SHORT COMMUNICATION

Spectrophotometric determination of total lactones in *Andrographis paniculata* Nees

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Abstract

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Spectrophotometric determination of total lactones in *Andrographis paniculata* Nees
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A spectrophotometric method for determination of total lactones in *Andrographis paniculata* was established by using dinitrobenzoic acid and potassium hydroxide solutions as colour forming agents. The absorbance of the solution was determined at 536 nm. The linearity range was 12-72 $\times 10^{-6}$ g.ml^{-1}. The detection limit was 1.2 µg, the quantitation limit was 4.23 µg. The intraday variation had an average of slope 6082.97 g.ml^{-1}, % RSD 0.10; an average intercept 0.2786, %RSD 3.66 (n=3). The interday variation had an average of 6146 g.ml^{-1} with the %RSD of 6.30 and an average intercept 0.2628, %RSD 4.95 (n=4). The coefficients of determination were 0.998-0.999. The total lactones content, calculated as andrographolide, determined by this method was 8.61±0.52% (n=4) and by the official method, Thai Herbal Pharmacopoeia, was 8.12±0.34% (n=2). The results of the two methods do not differ significantly at P=0.05 (P(|t|≥0.903 = 0.53)

Key words : *Andrographis paniculata*, total lactones, spectrophotometric determination, colorimetry

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บทคัดย่อ

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การตรวจหาปริมาณโตตอลแลคโทนส์ในฟ้าทะลายโจรโดยวิธีการตรวจวัดทางสเปคโตรโฟโตเมตรี

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การพัฒนาวิเคราะห์โดยวิธีการตรวจวัดทางสเปคโตรโฟโตเมตรี เพื่อใช้หาปริมาณโตตอลแลคโตนรวมในฟ้าทะลายโจร ทำโดยอาศัยหลักการทำให้เกิดสีระหว่างแลคโตนกับสารละลายไดไนโตรเบนโซอิกและสารละลายโปแตสเซียมฮัยดรอกไซด์ จากนั้นวัดการดูดกลืนแสงด้วยเครื่องสเปคโตรโฟโตมิเตอร์ ที่ความยาวคลื่น 536 นาโนมิเตอร์ ความเข้มข้นที่แปรผันเป็นเส้นตรงกับค่าการดูดกลืนแสงอยู่ในช่วง 12-72 x 10^{-6} ก.มล$^{-1}$ ปริมาณสารที่ตรวจพบได้ต่ำสุดคือ 1.2 ไมโครกรัม ปริมาณต่ำสุดที่ตรวจหาปริมาณได้คือ 4.23 ไมโครกรัม ความเบี่ยงเบนภายในวัน ค่าความลาดชันเฉลี่ย 6082.97 ก$^{-1}$.มล. %ความเบี่ยงเบนมาตรฐานสัมพันธ์ 0.10; ระยะแกนตัด 0.2786, %ความเบี่ยงเบนมาตรฐานสัมพันธ์ 3.66 (n=3) สำหรับความเบี่ยงเบนระหว่างวันมีค่าความลาดชันเฉลี่ย 6146 ก$^{-1}$.มล. %ความเบี่ยงเบนมาตรฐานสัมพันธ์ 6.30; ระยะแกนตัด 0.2628, %ความเบี่ยงเบนมาตรฐานสัมพันธ์ 4.95 (n=4). ค่าสัมประสิทธิ์สัมพันธภาพ 0.998-0.999 ปริมาณแลคโทนรวมที่ตรวจวัดโดยวิธีนี้คือ 8.61±0.52% (n=4) และโดยวิธีมาตรฐานตาม Thai Herbal Pharmacopoeia คือ 8.12±0.34% (n=2) ผลการวิเคราะห์จาก 2 วิธีไม่มีความแตกต่างกันอย่างมีนัยสำคัญที่ความเชื่อมั่น 95% (P(|t|≥0.903 = 0.53)

ภาควิชาเภสัชเคมี คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002

Andrographis paniculata Nees is widely used as an antipyretics, antibacterials and antivirals etc. (Boonyaprapasara, N. and Chokecharoenporn, W., 1997). The constituents found in the plants were diterpene lactones and their glycosides, i.e. andrographolide, deoxyandrographolide, 11,12-didehydro-14-deoxyandrographolide, neoandrographolide. Flavonoids were also reported to be found in this plant (Rao *et al*., 2004). Andrographolide is the main component and is believed to be the active constituent for biological activities and represents as an identity indicator for the plant. In Thai Herbal Pharmacopoeia, the total lactones in the plant was determined by an acid-base titration (Thai Herbal Pharmacopoeia, 1995). The method takes about at least 6 hours to determine each sample. The HPLC methods using a reversed phase column and mobile phase containing methanol-water or acetonitrile phosphate buffer (Burgoes *et al*., 1999 and Kumaran *et al*., 2003) were used to determine the andrographolide in rat and rabbit serum. However, the determination is only for andrographolide content. Zhao *et al*., 2002, determined andrographolide, deoxyandrographolide and neoandrographolide by capillary electrophoresis, which required a rather sophisticated instrument. The main problem of the HPLC and capillary electrophoresis analysis is the commercial unavailability of standards except andrographolide. It is the main hindrance for quality control of the plant for herbal drug dealers.

In this work, a spectrophotometric method for determination of total lactones in *Andrographis paniculata* was established by using andrographolide as the standard lactone, and dinitrobenzoic acid and potassium hydroxide solutions as colour forming agents. This is a fast, simple and convenient technique. It is a method most suitable for local medicinal herbal industries.

Materials and Method

Andrographolide used as a standard was obtained from Sigma. Dinitrobenzoic acid and potassium hydroxide were analytical grade. Alcohol was 95% ethanol obtained from the Thai Govern-

ment Distiller. The spectrophotometer was a Shimadzu's Model UV-VIS Mini-1240, the cuvette was a thick wall quartz window 1- cm thick with the minimum volume 0.150 ml.

1. Solutions

1.1 Stock standard solution: a 0.24% w/v of andrographolide solution in alcohol.

Standard solutions: A series of standard solutions containing andrographolide, 1.2-72 µg. ml^{-1} were prepared from the stock standard solution.

1.2 Dinitrobenzoic acid solution: 2% solution of dinitrobenzoic acid in alcohol.

1.3 Potassium hydroxide: a freshly prepared 5.7% of potassium hydroxide in alcohol.

1.4 Sample solutions: a.- Eighty mg of *Andrographis paniculata* fine powder was sonicated with 50 ml of alcohol in a 50 ml volumetric flask for at least 30 minutes.

b.- Eighty mg of *Andrographis paniculata* fine powder and 40 mg of decolorizing charcoal were sonicated with 50 ml of alcohol in a 50 ml volumetric flask for at least 30 minutes. The solution was filtered and 200 µl filtrate was used for the analysis.

2. Procedure

A 200 µl of standard or sample solution was transferred in to the cuvette, 50 µl of dinitrobenzoic acid and 50 µl of potassium hydroxide solution were added, respectively. The mixture was mixed gently and immediately placed in the sample holder. The absorbance was recorded at the maximum reading which usually occurred after 3-5 minutes. Blank reagents and the blank sample were measured to correct the absorbancy.

3. Validation of the method

The limit of detection and limit of quantitation were determined from the $3.s_0$/slope and $10.s_0$/slope respectively, where s_0 is the intercept (ICH Guidelines 1996). The precision was determined by repeating the experiment trice in a day for intraday variation and once a day in 3 days for interday variation. The accuracy was validated by comparing with the official method, Thai Herbal Pharmacopoeia.

Results and Discussion

When dinitrobenzoic acid and potassium hydroxide solutions (Kedde's reagents) were added to the andrographolide, a pink colour was formed. This reaction is used for a screening test for cardiac glycosides. The reaction is adapted for quantitative determination. In this method, the reaction mixture, andrographolide and reagents were mixed in the cuvette and allowed to wait for the maximum absorption to take place, which was around 3-5 minutes. The absorbance was scanned from 700-400 nm. Figure 1 shows the spectra of the resultant solution of andrographolide 36 µg.ml^{-1}, solvent and reagent blank. Figure 2 shows the spectra of the plant extract with and without decoloring agent.

1. Accuracy

The accuracy of the method was evaluated by comparing with the official method, the Thai Herbal Pharmacopoeia. The total lactones content deter-mined by this method was 8.61+0.52% (8.72, 7.85, 8.94 and 8.93%) whereas that results obtained from Thai Herbal Pharmacopoeia method was 8.12% (8.19 and 7.18%). The paired t-test, using SPSS 9.0 for Window, gave $t = 0.903$, $P = 0.53$ (two-tailed). The Thai Herbal Pharmacopoeia method is time-consuming and the titration end point is quite difficult to determine. The end point detection needs some experience of the analyst. In the method, the total lactones were extracted from *Andrographis paniculata* powder by refluxing with alcohol. The interfered components of the extract were eliminated by treating with activated charcoal, lead subacetate and sodium sulfate. The extract was then digested with a known amount of sodium hydroxide standard solution, thus the lactones were hydrolysed to hydroxyl acid and, in turn, interacted with the standard sodium hydroxide solution. The excess sodium hydroxide was then determined with the standard acid.

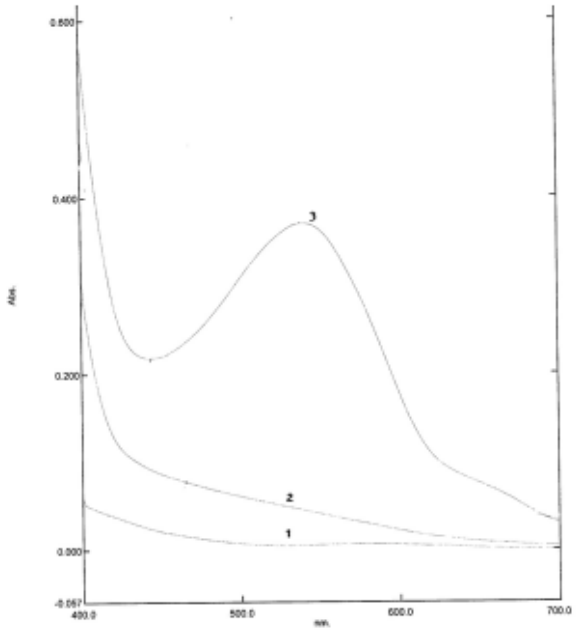

Figure 1. Visible spectra of (1) 95% ethanol; (2) 200 µl ethanol and reagents and (3) standard andrographolide 36 µg ml^{-1}, 200 µl and reagents.

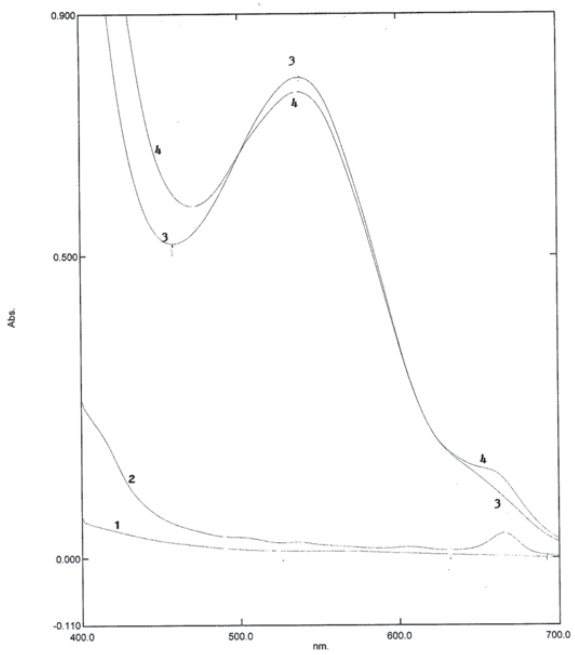

Figure 2. Visible spectra of (1) *Andrographis paniculata* and charcoal alcohol extract; (2) *Andrographis paniculata* alcohol extract; (3) solution of 1 after adding coloring reagent and (4) solution of 2 after adding reagents.

2. Precision

The intraday variation slope was 6082.97 g^{-1}.ml, % RSD 0.10, intercept was 0.2786, %RSD 3.66 (n=3) . The interday variation slope was 6146 g^{-1}.ml, %RSD 6.30 and intercept was 0.2628, %RSD 4.95 (n=4), respectively. The coefficients of determination were 0.998-0.999.

3. Linearity

A series of standards was measured from 1.2 -72 µg.ml^{-1}. It was found that the linearity range was 12-72 µg.ml^{-1}, with the regression of 0.998-0.999. The detection limit was 1.2 µg, the quantitation limit was 4.23 µg.

4. Factors affecting the spectroscopic absorption

The spectrophotometric determination of a plant is very difficult to determine, due to the interference from indigenous substances. However, for this method the colour formed is pink, thus the yellow and green colour from the plant exerts no effect. Decolourisation of the sample (80 mg) with activated charcoal (40 mg) in 50 ml of alcohol gave a colourless solution, this was confirmed by scanning the solution at the same wavelengths (see the spectrum shown in Figure 2). The solution of Andrographis paniculata 80 mg in 50 ml 95% alcohol is pale greenish yellow, while the red colour is absorbed around 536 nm thus the green and yellow colour should not interfere. To avoid the possible interference from the indigenous substances, the absorbance of the sample at 536 nm was measured before the addition of the reagents.

5. Stability of the reagents

The 2% dinitrobenzoic acid solution in 95% ethanol turned to very pale yellow within 2 weeks, whereas the potassium hydroxide solution in 95% alcohol turned from pale yellow to light brown

within 2 days. There was no change in appearance of solution of potassium hydroxide and dinitrobenzoic acid in absolute alcohol within 6 months. However, the freshly prepared solutions in 95% and absolute alcohol did not give different result, thus for an economic reason, the freshly prepared solution in 95% alcohol were used.

6. Concentration of andrographolide and reagents

If 2.0 ml, instead of 0.2 ml, of the sample or standard of the same concentration was used for the reaction, the sensitivity decreased. Increment of the reagents concentration increased the reaction rate but solution usually became turbid.

7. Solvent for sample and standard

Acetonitrile was found to give a bright violet colour to dinitrobenzoic acid and potassium hydroxide. Andrographolide in methanol or chloroform does not form the colour.

Conclusion

The reaction between the Kedde's reagents and andrographolide is fast and rapid compared to the titration method described in the Thai Pharmacopoeia. The HPLC method requires a more sophisticated instrument, pure components as standards. This method is suitable for determination of total lactones in *Andropgaphis paniculata* especially for the local herbal industries for a rapid result and comparable to the titration method.

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