Talanta 85 (2011) 1634–1641

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Identification and quantification of nucleosides and nucleobases in Geosaurus and Leech by hydrophilic-interaction chromatography

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ARTICLE INFO

Article history:
Received 9 April 2011
Received in revised form 22 June 2011
Accepted 22 June 2011
Available online 29 June 2011

Keywords:
Hydrophilic-interaction chromatography
Nucleosides
Nucleobases
Geosaurus
Leech

ABSTRACT

A simple hydrophilic-interaction chromatography (HILIC) method was developed for the identification and quantification of 14 nucleosides and nucleobases, namely cytosine, uracil, cytidine, guanine, hypoxanthine, xanthine, uridine, thymine, inosine, guanosine, thymidine, 2′-deoxyadenosine, 2′-deoxyinosine and 2′-deoxyuridine in two traditional Chinese medicines, Geosaurus and Leech. The separation was achieved on a TSKgel Amide-80 column (150 mm × 2.0 mm, 3.0 μm) with a mixture of acetonitrile and 10 mM aqueous ammonium acetate as the mobile phase at a flow rate of 0.2 mL/min. The temperature was set at 30 °C and UV detection wavelength was set at 260 nm. All calibration curves showed good linearity (R^2 > 0.9957) within the test ranges. The overall intra- and inter-day RSD ranged from 0.4 to 3.4% and from 0.7 to 3.3%, respectively. The LOD and LOQ were in the range of 0.07–30.49 ng/mL and 0.26–60.98 ng/mL, respectively. The repeatability of the method was in the range of 2.2–5.8% for Geosaurus and 1.4–5.5% for Leech. The recoveries of the samples were in the range of 91.4–100.9% for Geosaurus, and 91.9–99.3% for Leech. The established method was applied successfully for the analysis of nucleosides and nucleobases in 22 commercially available samples collected from different regions in China and Japan. Our data showed that HILIC had advantages as a useful tool for the study of the bioactive components in Geosaurus and Leech as well as their quality control, and could therefore be used for the determination of the analytes in pharmaceutical products and biological fluids.

1. Introduction

The annelids of Geosaurus (*Pheretima aspergillum* (E. Perrier), *Pheretima vulgaris* Chen, *Pheretima guillelmi* (Michaelsen), *Pheretima pectinifera* Michaelsen) and Leech (*Whitmania pigra* Whitman, *Hirudo nipponica* Whitman, *Whitmania acranulata* Whitman) are valued traditional Chinese medicines (TCMs) which are listed in the Pharmacopoeia of the People's Republic of China [1,2]. Both Geosaurus and Leech preparations, especially their aqueous extracts, have been used widely for medical purposes, and have shown to exert various effects such as blood-activating, stasis-dissolving, antipyretic and diuretic effects base on a combination of multiple mechanisms. However, the bioactive components of the medical products of Geosaurus and Leech and their related activities are still not fully understood.

Recently, nucleobases and nucleosides have been proven as important bioactive compounds involved in multiple biological activities such as anti-platelet aggregation, anti-arrhythmic and anti-seizure effects [3–6], and have also been used as markers in the quality control of several TCMs, such as *Ganoderma lucidumn* and *Cordyceps sinensis* [7,8]. Therefore, the purpose of this study was to quantitatively and qualitatively analyze the nucleobase and nucleoside compounds from the water extracts of Geosaurus and Leech.

The contents of nucleosides and nucleobases in biological fluids and herbal materials have been determined by a number of analytical methods including thin layer chromatography (TLC) [9], high-performance liquid chromatography (HPLC) [10–18], liquid chromatography–mass spectrometry (LC–MS) [19–23], ultra-performance liquid chromatography (UPLC) [24], capillary electrophoresis–mass spectrometry (CE–MS) [25,26], gas chromatography (GC) [27,28], capillary zone electrophoresis (CZE) [29,30], capillary electrochromatography (CEC) [31,32] and micellar electrokinetic chromatography (MEKC) [33,34], but many of these methods have disadvantages such as limited analytes [12,14,15,17–20,23,26–31,33], low sensitivity [9] or expensive instrumentation [19–23,25,26]. The establishment of a simple, efficient and sensitive method is thus required for the identification and quantification of nucleosides and nucleobases in TCMs.

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0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.06.056

The HILIC method was first developed by Alpert in 1990 as an alternative to reversed-phase liquid chromatography (RP-HPLC) [35]. In contrast to RP-HPLC where a hydrophobic octadecyl (C18) stationary phase is used, HILIC separation is based on the strong hydrophilic interaction of polar compounds with the hydrophilic polar stationary phase. It has consequently been shown that HILIC is suitable for the separation of a broad spectrum of hydrophilic compounds, including peptides, amino acids, oligonucleotides, carbohydrates and many other biologically important compounds [35–37].

In this study, both RPLC and HILIC method were used and compared for the identification and quantification of nucleosides and nucleobases in the samples of Geosaurus and Leech. In contrast to RP-HPLC, where the separation of hypoxanthine and guanine remains a problem [22], 14 nucleosides and nucleobases, including cytosine, uracil, cytidine, guanine, hypoxanthine, xanthine, uridine, thymine, inosine, guanosine, thymidine, 2′-deoxyadenosine, 2′-deoxyinosine and 2′-deoxyuridine could be identified and quantified simply and accurately by HILIC method. The investigated compounds of the collected Geosaurus and Leech samples in their aqueous extracts could be satisfactorily separated, and their contents were also compared.

2. Material and methods

2.1. Materials and chemicals

Nucleoside and nucleobase standards of cytosine, uracil, cytidine, guanine, hypoxanthine, 2′-deoxycytidine, xanthine, uridine, thymine, adenine, inosine, guanosine, 2′-deoxyguanosine, xanthosine, thymidine, adenosine, 2′-deoxyadenosine, 2′-deoxyinosine and 2′-deoxyuridine (the structures are shown in Fig. 1) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); ammonium acetate, acetic acid, monopotassium phosphate, ammonia solution (25%), hydrochloric acid, HPLC grade methanol and acetonitrile were also purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); Ultra-pure water was prepared using a Milli-Q Plus system (Millipore, Bedford, MA, USA).

Seventeen samples of Geosaurus and Leech were collected from Guangdong, Jiangxi, Sichuan, Guangxi, Shandong, Hebei, Anhui, Jiangsu Provinces, China, and 5 (G1, G8, L1, L4, L9) from Japan. All samples were authenticated by one of our authors, W.L. according to their morphological characteristics. The voucher specimens were deposited in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Toho University, Japan.

2.2. Preparation of standard solutions for linearity studies

Nucleoside and nucleobase standards were dissolved in methanol, except for inosine, adenosine, hypoxanthine, xanthine, adenine and guanine, which were dissolved in water, 0.1 mol/L hydrochloric acid, 1.2% ammonia solution and concentrated hydrochloric acid, respectively. The stock solutions were further diluted with methanol to obtain stocks at concentration of 1.0 mg/mL, and stored in a refrigerator at 4 °C.

2.3. Sample preparation

Nucleosides and nucleobases are generally extracted in water soluble form [14,16,19–21,25,38]. In this study, samples of Geosaurus and Leech were first dried at 40 °C for 2 h before being grinded into powder (approximately 20 meshes), and then prepared as 5% (g/mL) solution by dissolving 1 g of the powder with 20 mL Milli-Q water. The solution was then ultrasonic extracted (40 kHz, 240 W) for 2 h at room temperature followed by filtration. Five millilitres of the filtrate was vacuum-dried, and the residue was dissolved in 3 mL of mixture of water/methanol (1:1). After centrifugation at 3000 rpm for 10 min, the supernatant was then filtered through a 0.45 μm membrane filter prior to further analysis.

2.4. Instrumentation

Analysis were performed on a Waters Series 2950 (Waters Technologies, USA) liquid chromatograph system comprising a vacuum degasser, a quaternary pump, an autosampler, and a Photo-Diode Array (PDA) system. Data was collected and analyzed by Waters ChemStation software.

2.5. Chromatographic condition

For RP-HPLC, chromatograms were run on an YMC C_{18} (4.6 mm × 250 mm, 5 μm) column, and two sets of elution buffers were used as mobile phase. The system operated at 30 °C, and the PDA detection wavelength was set at 260 nm. The elution conditions were as follows:

(a) Mobile phase A = 20 mM monopotassium phosphate solution, B = acetonitrile, flow rate, 0.5 mL/min; a linear gradient of 1–6% B for the first 24 min; increased from 6 to 20% B from 24 to 35 min; increased from 20 to 70% B from 35 to 45 min; 75% B from 45 to 50 min; and a linear gradient of 75–50% B from 50 to 52 min.
(b) Mobile phase A = acetate–ammonium acetate (pH 3.5), B = acetate–ammonium acetate (pH 3.5)/acetonitrile (90/10), flow rate, 0.7 mL/min; 75% B isocratic for the first 20 min; a linear gradient from 1 to 70% B from 20 to 30 min; increased from 70 to 95% B from 30 to 40 min.

For HILIC, chromatograms were run on TSKgel Amide-80 (2.0 mm × 150 mm, 3 μm) column. Mobile phase including (A) acetonitrile and (B) ammonium acetate (10 mM, pH 6.9) was degassed ultrasonically before use. The flow rate and sample injection volume was 0.2 mL/min and 2 μL, respectively. The system operated at 30 °C, and the PDA detection wavelength was set at 260 nm.

2.6. Calibration curves

Standard stock solutions of the reference compounds were prepared and diluted to a series of appropriate concentration for the construction of calibration curves. At least 6 concentrations of each reference compound solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentration of each reference compound.

2.7. LOD and LOQ

The limits of detection (LOD) was defined as the lowest concentration resulting in peak heights of three times the baseline noise. The limits of quantification (LOQ) was defined as the lowest concentration resulting in peak heights of interest with S/N ratio higher than 10, with a precision of 15% and accuracy of 80–120%.

2.8. Precision, repeatability and recovery

Intra- and inter-day variations were chosen to determine the precision of the method. For intra-day variability test, the mixed standards solution was analyzed for six replicates ($n = 6$)