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Development and Validation of HPLC-PDA Assay method of *Frangula* emodin

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Abstract: *Frangula* emodin, (1,3,8-trihydroxy-6-methyl-anthraquinone), is one of the anthraquinone derivatives found abundantly in the roots and bark of a number of plant families traditionally used to treat constipation and haemorrhoids. The present study describes the development and subsequent validation of a specific assay HPLC method for emodin. The separation was achieved on a Waters Symmetry C18, 4.6 × 250 mm, 5 µm particle size, column at a temperature of 35 °C, with UV detection at 287 and 436 nm. An isocratic elution mode consisting of 0.1% formic acid and 0.01% trifluoroacetic acid as the aqueous mobile phase, and methanol was used. The method was successfully and statistically validated for linearity, range, precision, accuracy, specificity and solution stability.

Keywords: Emodin; anthraquinone; HPLC; assay; development; validation.

Introduction

A significant number of herbal medicinal products, despite their long tradition, do not fulfill the requirements imposed by European Union (EU) Directive 2004/24/EC on Traditional Herbal Medicinal Products, introduced in 2005[1], which states that companies manufacturing herbal medicinal drugs must show proof of the drug's efficacy and pharmaceutical quality. To maintain these products on the market, companies must supply scientific evidence that proves the quality, safety and efficacy of their products. *Frangula* emodin, (1,3,8-trihydroxy-6-methyl-anthraquinone) (Fig. 1), is one of the anthraquinone derivatives found abundantly in the roots and bark of Fabaceae (*Cassia* spp.), Polygonaceae (*Rheum*, *Rumex* and *Polygonum* spp.) and Rhamnaceae (*Rhamnus* and *Ventilago* spp.). These plants have been used traditionally in the Eastern world for their purgative effects to treat constipation and haemorrhoid [2, 3]. The laxative properties have been attributed to the active constituents of these plants, one of which is emodin.

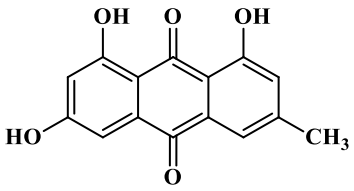

Figure 1. Emodin structure

Studies have shown emodin to display a number of biological activities such as anti-microbial[4], immunosuppressive[5], anti-diabetic[6] and anti-inflammatory activities[7, 8]. Moreover, emodin is thought to have anti-tumor properties. Studies have been performed both *in vitro* and *in vivo*, and results have shown emodin to have potential as a cytotoxic agent against a variety of human cancers including lung cancer[9], liver cancer[10], and gastric cancer[11].

Various methods have been reported in the literature for the analysis of emodin in a mixture of anthraquinones including thin layer chromatography[12], high performance liquid chromatography (HPLC) with ultraviolet-visible (UV–Vis)[13], photodiode array (PDA)[14], fluorescence[15] and mass spectrometric (MS)[16] detection. The disadvantage of running methods that were developed for the simultaneous determination of a number of anthraquinones is that in order to achieve sufficient separation between the peaks, they usually incorporate long run times. For routine analysis of emodin, a simple and rapid analytical method is preferred. Few reports of a specific validated UV spectrophotometric method for the assay quantification of *Frangula* emodin have been published to date[17, 18]. This study focused on supplying chromatographic information on the emodin molecule to meet the requirements imposed by the aforementioned directive, and enable further research and product development. The first objective was to establish the physicochemical properties of emodin that were relevant to the development of a chromatographic method in terms

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of sample preparation and HPLC analysis. Secondly, an assay HPLC method capable of identifying and quantifying emodin in the presence of related substances was developed and subsequently validated in accordance with the International Conference on Harmonisation (ICH) guideline Q2(R1) relating to the validation of analytical procedures[19].

Experimental Section

Instrumentation

A **Waters Alliance 2695 Separations Module**, consisting of an on-line degasser, quaternary pump, thermostatted sample compartment, auto-sampler, column heater/cooler and Waters 2998 PDA detector was employed. Data were acquired and processed using the Waters Empower 2.0 software. A C18 reversed phase column Waters Symmetry C18, 4.6 × 250 mm, 5 µm particle size, was used for the separation. A **Bruker AM250 Nuclear magnetic resonance (NMR) spectrophotometer** fitted with ^{1}H and ^{13}C probe of frequency 250.1 MHz for ^{1}H and 62.9 MHz for the ^{13}C probe with the corresponding software Bruker Aspect 3000 pc using 16K for ^{1}H NMR was used.

Software

ACD Chemsketch software version 10.00 Calculator Plugins were used to predict pK_a values and generate pK_a plots, Marvin 6.1.6, 2014, ChemAxon (http://www.chemaxon.com). Statistical analysis was performed using Microsoft Excel and SPSS 20.0 for Windows.

Chemicals and Reagents

HPLC gradient grade methanol and trifluoroacetic acid (TFA) (> 99.0% purity) were purchased from Sigma Aldrich. Formic acid (98% purity) was obtained from Fischer Scientific (UK.). Deionized water was purified using the ELGA Pure Lab Classic System with 0.2 µm filter, TOC 1 to 3 ppb and resistivity ≥ 18.2 MΩ-cm. The **emodin working standard** utilized during this research project was obtained from Applichem, Germany with the item code A2056. It was certified as being of HPLC grade and extracted from *Rhamnus frangula.* This working standard was used as the sample throughout the project. The reference standard of emodin of item code E7881 was obtained from Sigma Aldrich. The standard was extracted from *Frangula* bark and its HPLC purity was stated as being 96.10%.

Standard Solution

Working and reference standard solutions of emodin were prepared by dissolving accurately weighed amounts of emodin standard in 100% methanol, followed by 15 minute sonication and topping up to the mark to give a final concentration of 0.5 mg/mL. A similar standard concentration was selected in multiple studies[20, 21, 22]. The solutions were stored at 4 °C.

Method Scouting and Development

Several HPLC methods have been developed and validated for the analysis of anthraquinones. These methods primarily differ in the type of column used, mobile phase, flow rate and their applications. A literature survey was first performed in search of HPLC methods that included emodin as one of the analytes. Most methods were developed for the separation and determination of mixtures of anthraquinones present in various herbal extracts. Additionally they incorporated rather long runtimes to allow for the separation of the various components.

Methods that included phosphate buffers[23, 24] were excluded as phosphate buffers are incompatible with certain detectors, including mass spectrometry. Hence in order to allow the rapid transfer of methods from UV detection to MS detection, the chromatographic parameters were chosen to be both UV and MS compatible. Preliminary testing was performed to establish the pK_a of the emodin molecule and also to identify the UV wavelengths at which the emodin molecule absorbed most intensely.

The shortlisted methods (Systems 1, 2 and 3), which were selected based upon the results of the preliminary testing, were attempted on the Waters Alliance HPLC injecting only the emodin working standard. The resultant chromatograms were examined for sensitivity, efficiency and separation characteristics. The retention time, peak area, United States Pharmacopeia (USP) plate count and USP tailing were measured. The method that produced the best results for the emodin peak (System 3) was further optimized for column temperature, detection wavelength and mobile phase composition to obtain specificity of the emodin peak in the shortest run time possible (Systems 4, 5 and 6). A small quantity of TFA was incorporated into the mobile phase to sharpen peaks and as a result improve peak efficiency and resolution. In order to determine the optimal TFA concentration that was compatible with the emodin molecule without exceeding the pH limit recommended by the column, two concentrations of TFA were analyzed on the HPLC (Systems 7 and 8) and the resultant chromatograms were examined for efficiency of peaks. Specificity and the optimal detection wavelength were determined by performing peak purity testing using the PDA detector. The optimized method was then validated in accordance to the International Conference of Harmonization (ICH) guideline Q2(R1)[19].

Table 1. Summary of HPLC methods investigated during method scouting and development.

System	Mobile Phase Constituents (A:B)	Isocratic/Gradient Profile	Flow Rate (mL/min)	Column Temp. (°C)	UV λ (nm)	Retention Time (mins)	Reference
1	Water : methanol	Gradient 0 mins: 55%A 0 to 6 min: 55% A 6 to 9 min: 25% A 9 to 18min: 0% A 18 to 25min: 0% A	1.0	25	436	21.090	[18]
2	0.1% (v/v) Formic acid : methanol	Gradient 0 mins: 28% A 0 to 5 min:20% A 5 to 10 min: 15% A 10 to 14min: 10% A	1.0	50	N/A	21.871	[25]
3	0.1% (v/v) Formic acid : methanol	Isocratic 15% A : 85% B	1.0	25	254	9.629	[26]
4	0.1% (v/v) Formic Acid : methanol	Gradient 0 mins: 28% A 0 to 10 min:28% A 10 to 21 min: 15% A 21 to 30 min: 10% A	1.0	35	254	19.408	Optimization of System 2
5	0.1% (v/v) Formic Acid : methanol	Gradient 0 mins: 55%A 0 to 6 min: 55% A 6 to 9 min: 25% A 9 to 18min: 0% A 18 to 25min: 0% A	1.0	35	254 436	17.051	Optimization of Systems 1 and 2
6	0.1% (v/v) Formic Acid : methanol	Isocratic 10% A : 90% B	1.0	35	254 436	6.425	Optimization of System 3
7	0.1% TFA : methanol	Isocratic 10% A : 90% B	1.0	35	254 436	5.985	Investigating the effect of TFA
8	0.01% TFA : methanol	Isocratic 10% A : 90% B	1.0	35	254 436	5.891	Investigating the effect of TFA
9	0.01% TFA 0.1% formic acid : methanol	Isocratic 10% A : 90% B	1.0	35	254 436	5.905	Chosen optimized chromatographic conditions

Results and Discussion

Preliminary study of the emodin molecule

The pK_a of emodin generated using ACD Chemsketch software version 10.00, was found to be 6.39 with an error of 0.2. This value appears to be a combination of the dissociation constants pK_{a1} and pK_{a2} which were reported as being 5.70 and 7.94 respectively[27]. From this information it was deduced that an acidic mobile phase was preferable with a pH lower than 4.70 in order to have the emodin in a single ionization form. Due to the molecule's acidic functionality, the emodin would be in its unionized form at pH's lower than 4.70 and moreover in a 'robust pH zone' which is vital for reproducible retention times.

The UV spectrum of the emodin working standard dissolved in methanol (Fig. 2) gave a preliminary idea of the wavelengths that showed the highest absorption for emodin which is an important factor to consider when deciding on the detection wavelength for the HPLC method. A spectrum of pure methanol was also included as a blank to ensure that no interferences from the diluent were present. The electronic absorption spectrum of emodin was measured in methanol at room temperature in the wavelength region 200 to 700 nm. Five prominent bands were identified with maxima at 221, 252, 265, 288 and 435 nm. The spectrum matched the spectrum reported by Marković et al.[28]. The broad band at 435 nm was reported to have a dominant charge transfer character in the same research study.

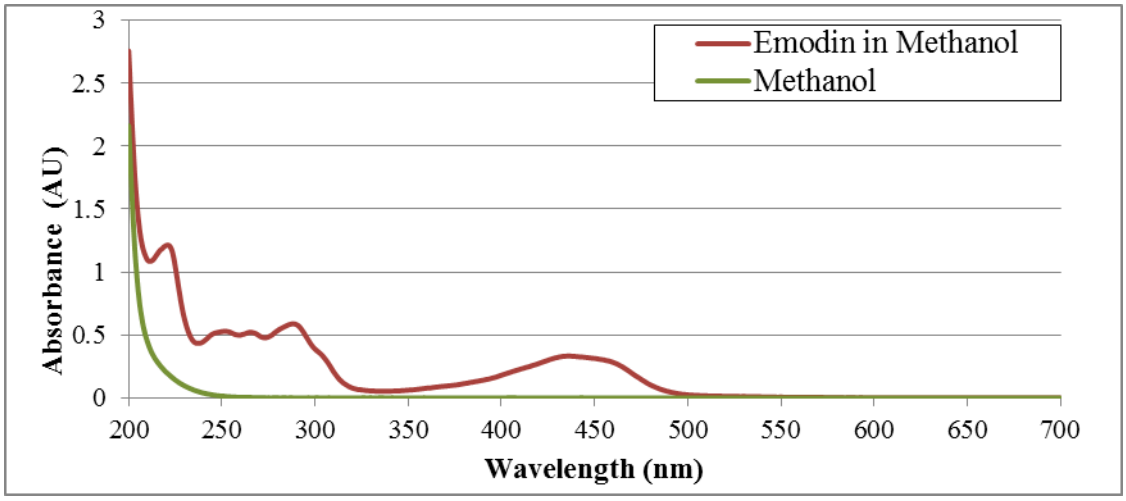

Figure 2. UV-VIS spectrum of the emodin working standard in methanol using the Shimadzu UV-Vis spectrophotometer

Method Development and Optimisation

The optimal chromatographic conditions for the HPLC assay method for emodin are listed in Table. 2, Full and Expanded integrated chromatograms are displayed in Fig. 3 (A) and 3 (B).

Table 2. HPLC method parameters for the assay determination of emodin (System 9).

Column	Waters Symmetry C18 250 x 4.6 mm, 5 µm
Mobile Phase	Mobile Phase A: 0.01% (v/v) TFA 0.1% (v/v) formic acid Mobile Phase B: 100% methanol HPLC grade
Isocratic Profile	10% MP A : 90% MP B
Flow rate	1.00 mL/min
Run Time	15 minutes
Column Temperature	35 °C
Sample Temperature	4 °C
Injection volume	10 µL
UV detection wavelength	287 nm, 436 nm and PDA Analysis (210-500 nm) Resolution: 1.2 nm
Diluent	100% HPLC grade methanol
Solution Filters	0.45 µm Pall GHP filters of code PN-4562
Test concentration	0.5 mg/mL

On injecting the working standard, four peaks were eluted, one of which was more significant than the other three (Fig. 3). An injection of the reference standard of emodin indicated that the emodin peak was expected to elute at approximately 5.9 minutes. The peaks were labelled emodin, Unknown 1, 2 and 3, all of which had excellent USP Tailing and USP plate count. The total run time of the system was 15 minutes which was considerably short. The chromatograms at 287 nm and 436 nm were visually identical so only chromatograms for the 436 nm wavelength are reported in this paper.

The method was run at dual wavelengths, 287 nm and 436 nm, as they provided two distinctive insights into the performance of the analytical method. Investigation at 287 nm, albeit having slight interferences from the mobile phase additives which may reduce the robustness of the method, offers a broader view of the components in the sample since most samples absorb at low wavelengths. On the other hand, selecting a longer wavelength is more selective towards the drug substance and picks up less system peaks and degradation products. The result of both wavelengths was reported to relate both perspectives (Table. 3).

The peaks were all baseline resolved from each other with a resolution larger than 3.0 for all the four peaks. Nevertheless peak purity testing was performed using the Waters Empower software to ensure that the peaks were also spectrally pure. A max plot for a standard injection analysed using system 9 (Tables. 1 and 2) was generated and the results are displayed in Table. 4. The results indicated the emodin peak was spectrally pure, with

purity angle values lower than the purity threshold. Thus, the method was specific for emodin as per ICH criteria for specificity, namely, "the ability of the method to unequivocally assess the analyte in the presence of other components that may be expected to be present for example, impurities, degradation products and matrix components". The first two peaks in the chromatogram (Fig. 3) were spectrally impure (Table. 4) and were hence referred to as Unknown 1 and Unknown 2. The second major peak in the system, at retention time 10.222 minutes (Table 4) was labelled Unknown 3, and with purity angle values below the threshold, was also found to be spectrally pure. All three unknown peaks were most likely impurities or matrix components. The next section describes tests that were performed in an attempt to identify the unknown peaks.

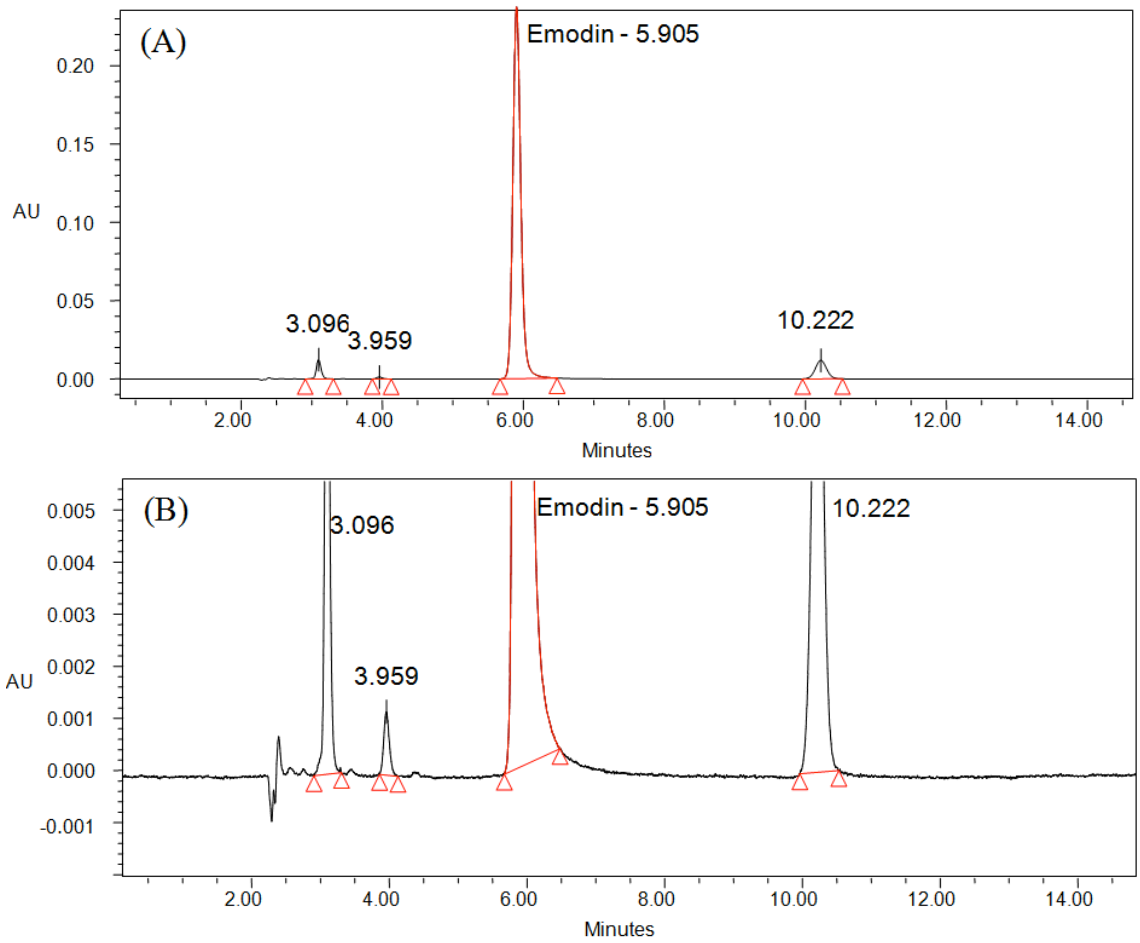

Figure 3. Full (A) and expanded (B) chromatograms obtained using chromatographic System 9 at 436 nm; Injection peak at 2.361minutes.

Table 3. Chromatographic profile obtained for the optimised HPLC assay method for emodin at 254 nm and 436 nm.

At 287nm						
Peak number	**Retention time (mins)**	**Peak name**	**Area (µv*sec)**	**% Area**	**USP Plate count**	**USP tailing**
1	3.096	Unknown 1	101984	2.77	10111	1.160
2	3.959	Unknown 2	10295	0.28	10752	1.150
3	5.905	Emodin	17366864	91.36	15941	1.170
4	10.222	Unknown 3	205965	5.59	18908	1.021
At 436nm						
Peak number	**Retention time (mins)**	**Peak name**	**Area (µv*sec)**	**% Area**	**USP Plate count**	**USP tailing**
1	3.096	Unknown 1	59603	2.89	10115	1.150
2	3.959	Unknown 2	6959	0.34	11719	1.203
3	5.905	Emodin	1863743	90.34	15958	1.168
4	10.222	Unknown 3	132767	6.44	18937	1.020

Table 4. PDA Peak Purity Output obtained using chromatographic System 9

Peak number	Retention time (min)	Peak name	Purity angle	Purity threshold	Spectral status
1	3.096	Unknown 1	2.193	0.469	Impure
2	3.959	Unknown 2	15.182	2.417	Impure
3	5.905	Emodin	0.217	0.261	Pure
4	10.222	Unknown 3	0.225	0.402	Pure

Identification of impurities

Identification of the unknown peaks that had eluted in the optimised system was attempted. The UV absorption spectra for the four individual peaks (Fig. 4) were extracted using the Empower PDA software.

On comparing the four absorption spectra, several similarities were observed. The peaks and troughs of the spectra, as well as the λ_{max} values, were similar to each other suggesting that all three unknown peaks were anthraquinones, albeit with different side groups.

A common observation made from most systems that were investigated is that one of the unknown peaks that eluted after emodin was always present in the approximate range of 6 to 7.5 % area of the total peak area. This was a good indication that the peak in these systems represented the same impurity or degradation product. Since this component was the second most abundant component in the sample it was important to attempt to identify its structure.