Simultaneous determination of two biflavones in Biyanling tablets by HPLC

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To develop analytical methods for simultaneous determination of two major biflavones, amentoflavone and heveaflavone, in Biyanling Tablets, thereby providing methodological reference for quality control of anticancer drug Biyanling Tablets. Diamonsil C₈(4.6 mm × 250 mm, 5 µm) column is used; elution is performed with a mobile phase of acetonitrile (B) –0.5% acetic acid solution (A) by a gradient of 0~4 min, B: 35%→45%; 4~20 min, B: 45%→50%, detection wavelength 270 nm, flow rate 1.0 mL/min, and column temperature 30°C. Amentoflavone and heveaflavone show good linear relationships (r ≥0.9998 and 0.9996) within a range of 1.56~100 µg/mL, average recoveries are 98.2% and 96.5%, with RSDs of 2.1% and 2.4% (n = 6), respectively. The method is simple, fast and reproducible, which provides a quantitative analytical method for quality control of Biyanling Tablets.

Keywords: Biyanling, Biflavone, Determination, HPLC, Simultaneous

Biyanling Tablets are a compound preparation made from Herba Selaginellae Doederleinii, Radix Sophorae Subprostratae, Poria cocos, Radix Trichosanthis, etc.¹, which has heat clearing, detoxifying, hard lump resolving, node dispelling, Qi supplementing and Yin nourishing actions. It is used for symptoms like chest and diaphragm wind-heat, phlegm-fire depression, heat-toxin up invasion, Qi consumption and body fluid impairment. Common symptoms include dry mouth, sore throat, dry and burning throat, hoarseness, headache, stuffy nose, purulent nasal discharge or blood in nasal discharge. It is also used in the treatment of acute and chronic pharyngitis and stomatitis, and as adjuvant treatment to radiotherapy and chemotherapy of nasopharyngitis and nasopharyngeal carcinoma²⁻³.

Nasopharyngeal carcinoma is a malignancy occurring in the apex area and side wall of nasopharyngeal cavity. It is one of the world's most prevalent malignancies, whose incidence tops among otolaryngologic malignancies. Common clinical symptoms include nasal congestion, blood in nasal discharge, ear fullness, hearing loss, diplopia, headache, etc. Occurrence and development of nasopharyngeal carcinoma are closely linked to PI3K/AKT signaling pathway, of which PI3K, AKT and pAKT are associated with the metastasis and prognosis of nasopharyngeal carcinoma⁴⁻⁸.

Biyanling Tablets are a potential cure for nasopharyngeal carcinoma, and biflavones are its active anti-cancer constituents⁹⁻¹². So, this paper determines the contents of amentoflavone and heveaflavone in Biyanling Tablets using HPLC, with the aim to effectively control their quality, and improve the original quality standard.

Materials

Agilent 1200 HPLC system (with quaternary low pressure mixing pump, autosampler, column oven and diode array detector), SBC1250 electronic balance (Nanjing Kademenqi Instrument Co., Ltd.); SSB-500A medical CNC ultrasonic cleaner (Zunyi Ultrasonic Instrument Co., power 200 W, frequency 40 kHz).

Amentoflavone and heveaflavone reference substances were purchased from Chengdu Must Biotechnology Co., Ltd. Purities of the above reference substances were validated to be over 99% by HPLC normalization. HPLC grade acetonitrile (Merck), acetic acid (Dalian Shenqi Chemical Reagent Co., Ltd.); purified water (laboratory-made); other reagents were all of analytical grade. All

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solvents were filtered through 0.45 µmol/L microporous membrane, and degassed ultrasonically. Biyanling Tablets were purchased from Beijing Fenghe Pharmacy.

Methodology and results
Preparation of solutions
Reference solution
Ten mg of amentoflavone and heveaflavone reference substances were accurately weighed, respectively, placed in 5 mL volumetric flasks, ultrasonically dissolved by adding 80% ethanol, diluted to the mark, and shaken well to give the reference stock solutions.

Test solution
After film coating of Biyanling Tablets was removed, the remaining was pulverized, and passed through a 60 mesh sieve. 1 gm of the resulting powder was accurately weighed, placed in a stoppered conical flask, added with 50 mL of petroleum ether (60–90°C), and ultrasonically extracted for 60 min (power 200 W, frequency 40 kHz). After discarding the supernatant, ultrasonic extraction was repeated once again. Drug residue was placed on filter paper, petroleum ether was evaporated to dryness at room temperature, and the remaining was transferred into a stoppered conical flask, added precisely with 50 mL of 70% ethanol, stoppered, weighed, ultrasonically extracted for 60 min (power 200 W, frequency 40 kHz), replenished with solvent, and filtered. 10 mL of the filtrate was accurately weighed, evaporated to dryness in a 58 °C water bath, redissolved in 80% ethanol, and diluted to the mark in a 10 mL volumetric flask for later use. Before injection analysis, the solution was filtered through a 0.45 µmol/L microporous membrane, and the subsequent filtrate was collected as the test solution.

Chromatographic conditions
Chromatographic conditions were optimized using the above test solution. Analysis was performed on Diamonsil C18 (4.6 mm × 250 mm, 5 µm) column; gradient elution conditions were optimized using acetonitrile (B)—0.5% acetic acid solution (A) as the mobile phase to be (0–4 min, B: 35% → 45%; 4–20 min, B: 45% → 50%); detection wavelength (270 nm), flow rate (1.0 mL/min), column temperature (30°C), and injection volume 10 µL. Chromatographic analysis results are shown in Fig. 1, amentoflavone and heveaflavone in Biyanling Tablets met the baseline separation conditions, in addition, chromatographic analysis time for single injection was less than 30 min.

Standard curve and limit of detection
Appropriate amount of two types of reference stock solutions under the section "Reference solution" was accurately weighed, placed on the same 2 mL volumetric flask, diluted to the mark with anhydrous ethanol, and shaken well to prepare the standard working solution containing the two constituents both with a concentration of 200 µg/mL. The above standard working solution was prepared by multiple dilution to standard serial working solutions (100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg/mL), and injected for analysis as per the chromatographic conditions under the section "Chromatographic conditions". Standard curve was plotted with concentration of reference substance (µg/mL) as the abscissa (X), and chromatographic peak area as the ordinate (Y), and the limit of detection (S/N≥3) and the limit of quantification (S/N≥10) were calculated, the results are shown in Table 1.

Precision test
An aliquot of reference solution (with concentrations of amentoflavone and heveaflavone both 12.5 µg/mL) was taken, and injected for 6 continuous times as per the chromatographic conditions under the section "Chromatographic conditions". Peak areas were recorded, and RSDs of amentoflavone and heveaflavone were calculated to be 1.5% and 2.0%, respectively.

Reproducibility test
Six aliquots of Biyanling Tablets with the same batch number were taken and prepared into six parallel test solutions following the method under the section "Test solution", and injected for analysis as per the chromatographic conditions under the section "Chromatographic conditions". RSDs of average amentoflavone and heveaflavone contents were measured to be 1.2% and 1.5%, respectively.

Stability test
An aliquot of test solution was taken, placed at room temperature, and injected once every 0, 2, 4, 8, 10, 24, 36 and 48 hrs for analysis as per the chromatographic conditions under the section "Chromatographic conditions". Peak areas were recorded, and standard curves were employed to calculate the RSDs of average contents of
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amentoflavone and heveaflavone to be 0.2% and 0.5%, respectively. The results showed good stability of sample solutions within 48 hrs.

Sample content determination

Test samples were prepared into test solutions as per the section "Test solution", and injected for analysis as per the chromatographic conditions under the section "Chromatographic conditions", followed by calculation of amentoflavone and heveaflavone contents in the samples by external standard method. The results are shown in Table 2.

Recovery test

In six text solution, 1 gm aliquots of samples with known content were accurately weighed, placed in stoppered conical flasks, added separately with 200 µL of amentoflavone and heveaflavone reference solutions (with concentration both 500 µg/mL), prepared into test solutions following the method under the section "Test solution", and injected for analysis as per the chromatographic conditions under the section "Chromatographic conditions", followed by calculation of recoveries (Table 3).

Discussion

Selection of extraction method

Reflux extraction, Soxhlet extraction and ultrasonic extraction are investigated, respectively, in this experiment. Ultrasonic extraction is adopted owing to its high efficiency, simplicity and fastness. During the experiment, different extraction solvents (50%, 70% and 100% ethanol) used for test solution preparation, different solvent volumes (30, 50, 75 and 100 mL) and different extraction time (15, 30, 60 and 90 min) are investigated, respectively. The results reveal that amentoflavone and heveaflavone are extracted relatively completely when the samples are extracted ultrasonically (50 mL × 60 min × 1 time) with 50 mL of 70% ethanol.

Selection of detection wavelength

Detector used in this study is a diode array detector, UV spectra recorded shows absorption peaks of amentoflavone at around 210, 288 and 335 nm, and absorption peaks of heveaflavone at around 210, 268 and 345 nm. Taking into account the durability of analytical method (such as using an ordinary UV detector), a single wavelength of 270 nm is selected as the detection wavelength.

Selection of mobile phase

Mobile phase systems with different proportions of methanol–water and acetonitrile–water are tested; gradient elution with acetonitrile–water system can achieve good separation effect. Besides, addition of 0.2% acetic acid in water can effectively improve

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Standard curve</th>
<th>Linearity range (µg/mL)</th>
<th>R</th>
<th>LOD (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amentoflavone</td>
<td>Y=16.899X+12.512</td>
<td>1.56-100</td>
<td>0.9998</td>
<td>0.78</td>
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<tr>
<td>Heveaflavone</td>
<td>Y=6.213X+3.214</td>
<td>1.56-100</td>
<td>0.9996</td>
<td>0.78</td>
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</table>

<table>
<thead>
<tr>
<th>Sample batch No.</th>
<th>Amentoflavone (mg/gm)</th>
<th>Heveaflavone (mg/gm)</th>
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<tbody>
<tr>
<td>140701</td>
<td>0.110±0.003</td>
<td>0.093±0.004</td>
</tr>
<tr>
<td>140702</td>
<td>0.149±0.006</td>
<td>0.089±0.003</td>
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<tr>
<td>140703</td>
<td>0.138±0.002</td>
<td>0.119±0.001</td>
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Fig. 1—Chromatograms of sample (A) and reference (B): 1. amentoflavone, 2. heveaflavone

Table 1—Standard curve and limit of detection (n = 8)

Table 2—Content determination results (mg/g, n = 3)
the shapes of amentoflavone and heveaflavone chromatographic peaks, so acetonitrile–0.5% acetic acid system is selected.

Selection of biyanling tablets content determination indices

The author team's preliminary study isolated and purified two biflavones, amentoflavone and heveaflavone from Biyanling Tablets; and literatures have shown that these biflavones have good anti-cancer activities. Therefore, the above two biflavones are likely to be the major active constituents contributing to the anticancer effect of Biyanling Tablets. In this paper, the two major active constituents in Biyanling Tablets are selected as the content determination indices, which can to some extent objectively reflect the quality of Biyanling Tablets preparation, and thus lay the foundation for research and development of Biyanling Tablets as well as anti-cancer medication.

References

2 Writing Group of Compilation of National Herbal Medicine, Compilation of National Herbal Medicine, People's Medical Publishing House, (I), 1975, 240, 339, 340, 903.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Known content (mg)</th>
<th>Added amount (mg)</th>
<th>Total measured amount (mg)</th>
<th>Average recovery (%)</th>
<th>RSD (%)</th>
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<tbody>
<tr>
<td>Amentoflavone</td>
<td>0.120</td>
<td>0.1</td>
<td>0.216±0.002</td>
<td>98.2</td>
<td>2.1</td>
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<tr>
<td>Heveaflavone</td>
<td>0.098</td>
<td>0.1</td>
<td>0.191±0.003</td>
<td>96.5</td>
<td>2.4</td>
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