Detection of Phytomelatonin in Medicinal Plant Huang-qin Using High Performance Liquid Chromatography-Fluorescence Detector

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ABSTRACT

Melatonin is highly conserved molecules that not only exists in vertebrates, but also present in bacteria, unicellular organisms and in plants coined as “phytomelatonin”. Due to the complexity of the plant matrix and the vulnerability of melatonin, accurate detection of melatonin was proven to be a tough work. So far, various methods such as GC-MS, LC-MS, RIA, ELISA, IAC and HPLC were designed for the determination of melatonin in plant samples. However, residing disadvantages in each method easily led to either false positive or negative result. Comparatively, HPLC coupled to fluorescence detector has gained its popularity recently for its ease of analysis and also the high sensitivity and selectivity supported by FD. In the present study, a detection method was optimized for the rapid and accurate determination of melatonin in plant samples. The method was based on HPLC coupled to fluorescence detector. Separation was obtained using a C\textsubscript{18} column and a mobile phase composed of 40% methanol and 60% 50 mM Na\textsubscript{2}HPO\textsubscript{4}/H\textsubscript{3}PO\textsubscript{4} buffer at pH 4.3. Fluorescence intensity was monitored at λ = 348 nm while exciting at λ = 280 nm. Also, a careful pre-treatment of plant samples was implemented. The calibration curve was linear over the concentration ranges of 50 to 1000 ng/ml melatonin. The melatonin peak was successfully controlled at an early retention time within 15 min and the recovery rate was 92%. Consequently, 66 ng/g in the roots and 890 ng/g in the leaves of melatonin were detected in Huang-qin.

Key words: Phytomelatonin, HPLC, fluorescence detector, medicinal plants, Scutellaria baicalensis Georgi.

INTRODUCTION

Melatonin (N-acetyl-5-methoxy-tryptamine) was discovered from the pineal gland. It has also been found in retinae, Harderian gland and gastro-intestinal tracts in vertebrates, as well as in a wide variety of organisms. Phytomelatonin has been a new and popular terminology recently. Since the first complete publications showing that melatonin indeed existed in plants (Dubbelis et al., 1995; Hattori et al., 1995), there have been an ever-growing number of studies reporting the detection of this hormone in vegetables, cereals, fruits, seeds and even medicinal herbs (Reiter et al., 2007).

Examination of reports on melatonin in plant materials showed that the quantity of the indole varies widely according to the plant species studied (Table 1). Levels of melatonin in the range of ng/g to μg/g tissue were normally reported in medicinal plants and most of them are indigenous to China or the Mediterranean or alpine areas (Chen et al., 2003; Murch et al., 2004, 1997; Tettamanti et al., 2000). Surprisingly, large amount of melatonin such as leaves of Huang-qin was detected (7.11 μg/g) and flowers of St. John’s Wort (4.39 μg/g) (Murch et al., 1997).

The extraction and detection of melatonin from plant matrices proved to be uneasy (Poeggeler and Hardeland, 1994; Van and O’Neill, 2001). Unlike animal blood and urine, non-destructive and relatively clean samples can be assayed directly for the presence of melatonin and plants must normally be destructively sampled by extraction.
followed by extensive procedures before the hormone can be assayed (Poeggeler and Hardeland, 1994). In addition, researchers are urged strictly to apply preservative conditions of extraction, as well as, controlling the yield by the determination of recovery besides the appropriate application of internal and external calibration measures (Hardeland and Poeggeler, 2003; Hardeland et al., 2007).

So far, the methods for determination of melatonin in plants mainly contain GC-MS, LC-MS, RIA and HPLC. Although, RIA is well-known for its high sensitivity and efficiency, false positive results can be obtained due to the interference of the second metabolites in plant matrix (Harumi and Matsushima, 2000). GC-MS and LC-MS are commonly employed to identify the content of melatonin (Van and O’Neill, 2001). HPLC-ED and HPLC-FD are the common-used methods to detect melatonin in plant samples nowadays. Although, ED exhibits a higher sensitivity than FD, it demands high content of the organic solvent in mobile phase and the analyzed sample cannot be recovered (Hardeland et al., 2007). Also, the selectivity of ED is lower (Kolár and Macháčková, 2005). Thus, a simple, convenient, high-sensitivity and high-recovery methodology for the determination of melatonin is necessary. Comparatively, HPLC-FD seems to be most desirable amongst those advantages and disadvantages.

Herein, we have optimized a rapid and accurate methodology for the detection and quantification of melatonin from plant samples in our study. Additionally, this study aimed to give scientific basis to commercialization of natural melatonin products from melatonin-rich medicinal plants such as Huang-qin (Scutellaria baicalensis Georgi).

**MATERIALS AND METHODS**

**Plant materials**

The dried leaves and roots of *S. baicalensis* Georgi were purchased from local drug store, People’s Republic of China.

**Melatonin extraction**

Samples of plant tissues (whole leaves and roots) were grounded with liquid nitrogen and 20 g accurately weighed and then transferred to a 100 ml beaker. 50 ml of methanol (PubChem CID: 887) (SK Chemicals; Seoul, Korea) was added to each sample and extraction was achieved by sonication in an ultrasonic bath Powersonic 510 (Hwashin Technology; Seoul, Korea) for 45 min and incubation on an orbital shaker WiseMix SHO-1D (DAIHAN Scientific; Seoul, Korea) for 1 h at room temperature. After centrifugation by Supra 30 K (Hanil science industrial; Incheon, Korea) at 4,000 rpm for 10 min, the supernatant was filtered.
through a 0.2 um × 25 mm filter (Millipore; Bedford, MA, USA). The supernatant was evaporated in vacuum BuchiRotavapor R-205 (Buchilaborotechnik; Flawil, Switzerland) while the temperature was maintained below 35°C. Finally, the residues were resuspended with 2 ml methanol before injected to HPLC. The entire procedure was performed in a darkened room and all solutions were pre-cooled at 4°C before subjection to experiments.

Melatonin detection

A HPLC-FD HP 1100 series (Agilent technologies; Foster City, CA, USA) was employed to detect melatonin extracts from the samples of plant tissue. The HPLC system consisted of a reverse-phase column C18 110A (250 × 4.60 mm) (Phenomenex; Torrance, CA, USA) connected to the HPLC and equipped with the fluorescence detector G1321A (Agilent technologies). The mobile phase constituted of methanol (DUKsan pure chemical; Ansan, Korea): 50 mM Na₂HPO₄ (PubChem CID: 24203)/H₃PO₄ (PubChem CID: 1004) buffer (40:60, v/v; pH 4.3) (DAEJUNG chemicals and metals; Shiheung, Korea). The flow rate was set up at 1 ml/min. 20 μl analytical standard melatonin (PubChem CID: 896) (Sigma; St. Louis, MO, USA) and prepared samples were injected to the HPLC-FD for analysis. The excitation wavelength was selected at 280 nm while emission wavelength at 348 nm for the fluorescence conditions.

Quality control for the melatonin extraction and detection

To measure the recovery rate of our extraction procedures, melatonin standard with known concentrations (from 10 to 100 μM) went through all our extraction processes as the plant samples. Both the melatonin standard solutions and their respective elutes were measured using HPLC-FD and the values were compared to obtain the recovery rate. A melatonin standard curve within the range of 50 to 1000 nM was made to quantify plant melatonin content. Melatonin concentrations were calculated based on the HPLC peak areas.

Statistical analysis

All samples were extracted thrice and the data are the mean values of independent measurements.

RESULTS

Chromatogram of melatonin detection

Melatonin detection was performed using high performance liquid chromatography with a fluorescence detector. HPLC analysis of plant sample extracts was obtained with the modified method of Chen et al. (2003). Its identification was obtained by a parallel figure comparison of standard and sample chromatograms and also of samples with or without standard enrichment (Figure 1).

Peak of standard melatonin was spiked at a retention time of 12 min (Figure 1A) while in the samples of Huang-qin roots and leaves clear peaks showed at the same retention time (Figure 1B and C). 0.5 ml of 0.0001 mg/ml standard melatonin solution was mixed with 0.5 ml Huang-qin extracts before injection to identify the melatonin peak. An enhanced peak height was observed at the retention time of 12 min after HPLC analysis (Figure 1D).

Figure 1E and F showed the retention time of melatonin in plant samples from other studies also using HPLC (Chen et al., 2003; Iriti et al., 2006). Generally, it takes more than 20 min to finish the analysis. Compared with our methodology, we achieved a faster detection around 12 min of melatonin.

Melatonin quantification

A calibration curve with five different standard melatonin concentrations (0.00005, 0.0001, 0.0002, 0.0005 and 0.001 mg/ml) respectively, was built to quantify the melatonin content in the roots and leaves of Huang-qin (Figure 2). Consequently, 66 ng/g in the roots and 890 ng/g in the leaves of melatonin were detected.

Recovery rate determination

The recovery rate of our extraction procedures was measured as previously described in the materials and methods section. The results showed that it ranged from 89 to 96% and the average recovery rate was 92% (Table 2). Table 3 shows the melatonin recovery rate from different methods. Comparatively, our methodology is also considered to be a reliable one.

DISCUSSION

Huang-qin (S. baicalensis Georgi) is a medicinal species used extensively in Chinese prescriptions. It is used for the treatment of cancers, hepatitis, cirrhosis, jaundice, hepatoma, leukemia, hyperlipemia, arteriosclerosis and inflammatory diseases (Chang et al., 2002). Moreover, Huang-qin leaves are recommended as medicine for the treatment of ailments of the nervous system based on anecdotal and historical evidence. Thus, it is questioned whether melatonin can be present in this species with historical medicinal value and pharmaceutical
Figure 1. HPLC spectrums of melatonin detection. (A) Chromatogram of analytical standard melatonin (0.01 μg/ml); (B) Chromatogram of Huang-qin roots extracts; (C) Chromatogram of Huang-qin leaves extracts; (D) Chromatogram of Huang-qin leaves with standard melatonin enrichment; (E) Chromatogram of Babreum coscluea and (F) Chromatogram of grape skin extracts.

Figure 2. Relationship of the melatonin concentrations and the HPLC peak areas. \( y = 0.6841x + 4.3005 \). \( r = 0.99975 \).
preparations of plant leaves. Murch et al. (1997) found a high melatonin content of 7.11 μg/g in leaves of Huang-qin using a classical method from Poeggeler and Hardeland (1994). Thus, we considered Huang-qinas a desirable material for its potential high melatonin content.

Confusingly, by using our protocol, only 890 ng/g was detected in the commercial preparations of leaves of Huang-qin from China. In contrast, Chen et al. (2003) reported 179 ng/g of melatonin in the dried powder of Huang-qin. However, their material is Scutellaria amoena C.H Wright which is another member of this species and grows in the southwest of China. Furthermore, we also detected a considerable amount of melatonin as 66 ng/g in the commercial preparations of Huang-qin roots, which is the first report in this species.

As previously described in the introduction, melatonin analysis from plant matrix has been a complicated work due to a variety of factors leading to diverse results (Poeggeler and Hardeland, 1994; Van and O’Neill, 2001). Many authors focused on the differences in melatonin content, suggesting various hypotheses to explain the widely varying level. This can be attributed to extraction methods used to recover melatonin from plant material and those inherent to the particular molecular constituents of plants. Usually, intensive extraction procedures involve a high level of forming oxidants that can easily lead to the destruction of melatonin (Poeggeler and Hardeland, 1994). Murch et al. (1997) observed 15 and 30% loss of melatonin during the drying process when they compared the melatonin content of fresh leaves from fever few with freeze-dried and oven-dried leaves. This also may be a factor accounting for our detection of low melatonin content as compared to theirs.

In addition, the complex chemistry of plant tissues which often contain large amounts of carbohydrates, lipids and pigments may induce false positive and negative results (Van and O’Neill, 2001). Noticeably, the whole plant preparations of Huang-qin are complex mixtures of many thousands of small metabolites, which can be a factor affecting the detection results to be taken into account as well. Interestingly, the melatonin contents also vary within different tissues of plants. In Glycyrrhzy zauralenensis Fischer, various levels of melatonin were detected in the roots, seeds and stems (Kolář and Macháčková, 2005). It was reported by Datura metel L., where the average concentration detected in leaves was roughly 5-fold higher than that of unopened flowers (Hattori et al., 1995). In our study, we detected 10-fold melatonin content in leaves higher than in roots of Huang-qin.

Furthermore, some studies showed that there exist intraspecific differences among members of the same plant species. Hattori et al. (1995) reported 32.2 pg/g, while Dubbels et al. (1995) obtained 506 and 166 pg/g for the cultivars ‘Sweet 100’ and ‘Rutgers California Supreme’, respectively. Similar intraspecific variations were reported for grape with Nebbiolo and Croatina cultivars having the highest melatonin concentration (0.9 and 0.8 ng/g, respectively), and the Cabernet Franc cultivar having the lowest detected values (0.005 ng/g) (Iriti, 2009). We detected almost 6-fold higher melatonin in S. baicalensis Georgii than in S. amoena C.H Wright as reported by Chen et al. (2003).

The aforementioned variations in the amounts of melatonin in plant extracts can be also attributed to the physiochemical properties of melatonin. The level of this indole may vary artificially due to its co-extract and partition with chlorophyll and phenolic compounds, as well as, sublimation during vacuum drying and the susceptibility to destruction by the impurities commonly found in organic solvents (Van and O’Neill, 2001). Besides its vulnerability to light and heat, melatonin can be easily lost by binding to glass, polyvinyl-polylypyrrolidone, or nylon-membrane filters (Van, 1997). Therefore, in our experiments, we strictly down-controlled the temperature.

### Table 2. Melatonin recovery rates from the extraction procedures.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Melatonin (µg)</th>
<th>Melatonin measured (µg)</th>
<th>Recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>9.57</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>18.04</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>44.53</td>
<td>89</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>92.67</td>
<td>93</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>92</td>
</tr>
</tbody>
</table>

### Table 3. Melatonin recovery rates from different methodologies.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Methods</th>
<th>Recovery rate (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phototrophic</td>
<td>ELISA</td>
<td>95</td>
<td>Mercolini et al. (2008)</td>
</tr>
<tr>
<td>Human serum</td>
<td>IAC</td>
<td>95</td>
<td>Rolcik et al. (2002)</td>
</tr>
<tr>
<td>Medicinal plants</td>
<td>LC-MS</td>
<td>96</td>
<td>Chen et al. (2003)</td>
</tr>
<tr>
<td>Rhodnius prolulus</td>
<td>RIA</td>
<td>99</td>
<td>Gorbet and Steel (2003)</td>
</tr>
</tbody>
</table>
and process all the steps in a dark room with only a dim red light. Also, all the vessels used in extraction steps are disposable plastic-made materials. Moreover, the solvent used to extract melatonin from given plants may also be a factor: We tried to use ethanol and methanol as our extraction solvent in our study. Finally, in Huang-qin roots, 66 ng/g of melatonin extracted by methanol was detected while only 55 ng/g by ethanol proved methanol a better choice.

Overall, knowing the difficulty that melatonin extraction and quantification from plants involves, some analytical methodologies have recently been created in order to obtain rapid and accurate results. For example, Cao et al. (2006) designed a rapid and accurate method to detect and quantify melatonin in plant samples by means of LC-MS with ESI, APCI and APPI. The limit of detection of melatonin in the plant extract was 5 pg/ml and the limit of quantification was 0.02 ng/g. Mercolini et al. (2008) optimized an analytical method based on HPLC-FD for the determination of melatonin in red and white wine. Our optimized method is based on these studies focusing more on the avoidance of melatonin degradation, maximum melatonin extraction and experimental costs reduced. Hopefully, there will soon be methods specifically designed for plants avoiding the interfering difficulties encountered by researchers in the past.

Conclusions

The proposed method based on HPLC-FD was successfully applied to the analysis of melatonin in our plant samples in the current study. The native fluorescence of the analytes confers sensitivity and selectivity to the assay without the need for time-consuming and complicated derivatization procedures. Compared to the high-cost maintenance of LC-MS and GC-MS, the complicated preparation steps and the high-demand antibody of RIA, IAC and ELISA our method is more simple and economical. Moreover, the high recovery rate of 92% proves the reliability of our methodology. In conclusion, the present study has provided a rapid and reliable method for detection and quantification of melatonin in plant samples and this can be an interesting addition to current techniques in this field.

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