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Flow injection spectrophotometric determination of andrographolide from *Andrographis paniculata*

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Abstract

A simple flow injection colourimetric procedure for determining andrographolide was proposed. It is based on the reaction between andrographolide with 3,5-dinitrobenzoic acid, resulting in an intense purplish red complex with a suitable absorption at 536 nm. A standard or sample solution was injected into the 3,5-dinitrobenzoic acid stream (flow rate of 1.0 ml min^{-1}) which was then merged with potassium hydroxide stream with the same flow rate. Optimum conditions for determining andrographolide were investigated by univariate method. Under the optimum conditions, a linear calibration graph was obtained over the range 5.0–150.0 μg ml^{-1} and the detection limit was 1.50 μg ml^{-1} (3σ). The relatives standard deviation of the proposed method calculated from 10 replicate injections of 10.0 and 80.0 μg ml^{-1} andrographolide were 0.66% and 1.64%, respectively. The sample throughput was 50 h^{-1}. The proposed method has been satisfactorily applied to the determination of andrographolide in herb plant samples.

Keywords: Flow injection; Andrographolide; 3,5-Dinitrobenzoic acid

1. Introduction

Andrographis paniculata, commonly known as "King of Bitters", and belongs to the Acanthaceae family, is traditionally used as a medicinal herb to treat different diseases in India, China and Southeast Asia [1]. The structure of andrographolide was shown in Fig. 1. The typical medicinal component of *A. paniculata* is andrographolide [2]. This plant has been used traditionally for several applications such as active against various effect of snakebite [3], antimicrobial [4], antimalarial [5,6], anticancer [7] and help against HIV [8].

A simple and accurate analytical method is required for the quantitation of andrographolide, which are present in the medicinal plant. Several methods have been reported for the determination of andrographolide, including gravimetric [9,10], titrimetric [11,12], spectrophotometric [13], HPTLC [12,14,15], LC [16,17], electrokinetic chromatographic [18–20], and high-speed counter current chromatographic methods [21]. Some of these reported methods are expensive, time consuming, imprecise and require multiple steps of extraction and purification. Therefore, it is necessary to develope a simple, rapid and/or reliable method for quantitation of andrographolide which can be used for routine analysis. Flow injection technique seems promissing to fulfill these purposes. Flow injection method for the determination of andrographolide in *A. paniculata* using 3,5-dinitrobenzoic acid as complexing reagent has not been yet reported in the literature. There is only one method using 3,5-dinitrobenzoic acid as reagent for determination of amoxycillin in pharmaceutical preparations [22]. In the present work, a new flow injection spectrophotometric method is proposed for the determination of andrographolide in *A. paniculata*. The method is based on the spectrophotometric detection of the purplish red complex formed by the reaction between andrographolide and 3,5-dinitrobenzoic acid.

2. Experimental

2.1. Apparatus

The flow injection manifold consisted of a peristaltic pump (Eyela® MP3A, Tokyo Rikakikai Co. Ltd., Japan), the standard or sample solution was injected into the FI system via a four way

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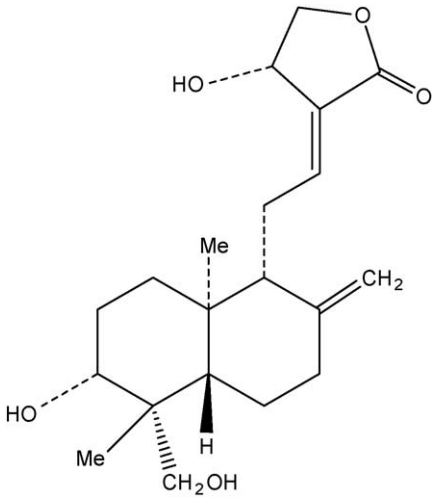

Fig. 1. The structure of andrographolide.

PTFE rotary valve with a 200 μl sample loop (Rheodyne® model 5041, Cotati, CA). PVC tubing (Elkay®, Galway, Ireland) with 0.8 mm i.d. was used as a flow line for 3,5-dinitrobenzoic acid solutions, and potassium hydroxide solution, and a Y-shaped connector was used for merging the reagent streams. A mixing coil used was made from PTFE tubing, 0.8 mm i.d. and 60 cm in length for the recommended configuration. The FI peaks were acquired by using a UV–vis detector (Thermo Separation Product®, TSP UV-2000, USA), coupled with a chart recorder (Kipp & Zonen® BD50, The Netherlands).

2.2. Reagents and solutions

All chemicals were of analytical reagent grade and were used without further purifications. Andrographolide (98%) was purchased from Aldrich (Germany). Stability of andrographolide was perfectly stable at room temperature [23]. The stock standard solution of andrographolide (500 μg ml^{-1}) was prepared by dissolving 0.0500 g of andrographolide in 95% of ethanol and diluting to 100 ml. Solutions of the desired concentrations were obtained by appropriate dilution of the stock solution to volume with 95% of ethanol.

The solution of 3,5-dinitrobenzoic acid (0.25%, w/v) was obtained by dissolving 1.2500 g of 3,5-dinitrobenzoic acid (Sigma, Germany) in 95% of ethanol and diluting to 500 ml. Solution (0.70%, w/v) of potassium hydroxide (Merck, Germany) was prepared by dissolving 3.5000 g in 95% of ethanol and diluting to 500 ml. All chemicals were prepared daily.

2.3. Recommended procedure

Using the two channel manifold as shown in Fig. 2, a 200 μl sample or standard solution containing andrographolide was injected into the reagent stream consisting of 0.25% (w/v) 3,5-dinitrobenzoic acid solution at the optimum flow rate of 1.0 ml min^{-1} which was then merged with a flowing stream of

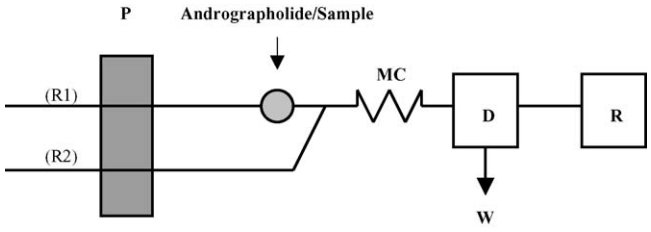

Fig. 2. Proposed FI manifolds; R1, 0.25% (w/v) of 3,5-dinitrobenzoic acid solution; R2, 0.70% (w/v) of potassium hydroxide solution; P, Pump; MC, Mixing coil; D, Detector; R, Recorder; W, Waste.

0.70% (w/v) potassium hydroxide with an optimum flow rate of 1.0 ml min^{-1}. Subsequently, the sample zone flowed through the 60 cm in length of reaction coil, where the complexation reaction occurred. The signal was monitored by spectrophotometric detection at 536 nm and the FI signal was recorded by mean of a chart recorder.

2.4. Sample preparation

For andrographolide extraction, about 1 g of andrographis herb or powdered plant of Fa-Tha-Lai-Chon capsules (commercially available in a drug store in Thailand) was accurately weighed and extracted separately with 20 ml 95% of ethanol for 30 min by ultrasonication. The extracts were filtered through Whatman No. 1 filter paper and 300 mg of decolourising charcoal were added to the filtrate, then it was stirred and again filtered. The filtrate was diluted with 95% of ethanol to volume to obtain the appropriate concentration for analysis.

3. Results and discussion

The proposed flow system was undertaken development of FI procedure for andrographolide determination based on the complexation between andrographolide (3-(2-(decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylenenaphthyl)ethylidine)dihydro-4-hydroxyfuran-2(3H)-one) [24–26] and 3,5-dinitrobenzoic acid in an alkaline solution resulting in purplish red soluble complex having an absorption maximum at 536 nm which was adopted from the Thai Herbal Pharmacopoeia method [12]. The experimental parameters dealing with the FI method development were optimized by an univariate method. The variable by variable method was applied to select the optimum conditions for the flow injection spectrophotometic determination of andrographolide.

3.1. Effect of absorption spectra

The absorption spectra of the coloured complex obtained by complexation reaction between andrographolide and 3,5dinitrobenzoic acid in an alkaline solution and the reagent blank were scanned over the range of 200–800 nm using a spectrophotometer. The absorption maximum of the complex was 536 nm (Fig. 3). In order to achieve the greatest sensitivity, measurements were made at 536 nm in further studies.

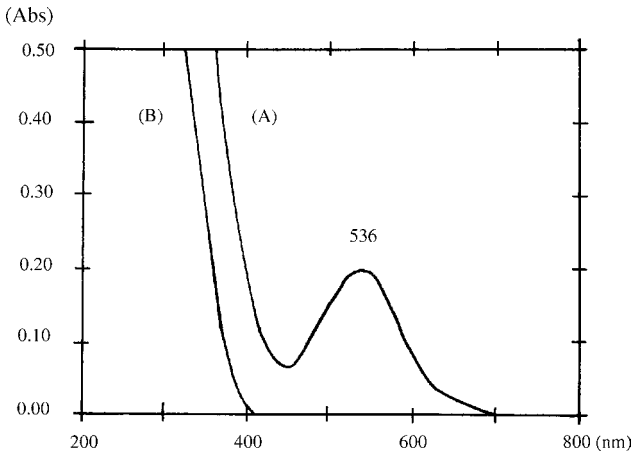

Fig. 3. Absorption spectra of (A) andrographolide and 3,5-dinitrobenzoic acid in an alkaline solution; (B) ethanol in reagent blank.

3.2. Effect of 3,5-dinitrobenzoic acid and potassium hydroxide reagent concentration

In general, the complexing agent concentration should be high enough to satisfy with the stoicheochemistry of the complex formation to make the reaction to be quantitative. The effect of varying concentrations of 3,5-dinitrobenzoic acid reagent solution between 0.03% and 0.50% (w/v) were examined. The greatest peak height was recorded when the concentration of 3,5-dinitrobenzoic acid solution was 0.25% (w/v) and was therefore chosen as optimum concentration to obtain the greatest sensitivity. Further increasing in 3,5-dinitrobenzoic acid concentration the peak height decreased gradually up to 0.50% (w/v) (Fig. 4).

The concentration of potassium hydroxide solution was optimized to obtain sufficient basicity of the reaction medium so that the complexation was effective. Various concentrations over the range 0.30–1.10% (w/v) were investigated. It was found that the peak height increased with increasing potassium hydroxide concentration and reached a maximum peak height at 0.70% (w/v), above which the peak height decreased. Thus, 0.70% (w/v) of potassium hydroxide was used subsequently to assess the greatest sensitivity (Fig. 5).

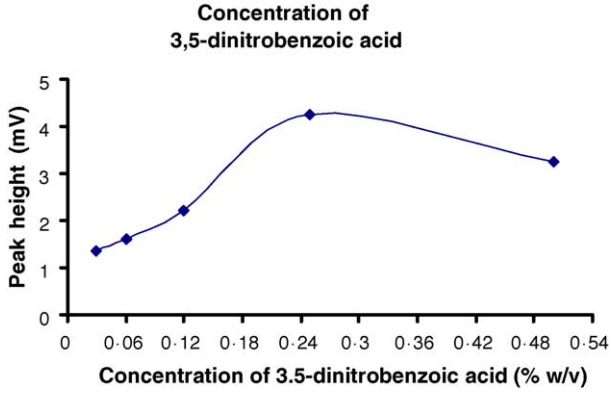

Fig. 4. Effect of 3,5-dinitrobenzoic acid concentration on the mean of peak height ($n = 5$) of 20 μg ml^{-1} andrographolide standard solution.

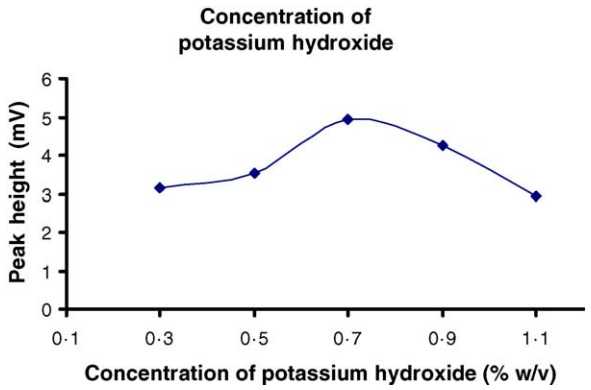

Fig. 5. Effect of potassium hydroxide acid concentration on the mean of peak height ($n = 5$) of 20 μg ml^{-1} andrographolide standard solution.

3.3. Effect of mixing coil length and injection loop volume

The mixing coil tubing length should be long enough to permit effective mixing of the reactants in which the chemical reaction to be favoured. This study was carried out at various mixing coil tubing lengths between 0 and 120 cm, injection loop volumes between 50 and 250 μl on the complexation produced were investigated. It was found that the peak height increased with the mixing coil length up to 60 cm, and the mixing coil lengths of 0, 30, 60, 90 and 120 cm provided the peak height of 2.22, 4.40, 7.60, 6.90 and 6.40 mV, respectively.

The sample injection volume must be large enough to contain sufficient amounts of the analyte to react with the reagent stream leading to the suitable response in the calibration range. The influence of the sample/standard volume on the absorbance was investigated by injecting the standard solution with varying volumes in the range 50–250 μl of 20 μg ml^{-1} andrographolide into the FI system. It was shown that peak height increased from 3.58 to 6.16 mV on increasing the injection volume from 50 to 250 μl. It was found that the peak height increased with the injection volume up to 200 μl, and the injection volume of 50, 100, 150, 200 and 250 μl produced the peak height of 3.58, 3.66, 4.96, 6.16 and 6.00 mV, respectively. The appropriate peak height was reached at 200 μl. The most suitable mixing coil length and injection loop volume values for further use were 60 cm and 200 μl, respectively.

3.4. Effect of 3,5-dinitrobenzoic acid and potassium hydroxide reagent flow rate

The effect of flow rate of 3,5-dinitrobenzoic acid solution and potassium hydroxide solution were investigated on the determination of andrographolide (20 μg ml^{-1}). The peak height increased from the flow rate of 0.50–1.75 ml min^{-1} for both streams. The peak height increased with increasing flow rate of each stream up to 1.00 ml min^{-1} of both 3,5-dinitrobenzoic acid and potassium hydroxide solution above which the peak height slightly decreased. Thus, 1.0 ml min^{-1} of 3,5-dinitrobenzoic acid and potassium hydroxide solution were regarded as the optimum flow rate (Fig. 6). At low flow rate, the dispersion is low and the sample throughput is low. At higher reagent flow rate,

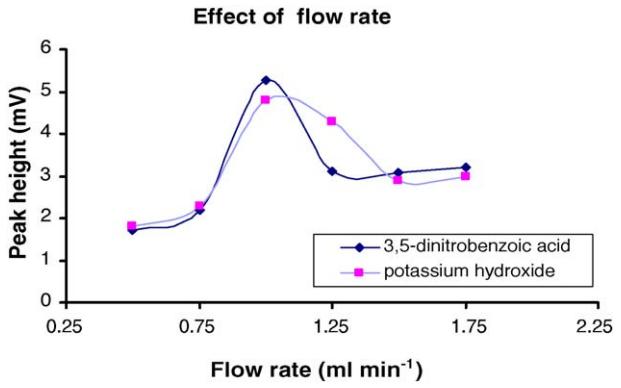

Fig. 6. Effect of flow rate on the mean of peak height ($n = 5$) of 20 μg ml^{-1} andrographolide standard solution.

the dispersion is too large, the sample throughput is high but this gives rise to high reagent consumption.

3.5. Analytical characteristics

Analytical characteristics for determination of andrographolide were studied under the optimum conditions (Table 1).

3.5.1. Calibration curve

Using the proposed FI manifold for determination of andrographolide under the optimum conditions, the linear calibration graph over the range of 5.0–150.0 μg ml^{-1} andrographolide was established which can be expressed by the regression equation $y = 0.3425 \pm 0.0017x - 0.2332 \pm 0.0699$ ($r^2 = 0.9994$, $n = 5$) where y represents the peak height in mV and x is andrographolide concentration in μg ml^{-1} after subtraction of blank. Thus, the amounts of andrographolide in *A. paniculata* can be quantified according to the above regression lines of equation. The detection limits is defined as the concentration of analyte that gives the signal that different from the blank by an amount equal to three times the standard deviation of the blank signal (3σ). It was found to be 1.50 μg ml^{-1}. The LOQ (10σ) was 5.0 μg ml^{-1} of andrographolide. The average molar absorption coefficient was found to be 0.7468×10^3 l mol^{-1} cm^{-1}.

Table 2
Effect of some excipients on the peak height of 20.0 μg ml^{-1} andrographolide standard solution

Excipients (μg ml^{-1})	Relative of peak height (%); $n = 5$
None excipient (andrographolide only)	100.00
Lactose (200)	100.00
Lactose (1000)	100.00
Magnesium sterate (200)	105.00
Magnesium sterate (1000)	107.50
Starch (200)	103.57
Starch (1000)	125.00
Magnesium hydroxide (200)	125.12
Magnesium hydroxide (1000)	135.17
Sucrose (200)	126.75
Sucrose (1000)	425.21

3.5.2. Possible mechanism

The reaction is still unclear but it is specific to the γ-lactone ring of the andrographolide. The lactone ring of andrographolide is normally stable or opens only very slowly at pH 7 or less in aqueous solution. This ring was opened readily in alkaline solution to produce the sodium salt compound of the corresponding hydroxy acid, from which the original lactone may nearly always be regenerated by acidification of the solution. This "pseudo-acid" behaviour is characteristic, and is the basis for what is easily the most widely used method for the detection of the lactone group [27,28]. It is possible that this intermediate compound was reacted with 3,5-dinitrobenzoic acid to produce an intense purplish red complex with a suitable absorption at 536 nm.

3.5.3. Reproducibility and accuracy

The relative standard deviation of the proposed method (peak height in mV) calculated from 10 replicate injections of 10.0 and 80.0 μg ml^{-1} of andrographolide were 0.66% and 1.64%, respectively. The recoveries were determined with the standard addition method in herb samples. Andrographolide (10.0 and 80.0 μg ml^{-1}) were added and mixed with 1.0000 g of a fine powder of *A. paniculata* or Fa-Tha-Lai-Chon, the herb sample

Table 1
Variables range studies and optimum conditions for determination of andrographolide

Parameters studied	Range studied	Optimum
Wavelength (nm)	200–800	536
3,5-Dinitrobenzoic acid concentration (%, w/v)	0.03–0.50	0.25
Potassium hydroxide concentration (%, w/v)	0.30–1.10	0.70
Mixing coil length (cm)	0–120	60
Sample injection volume (μl)	50–250	200
Flow rate of 3,5-dinitrobenzoic acid (ml min^{-1})	0.5–1.75	1.0
Flow rate of potassium hydroxide (ml min^{-1})	0.5–1.75	1.0
Detector: UV–vis detection unit		
Rise time (s)	–	1.0
Display	–	AU
Range (AUFS)	–	0.10
Temperature	–	Room temperature

Table 3
Accuracy of propose FI method for determination of andrographolide

Samples	FI-proposed method				Thai pharmacopoeia method
	Andrographolide content[a] (%, w/w)	Added ($\mu g\,ml^{-1}$)	Found ($\mu g\,ml^{-1}$)	Recovery[a] (%)	Andrographolide content[a] (%, w/w)
A. paniculata (1)	12.13	10.0	9.76	97.60	11.98
		80.0	78.42	98.02	
A. paniculata (2)	12.94	10.0	9.78	97.80	12.75
		80.0	78.65	98.31	
Mean	12.54			97.93	12.36
Fa-Tha-Lai-Chon (1)	10.10	10.0	9.87	98.70	9.92
		80.0	77.96	97.45	
Fa-Tha-Lai-Chon (2)	10.60	10.0	9.82	98.20	10.47
		80.0	78.13	97.66	
Mean	10.35			98.00	10.20

[a] Average from triplicate.

was extracted and analyzed using the proposed method. The percentage recoveries of 10.0 and 80.0 $\mu g\,ml^{-1}$ ($n = 12$) of andrographolide were found to be 97.60–98.70% and 97.45–98.31%, respectively, indicating that the proposed method could provide acceptable extraction efficiency and recovery of this analysis method was good.

3.5.4. Interferences

Effects of some possible excipients in commercial formulations (lactose, magnesium sterate, starch, magnesium hydroxide and sucrose) were investigated for the maximum w/w ratio of interfering to andrographolide up to 50:1. Synthetic sample solutions containing 20.0 $\mu g\,ml^{-1}$ andrographolide and different concentrations of interferences were tested, and peak heights obtained were recorded. Interestingly, lactose, magnesium sterate and starch had no significant effect on the determination of andrographolide. The most serious interferences were from magnesium hydroxide and sucrose (Table 2).

3.5.5. Analysis of andrographolide from A. paniculata

The recommended method has been applied to the determination of andrographolide in herb plant samples and Fa-Tha-Lai-Chon capsules (commercially available in a drug store in Thailand). The extracts of *A. paniculata* and Fa-Tha-Lai-Chon capsules were determined under the optimum conditions mentioned above, and the calculated contents of andrographolide were shown in Table 3. The mean contents of andrographolide from *A. paniculata* were found to be 12.54% (w/w) and 12.36% (w/w) by using this proposed method and the Thai Herbal Pharmacopoeia method, respectively. The average results for Fa-Tha-Lai-Chon capsules were 10.35% (w/w) and 10.20% (w/w), respectively.

4. Conclusion

The proposed FI spectrophotometric method has proved to be simple and uses inexpensive instrumentation for andrographolide determination. The linearity of the calibration graph is in the useful concentration range for quantitation of andrographolide in *A. paniculata*. The detection limit of this method was reasonable accepted. The method developed is simple, economic, rapid, providing a good sample frequency of 50 h^{-1}, and is especially suitable for quality control in pharmaceutical plants.

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