from an analytical instrument, such as TLC, HPLC, or FTIR without the necessity to characterize all compounds. Therefore, this approach will give a comprehensive representation of all compounds in the herb materials for evaluating the quality in order to guarantee the consistency of the raw materials and the herbal products. Accordingly, fingerprint analysis has the advantages to show all metabolites detected in a sample and their relative concentration proportion [6]. High-performance liquid chromatography (HPLC) is regarded as a popular analytical instrument applied to develop fingerprint of herbal medicines due to precision, sensitivity, and reproducibility. It is not limited by the volatility or stability of the sample compounds. Therefore, HPLC can be used to analyze almost all compounds that present in the herbal medicines [7].

In this study, we developed a fingerprint analysis method by using HPLC for quality control of glucofarmaka antidiabetic jamu. In addition, we also performed metabolite profiling using LC-MS (Liquid Chromatography-Mass Spectrometry) for identifying metabolites present in glucofarmaka jamu that can be used for further studies to find a marker compound of this jamu.

EXPERIMENTAL SECTION

Materials

Sembung leaves (Blumeabalsamifera), brotowali stems (Tinosporacrispa), ginger rhizomes (Zingiberofficinale), and pare leaves (Momordicacharantia) were used to make glucofarmaka antidiabetic jamu. All samples were obtained from Biopharmacu Research Centre, Bogor Agricultural University and identified in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, Indonesia. Voucher specimens of all samples were deposited at Biopharmacu Research Center, Bogor Agricultural University, Indonesia (BMK 0043052015–BMK 0046052015). Distilled water, methanol, and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Ekicrodisc 25R filter 0.45 µm was purchased from Gelman Science Inc. (Tokyo, Japan).

Instrumentation

HPLC LC-20A series (Shimadzu, Tokyo, Japan) with diode array UV detector, Shim-pack column VP-ODS C18 (150 mm x 4.6 mm i.d., particle size 4.6 µm) (Shimadzu, Tokyo, Japan) was used for HPLC fingerprint analysis. UPLC-QTOF-MS Waters Xevo G-2S using C18 column (50 mm x 2.1 mm i.d., particle size 1.7 µm) was used for identification of metabolites present in glucofarmaka antidiabetic jamu. MZmine 2.14,2 version software was used for data analysis from UPLC-QTOF-MS.

Procedure

Preparation of antidiabetic jamu extract

All samples were dried and pulverized prior to use. Powdered samples were weighed and mixed as the appropriate composition of the jamu formula to give 400 g total weight. Subsequently, the powder was poured into 5 L flask and added with 2800 mL of aquadest and boiled for 1 h. The extract was filtered by using a gray cloth to obtain a filtrate. The extraction was repeated twice. The resulting jamu filtrate was collected and concentrated using vacuum evaporator.

Optimization of HPLC fingerprinting of antidiabetic jamu

About 6 g of a powder mixture of all samples was diluted with 40 mL of methanol p.a and then sonicated for 2 x 15 min. After that, the extract was filtered through 0.45 µm membrane filter. This solution was injected into HPLC with an injection volume of 20 µL. The column temperature was set to 35 °C with a flow rate of the mobile phase is 1 mL/min. The parameters varied in the optimization of HPLC fingerprint analysis were the type and the composition of the mobile phase, and the length of analysis time. The wavelengths used were 210, 254, and 280 nm. A mixture of water and an organic modifier (acetonitrile or methanol) was used for mobile phase. First, elution was performed with a linear gradient of 5–95% organic modifier. Next, the initial and the final points of the organic modifier were determined by the elution profile obtained in the first step [8].

Analytical performance of HPLC fingerprint method

Analytical performance of HPLC fingerprint was determined by using chromatograms of glucofarmaka jamu extract. The parameters used for evaluation were
precision, reproducibility, and stability expressed as relative standard deviation value (RSD) of the relative retention time (RRT) and relative peak area (RPA) towards reference peak. The precision test was based on the RSD value of the RRT and the RPA of 5 injections of the same sample solution. Reproducibility test was performed by injecting 5 sample solutions prepared independently. The stability test was done by analyzing the sample solutions at 0, 3, 6, 24, and 48 h after prepared at room temperature.

**Sample preparation and metabolite profiling using LC-MS**

About 200 mg of glucofarmaka jamu extract powder was diluted with 10 mL of methanol p.a, sonicated for 5 min and filtered by using 0.45 µm membrane filter. 5 µL sample was injected into LC-MS. Mass Spectrometer was operated in negative ion mode with approximation at m/z 30 to 700. ESI parameters used include the capillary temperature at 120 °C, 50 L/h gas injection, +2.9 kV potential source, temperature source at 41 °C. The eluent used was 10–90% ACN-water for 10 min in gradient elution system.

**Data analysis**

LC-MS output was converted into NetCDF format so that it could be easier to process. Then, LC-MS chromatogram was processed by MZmine software to change the chromatogram data into a mass array. MZmine data processing was carried out in some steps, i.e. filtering and baseline correction, peak detection, deisotoping, alignment, gap filling, and normalization [9]. The mass arrays are given contained information of the detected peak mass, retention time, intensity, and normalized peak area.

**Interpretation and metabolite identification of glucofarmaka jamu extract**

Identification of metabolite from the output data of LC-MS was carried out by comparing m/z of the mass array to the metabolite compound listed in KNApSAcK database and comparing the mass fragmentation patterns with the mass fragmentation pattern of the predicted compounds in literature.

**RESULT AND DISCUSSION**

**Optimization of HPLC Fingerprint of Antidiabetic Jamu Extracts**

Chromatographic fingerprint analysis can be used as a quality control method of plant extracts. In this study, HPLC fingerprint was used to develop a quality control method for glucofarmaka antidiabetic jamu. Optimization of the HPLC condition was conducted in order to obtain a good separation profile of chemicals present in the samples. The samples used in this work are powder sample of each plant, a mixture of 4 plants powder sample as the ingredients of the glucofarmaka jamu formula and water extract of the glucofarmaka antidiabetic jamu. A good fingerprint profile will show the greatest amount of well-separated peaks on the chromatogram with short analysis time [10].

In the initial step of optimization, the sample solution was eluted in HPLC using an organic modifier, i.e. ACN or MeOH (5–95%), for 50 min. The detection wavelengths used were 210, 254, and 210 nm. The 210 nm wavelength was chosen based on its typical usage in HPLC analysis for 3 plants of jamu constituents, i.e. sembung [11], brotowali [12], and pare [13]. The 254 nm wavelength was used because most of the organic compounds would have aromatic rings that will absorb radiation at this wavelength. Meanwhile, 280 nm detection was conducted because ginger extract shows excellent chromatogram profile at
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Fig 3. HPLC chromatogram of simplicial mixture with the organic modifier percentages of 5–95% (a); 5–70% (b); and 10–80% (c) of CAN

This wavelength [14]. Among these detection wavelengths, the best result was obtained at 210 nm (Fig. 1). At this wavelength, the obtained number and the intensity of the peaks were higher than others and gave the best resolution. The use of organic modifiers, such as ACN, gave better chromatogram profile than MeOH. Fig. 2 showed that the chromatogram baseline of the MeOH-water mobile phase tended to rise as the MeOH percentage increased. It is believed that the MeOH will absorb more UV radiation at the wavelength of 205–235 nm as its concentration increases [15]. Thus, the 210 nm wavelength and ACN organic modifier were used in the optimization step.

Next, the upper and lower limits of ACN percentage in mobile phase were varied. As can be seen in Fig. 3, 5–70% ACN gave fairly good separation, but some peaks did not appear. This could be due to the compounds were retained in the column and could not be eluted with the ACN concentration below 70%. The 10–80% ACN percentage gave better chromatogram profile but some peaks did not appear. The 5–95% ACN percentage gave the best amount of peaks. Therefore, the upper limit of ACN percentage was determined at 95%. Some peaks which shown at the retention time below 2 min were not affected significantly by the modification of ACN lower limit, from 5 to 10%. It is expected that those peaks come from compound components which did not retain in the column. According to literature, the peaks with a retention time of 2 min or less is expected to come from unretained compounds [16]. Thus, the resolution was not affected by the percentage of the organic modifier. Consequently, the concentration of the composition of ACN organic modifier used was 10–95%.

The analysis time length parameter is one of the important factors which should be considered in the development of HPLC fingerprint method. The longer the analysis time, the better the separation and resolution that overlapped peaks will be separated well. On the other hand, it will not be efficient if this method applied to many samples. Thus, it is required to find the optimum time to compromise them. As shown in Fig. 4, the obtained higher resolution and the peak number increase as the length of analysis time increases. The best chromatogram profile was obtained at the analysis time of 90 min. However, it was too long and not efficient to be applied. The fairly excellent compromise result was obtained at the analysis time of 60 min which gave a fairly good peak resolution and acceptable analysis time period.

The optimized values of the HPLC fingerprint profile
Developed HPLC Fingerprint Analysis Method

Evaluation of the Analytical Performance of the Developed HPLC Fingerprint Analysis Method

Table 1. List of prediction secondary metabolite compounds in the glucofarmaka jamu

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>[M-H]</th>
<th>Alleged Compound</th>
<th>RT (min)</th>
<th>[M-H]</th>
<th>Alleged Compound</th>
</tr>
</thead>
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<td>135.0297</td>
<td>Tricyclene</td>
<td>6.6394</td>
<td>351.0871</td>
<td>Diacetoxy-[4]-gingerdial</td>
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<td></td>
<td></td>
<td>3-Carene</td>
<td></td>
<td></td>
<td>[10]-Gingerdiol</td>
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<td></td>
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<td></td>
<td></td>
<td>Camphene</td>
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<tr>
<td></td>
<td></td>
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<td>6.1061</td>
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<td>Unidentified</td>
<td>4.1916</td>
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<td>Momordicoside L</td>
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</table>

The parameter from the mixture of jamu raw material were applied to the antidiabetic jamu extract and each plant extract of the jamu constituents. There were 20 peaks that could be detected at the glucofarmaka jamu chromatogram and could be used as fingerprint markers. The peak number was counted by referring to Blesner criteria (2006), i.e. S/N ratio ≥ 3 and the resolution ≥ 1.5 [17]. According to the obtained fingerprint profile (Fig. 5), the result was considered as excellent for HPLC fingerprint of jamu extract and every constituent plant showed a good chemical separation.

**Evaluation of the Analytical Performance of the Developed HPLC Fingerprint Analysis Method**

Evaluation of analytical performance is the verification process to make sure that the chosen condition for analysis is appropriate for the purpose. The results of the evaluation can be used to consider the quality, reliability, and consistency of the resulting analysis data [18]. Analytical performance of HPLC fingerprint analysis method was evaluated by RSD values of RRT and RPA towards a single reference peak. The chosen reference peak was the peak at the retention time of 20 min, which is in the intermediate position and area. The precisions of the RSD values of the RRT and the RPA were less than 0.3% and 3.9%, respectively. For the reliability parameter, the RSD values of the RRT and the RPA were less than 0.3% and 4.7%, respectively. Based on these results, it was indicated that the developed method was adequate, valid, and applicable.

**Metabolite Profiling of Glucofarmaka Antidiabetic Jamu Extracts**

Secondary metabolite identity information of the jamu extract can be obtained from LC-MS data which combines the chromatography separation principles and mass spectrometry measurement. This data contain retention time, the peak intensity of LC chromatogram, m/z values, and compound fragmentation pattern. However, the data size is too large and complex that it requires software with a certain
algorithm to simplify the raw data of LC-MS. There is some common software to process LC-MS data, such as MarkerLynx™, MZmine, and XCMS. In this study, the Java programming language-based and open licensed MZmine was used. One of the advantages of MZmine is the ability to handle raw data format so that it can process data from various types of LC-MS instrument from different producers [19].

Actually, a single peak on the LC-MS chromatogram can represent more than one compound. Thus, the comprehensive integration of m/z data from the mass spectrum is required to identify the compounds in the sample. Data processing results using MZmine are shown in mass array form. There were 47 features of LC-MS peaks obtained successfully after the data processing of the jamu extract (Table 1). Among those peaks, 22 of them could be identified as the predicted compounds. Some of the resulting peaks of the LC-MS analysis could not be identified because of the limited available information in the LC-MS database until this research was reported.

At the base peak chromatogram (Fig. 6), the highest intensity peak with retention time of 6.58 min (m/z 409.165) was expected as shogasulfonic acid C compound from the ginger. The second highest intensity peak (m/z 149.061) was not able to be identified. The peak with retention time of 6.11 min (m/z 395.149) was expected as tinocordiside from brotowali.

As an effort to identify the marker compound in the jamu extract, gingerol and shogao internal standards (6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol compounds) were added to the jamu extract because they are commonly used as the marker for ginger extracts, which is one of this antidiabetic jamu constituent plants. After adding the internal standards, the peak area increased significantly at the retention times of 31 and 39 min (Fig. 7). The marker compound peaks which eluted at those retention times were 6-gingerol and 6-shogaol, respectively. In this case, the peak of the LC-MS in the metabolite profiling data analysis at the retention time of 31 min was expected as 6-gingerol. This expectation was supported by the MS² fragmentation data of LC-MS which showed the presence of this compound in the jamu extract. Fragments with m/z of 293.1798; 275.2017; 96.9599; and 79.9575 were suitable with the 6-gingerol fragmentation based on the MassBank database.

CONCLUSION

The optimum conditions of HPLC fingerprint profile of raw material mixture, single extract of the plant, and glucofarmaka antidiabetic jamu extract were obtained with 10–95% ACN-water as the mobile phase for 60 min at the wavelength of 210 nm. An analytical
performance test conducted to this method gave a good result. The peak in the jamu extract chromatogram with a retention time of 31 min is expected to be 6-gingerol potential to be a marker compound.

ACKNOWLEDGEMENT

We gratefully acknowledged Mrs. Erna Dwiyanti for her help in preparation of this manuscript. Hanifullah Habibie also gratefully acknowledged to PT Adaro Indonesia who has given the master scholarship at the Bogor Agricultural University, Indonesia.

REFERENCES


