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High-performance liquid chromatographic determination of dehydroabietic and abietic acids in traditional Chinese medications

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Abstract

In Asia, there is still a high usage of traditional Chinese medicament by the general population. Some patients with contact dermatitis to these medicaments have been found to be sensitive to colophony on patch testing. Dehydroabietic acid (DHAA) and abietic acid (AA) are the main components of colophony and believed to be the agents responsible for skin sensitization. This paper describes a reliable high-performance liquid-chromatographic method for determining these two resin acids in ointment samples. The samples were either pretreated with diethyl ether or treated with acetonitrile directly by ultrasonication for 30 min. One volume of this sample was added to an equal volume of water and purified by solid-phase extraction. The mobile phase used was methanol–water–phosphoric acid (87:13:0.02, v/v) and the flow-rate was 1 ml/min. DHAA and AA were detected at 4.3 and 6.3 min with ultraviolet detection at wavelength 200 and 239 nm, respectively. However, fluorimetric detection with an excitation wavelength of 225 nm and emission wavelength of 285 nm, provided more selective determination of DHAA. The detection limits for DHAA and AA were 1 ng. Analytical recovery generally exceeded 90%. We analyzed nine types of commonly used topical Chinese medicaments and two types of Western medical ointments in Singapore. The results showed that most of these medicaments contain colophony below 5 ppm ($\mu g\ g^{-1}$). Only one Chinese medicament contained >70 ppm of both allergens and one of the Western medical ointments contained 0.2% of DHAA and 2.2% of AA.

Keywords: Pharmaceutical analysis; Colophony; Dehydroabietic acid; Abietic acid; Organic acids

1. Introduction

Colophony, a natural resin from species of the family *Pinaceae*, is a common skin and respiratory sensitizer. It is found in many consumer products, such as medicaments, cosmetics and industrial materials, e.g., flux, paints and adhesives. Resin acids found in rosin are generally of the abietic and pimaric types. The oxidation products of abietic (AA) and dehydroabietic (DHAA) acids are believed to be the principal sensitizers in colophony [1,2].

Traditional Chinese medicaments are currently freely available as over the counter items to the general public in many Asian countries. The herbal oils are used as soothing balms or oils for general purposes, including headache, abdominal colic and skin rash. The herbal orthopedic lotions are used for muscle sprains and strains. Many skin ointments contain mainly salicylic acid and are used as antifungal medicaments. Some patients with contact dermatitis to these medicaments have been found to be sensitive to colophony on patch testing. The identification and confirmation of the presence of the two resin acids DHAA and AA in these medicaments will help skin sensitive patients seeking colophony-free alternatives. Therefore, a method was developed

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to measure the amount of DHAA and AA in medicated oil and ointment samples.

Although a few methods using gas chromatography [3–5] and high-performance liquid chromatography (HPLC) [1,6–8] to detect colophony are described in the literature, the analysis of colophony in medicament samples has not been reported previously. Among the established methods, a recent HPLC method for the detection of DHAA and AA in Bindi adhesives, is considered the most sensitive and reliable [9]. This method is superior as its fluorimetric measurement of DHAA is at least 100 times more sensitive than those using UV absorbance detection method. We modified the latest method for the analysis of the two resin acids in some of the most commonly used Chinese medicaments in South–East Asia. The optimum conditions for separation and detection with UV spectrometry were further investigated in the present study.

2. Materials and methods

2.1. Reagents and chemicals

Standards of the analytes of interest were purchased from two different chemical companies: DHAA from Helix Biotech (Richmond, Canada) and AA from Wako (Tokyo, Japan). Orthophosphoric acid, HPLC-grade methanol, diethyl ether and acetonitrile were obtained from Merck (Darmstadt, Germany). Distilled and deionized water was used for the preparation of all solutions. The mobile phase used was methanol–water (87:13, v/v) containing 0.02% (v/v) phosphoric acid.

2.2. Standard preparation

The stock standards of DHAA and AA were prepared from 10 mg of the respective acids dissolved in 9 ml of acetonitrile and 1 ml of water. A second stock solution containing each 50 $\mu g\ ml^{-1}$ of DHAA and AA were prepared by diluting the first stock solution with the same solution. Working standards for calibration were prepared in mobile phase with concentrations ranging from 0.05 to 5 $\mu g\ ml^{-1}$ of DHAA and AA. The sample injection volume was 20 μl.

2.3. Sample preparation

The solid-phase extraction (SPE) column used for sample cleaning was a 1 ml tube column containing 100 mg of C_{18} packing material (Whatman, Clifton, NJ, USA). The column was preconditioned by rinsing with 1 ml of methanol and followed by 1 ml of water. Nine Chinese and two Western medicated oil and ointment samples from different manufacturers were purchased from medical halls and departmental stores in Singapore. For oil samples, 100 μl (around 80–100 mg) was added to 900 μl of acetonitrile. For the ointment samples, 50 mg was dissolved in 0.2 ml of diethyl ether before addition of 0.8 ml of acetonitrile. After ultrasonication for 15 min, the samples were centrifuged at 15 000 *g* for 2 min. An aliquot of 0.6 ml of sample was mixed with 0.6 ml of water. The mixture was then centrifuged at 15 000 *g* for 2 min and 1 ml of the supernatant was allowed to percolate through the preconditioned SPE column. The column was washed with 1 ml of acetonitrile–water (40:60, v/v) and effluent was discarded. The analytes were then eluted with 1 ml of mobile phase. An aliquot of 20 μl of the collected eluate was used for HPLC analysis. The results are presented in $\mu g\ ml^{-1}$ with a correction of dilution factor 2. The amounts of DHAA and AA contained in each oil and ointment samples were calculated and presented in ppm ($\mu g\ g^{-1}$).

2.4. Chromatography

The HPLC system used consisted of a Hewlett–Packard Model 1050 quaternary pumping system (Palo Alto, CA, USA), a Gilson Model 231-401 autoinjector (Villiers-le-Bel, France), a Model RF-535 fluorimetric detector (slit width 15 nm) (Shimadzu, Kyoto, Japan) and a variable-wavelength detector (Model HP1050; Hewlett–Packard). A Waters photodiode array detector (Model 996) was used for peak purity test and MILLENNIUM 2010 software was used for peak identification and integration (Milford, MA, USA). The chromatographic separation was performed on a guard and analytical cartridges system (PartiSphere 5 C_{18}, 5 μm, 110× 4.6 mm I.D.; Whatman). A Whatman solvent IFD disposable filter device was used for in-line filtration and degassing of the organically based mobile phase.

The flow-rate was 1.0 ml/min. DHAA and AA were detected with ultraviolet detection at wavelengths 200 and 239 nm, respectively. DHAA was also detected by fluorimetry with excitation wavelength set at 225 nm and emission wavelength at 285 nm.

3. Results and discussion

3.1. Chromatographic performance and sensitivity

HPLC determination of resin acids is generally carried out by HPLC with UV absorbance detection [1,6–8]. This is because AA is not detected by fluorimetry under the conditions described for DHAA [9]. In our earlier report, it was suggested that good chromatographic performances of DHAA and AA could only be obtained if they were eluted with more than 80% (v/v) of methanol or 75% (v/v) of acetonitrile. The high resolution was achieved by gradient elution with the variation of methanol composition during an analysis [9]. In our present study, we found that using isocratic elution with methanol–water (87:13) containing 0.02% (v/v) of phosphoric acid provided adequate efficiency for the analysis of the medicament samples. Our earlier method also reported that addition of formic acid to the mobile phase could enhance the efficiency of chromatographic performance of AA on C_{18} column. Using the earlier method, the absorption maxima of DHAA and AA were at 267 and 238 nm, respectively. However, at UV 267 nm, the detection sensitivity for DHAA measurement was found 140 times lower than that using fluorimetric detection method. In our present investigation, we observed that the maximum UV absorption for DHAA was at 200 nm and was about 100 times more sensitive than at 267 nm. This was because, when using phosphoric acid, the background UV absorption at 200 nm was much lower when compared with the use of formic acid as modifier. Using the present chromatographic conditions, DHAA of pure aqueous standards solution (2.5 $\mu g\ ml^{-1}$) was detected at 4.3 min with UV 200 nm (Fig. 1a) and AA was detected at 6.3 min with UV 239 nm (Fig. 2a). As shown in Fig. 3a, the peak height of the same standards detected by fluorimetry as described in earlier report (excitation 225 nm, emission 285 nm) was only one and half times higher than the peak determined by UV 200 nm (Fig. 1a). Thus, the sensitivity of UV detection at 200 nm was compatible with fluorimetry. The lowest detection limits (signal-to-noise ratio 3) for DHAA and AA by using spectrometry were 0.1 and 0.05 $\mu g\ ml^{-1}$, respectively. When using fluorimetry, the

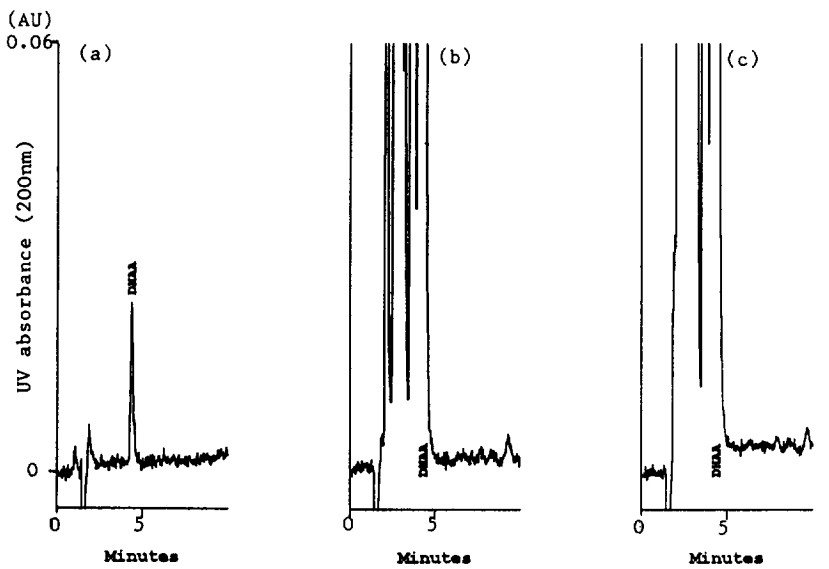

Fig. 1. Chromatograms of (a) a pure standard (2.5 $\mu g\ ml^{-1}$ of DHAA and AA), (b) a blank sample (White Flower Embrocation) and (c) same sample supplemented with 5 $\mu g\ ml^{-1}$ of DHAA and AA, were determined at UV 200 nm. The injection volume was 20 μl.

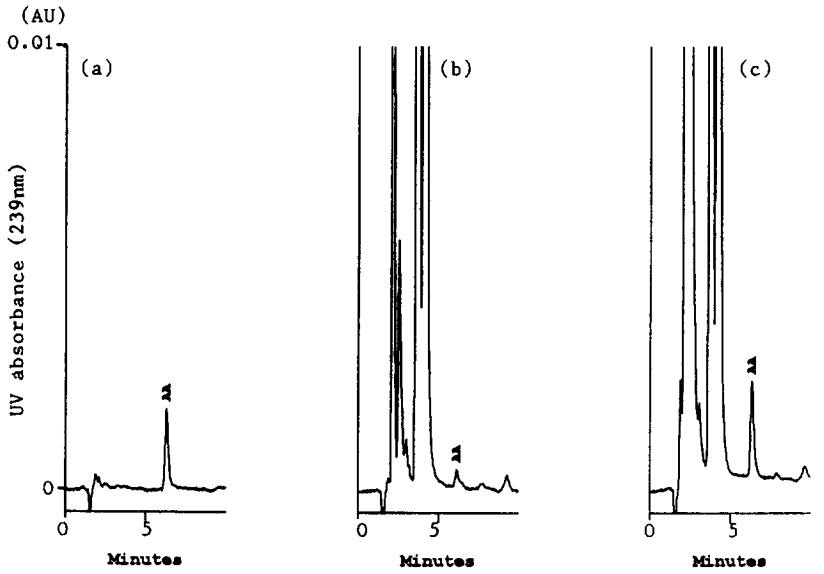

Fig. 2. Chromatograms of (a), (b) and (c) from the same samples as in Fig. 1, but determined at UV 239 nm.

lowest detection limit was 0.05 μg ml^{-1} for DHAA with a sample size of 20 μl. The total analysis time was only 12 min per injection which included a 6 min allowance for column cleaning prior to the next injection.

3.2. Matrix interference and specificity

As matrix interferences varied among individual products of different manufacturers, baseline separation for every sample was difficult to achieve. The

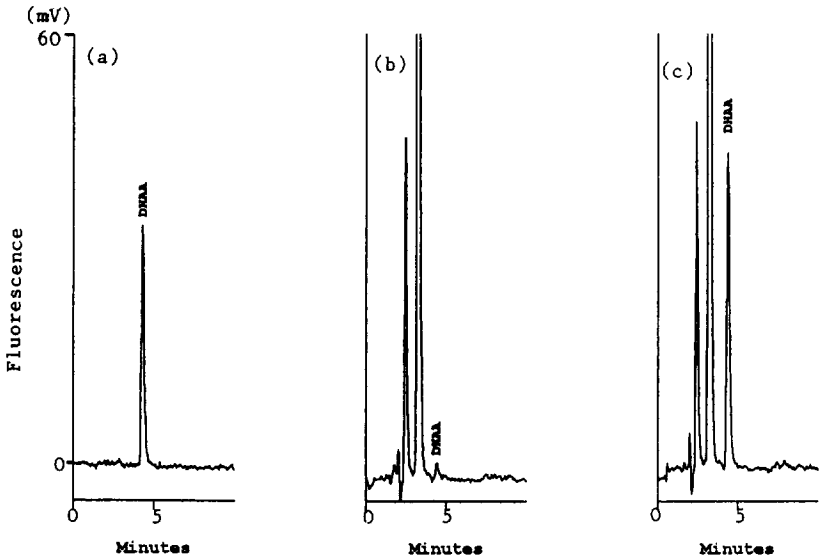

Fig. 3. Chromatograms of (a), (b) and (c) from the same samples as in Fig. 1, but determined by a fluorimetric method (excitation 225 nm, emission 285 nm).

condition described above was considered the best for the present analysis. The peak purity tests have been carried out for every sample analyzed. No evidence of coelution was found for most samples especially for AA measurement. The analysis was also free from interference by the other two types of major resin acids, pimaric and isopimaric acids. This is because these resin acids are insoluble in the mixture of methanol and water, and were separated from analytes during the sample treatment. The chromatograms of a blank sample (white flower embrocation) and its spiked (+5 μg/ml) sample determined at UV 239 nm are showed in Fig. 2b and c, respectively. AA was detected free from interference and having identical retention time as compared with the aqueous standard (Fig. 2 a). However, when UV was used for the determination of DHAA in three types of medicated oil samples, the results showed massive interference. As shown in Fig. 1b and c (same samples as in Fig. 2b and c) with chromatograms reviewed at 200 nm, DHAA coeluted with unknown components. However, good resolution of the same injection could be obtained when using fluorimetric detection (Fig. 3b and c, suggesting that fluorimetric measurement is still considered a more selective and reliable method for DHAA analysis.

3.3. Linearity

The calibration was carried out using the external standard method. The calibration curves were linear for concentrations of DHAA and AA in the range of 0.05–5 μg ml^{-1}. Typical regression equations and correlation coefficients (r) were $y=-0.04+8.43\cdot10^{-5}x$ ($r=0.99$) for DHAA and $y=0.02+6.57\cdot10^{-4}x$ ($r=0.99$) for AA, where y is the concentration of analytes (μg/ml) and x is the peak height (μV). The relative standard deviations (RSDs) of slope and linearity of calibrations for between-day analysis ($n=3$) were 0.7% and 0.2% for DHAA and 2.8% and 0.05% for AA; respectively.

3.4. Sample preparation, recovery and reproducibility

Traditional topical Chinese medicaments usually contain an assortment of plant extract, such as menthol, camphor, salicylates, eucalyptus (leaf extract) and cinnamon (bark extract). The concentrations of these components vary between different samples and manufacturers. After testing the solubility of DHAA and AA, it was found appropriate to precipitate some of these matrix components by adding equal volume of water into the sample pretreated with acetonitrile. The precipitate was removed after centrifugation. The sample was further cleaned-up with the SPE technique. The conditions and clean-up procedures have been worked out for maximum recovery. In our earlier report, the reliability and reproducibility of the method for low concentrations (<0.5 μg ml^{-1}) analysis, had been studied intensively [9]. As some of the medicament samples may have rather high concentrations of these two resin acids, in the present study we spiked the sample with concentrations of 1 and 5 μg ml^{-1} of DHAA and AA, for the determination of recovery, within-assay and day-to-day precision. The mean recovery of added concentrations was 94% for DHAA and 98% for AA. The RSDs of within-assay and day-to-day precision were generally less than 4 and 15%, respectively, for both DHAA and AA analysis ($n=3$).

3.5. Quantification of DHAA and AA in medicament samples

DHAA and AA can be found in various types of contact adhesives and facial cosmetics and can cause contact allergy [7–10]. The quantification of these resin acids in actual contact items would be helpful for clinical diagnosis and management. From our study, we observed that the content of these resin acids in two to three types of Chinese medicament samples were below the detection limit. However, a few of the samples analyzed contained more than 5 ppm of the resin acids. These samples were re-analyzed with further dilution of the specimens. The results indicate that most of the medicament samples contain 0.6 to 71.3 ppm of DHAA and 0.5–71.1 ppm of AA (Table 1). For method verification, we also analyzed two types of Western medical ointments. It was found that the concentrations of DHAA and AA in one of the samples contained about 0.2% of DHAA and 2.2% of AA.

In summary, the optimum analytical conditions for

Table 1
Dehydroabietic acids (DHAA) and abietic acid (AA) in medicament samples

Sample	DHAA (ppm)	AA (ppm)
Chinese medicament		
Axe brand universe oil (Singapore)	0.6	1.0
Eagle brand medicated oil (Singapore)	71.3	71.1
Green grass oil (Singapore)	1.8	3.5
Tiger oil (Singapore)	ND	0.5
Tiger balm ointment (Singapore)	ND	ND
Tjin koo lin oil (Singapore)	2.9	6.8
White flower embrocation (Hong Kong)	3.9	9.5
Wong cheung wah yu yee oil (Hong Kong)	ND	1.8
Zheng gu shui (Guangxi, China)	1.4	1.3
Western medicament		
Vicks VapoRub (USA)	1.8	1.7
Zam-Buk ointment (UK)	1687.7 (0.2%)	21 934.8 (2.2%)

ND=none detectable or below the detection limit of 0.4 ppm for DHAA and AA.

DHAA and AA were investigated using HPLC with UV spectrometry and fluorimetry in the present study. The results showed that fluorimetric detection is a more sensitive and specific method for DHAA analysis. However, if matrix interference is not a major problem, such as in the analysis of soldering flux, detection of both allergens could be achieved by using a wavelength programmable UV detector. It could be considered more convenient and cost effective. The proposed method has wide applications. Beside medicaments, it has been carried out for colophony determination in industrial materials such as soldering flux (unpublished data) and consumer items such as Bindi cosmetics (a type of Indian cosmetics) in our laboratory. These analyses will benefit colophony sensitive patients and workers who are in contact with colophony containing materials, seeking colophony free alternatives.

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