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A simple method for the simultaneous determination of guanine, xanthine and hypoxanthine by RP-HPLC with UV detection

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Abstract : A simple, rapid and accurate method for the simultaneous determination of guanine, xanthine and hypoxanthine has been developed. The quantitative determination of these bases was accomplished by reverse phase high-performance liquid chromatography (RP-HPLC) with UV detection based on their different retention time without chemical suppression. Separation in the order of guanine, hypoxanthine and xanthine were obtained on ODS hypersil column using mobile phase containing 1% ACN in acetic acid (50 mM), ammonium acetate (85 mM) and 0.5% methanol at 0.5 ml min^{-1} flow rate. A portion of the natural DNA isolated from *Avicennia marina* was spiked with known amount of hypoxanthine and its concentration was found to be 1.4 μM.

Keywords : Xanthine, hypoxanthine, retention factor, RP-HPLC.

Introduction

The atmospheric pollution due to the occurrence of NOx sourced from fossil fuel used in the energy sector and agricultural biomass burning is of increasing global concern to public health and plant functioning. Nitrous acid (HNO_2) is present in very small quantities (1–5 ppbv) and usually makes up only a small fraction of the total amount of gaseous nitrogen oxides present in the atmosphere and HNO_2 build up can be attributed to the heterogenous NO_2 to HONO conversion and the OH + NO reaction[1,2]. Nitrous acid readily produces transitions by replacing amino groups on nucleotides with keto group ($-NH_2$ to $=O$). The result is that adenine (A) is converted to hypoxanthine (Hx), and guanine (G) to xanthine (Xa). Transition mutation results, for hypoxanthine forms pair with cytosine instead of thymine, the original base paired with adenine. Xanthine, however, pair with cytosine just as guanine does. The replacement of guanine with xanthine does not cause changes in base pairing. Occurrence of hypoxanthine above normal level (5 μM) is found in plasma and urine of the patient suffering from xanthinuria[3,4].

Thus, considering the relatively complicated mobile phase of RP-HPLC, it is imperative to develop a simple and rapid method for the simultaneous determination of hypoxanthine along with other bases in DNA. The problem is to achieve separation of hypoxanthine from guanine and xanthine. A number of methods for the analysis of free bases, nucleosides and nucleotides by HPLC have been described in the literature[5,6]. Isocratic separations on ion-exchange columns[7–9] have proved to be more time consuming than analysis performed by reversed-phase HPLC[10,11]. Zheng and Row[12] showed that use of ionic liquids could resolve partially or completely the overlapped peaks of guanine and hypoxanthine. The purpose of the present study is to investigate the potential method for the separation of guanine (G), hypoxanthine and xanthine with out using any ionic salts or modifier. This paper represents a simple RP-HPLC technique using two organic solvent in different proportion and adjusted column flow rate. The technique takes advantage of the resolving capabilities of reverse phase chromatography reagents to separate rapidly and completely the purines of interest resulting in symmetrical, fairly narrow peaks that are easily quantified.

Results and discussion

The resolution of Hx, Xa and G achieved with commonly used mobile phase containing 3% acetonitrile, 50 mM acetic acid and 85 mM ammonium acetate was not

achieved. In an attempt to study the effect of organic solvent nature and concentration, ACN and MeOH were tested at different concentrations as organic modifier for further study. Among the all mobile phase attempted, better results were obtained using 50 mM acetic acid and 85 mM ammonium acetate with incorporation of 0.5% methanol and 1% acetonitrile for the separation of Hx, Xa and G at the flow rate of 0.5 ml min^{-1} (Fig. 1).

ACN was selected as organic modifier for several previous study[15]. The retention factor of all studied com-

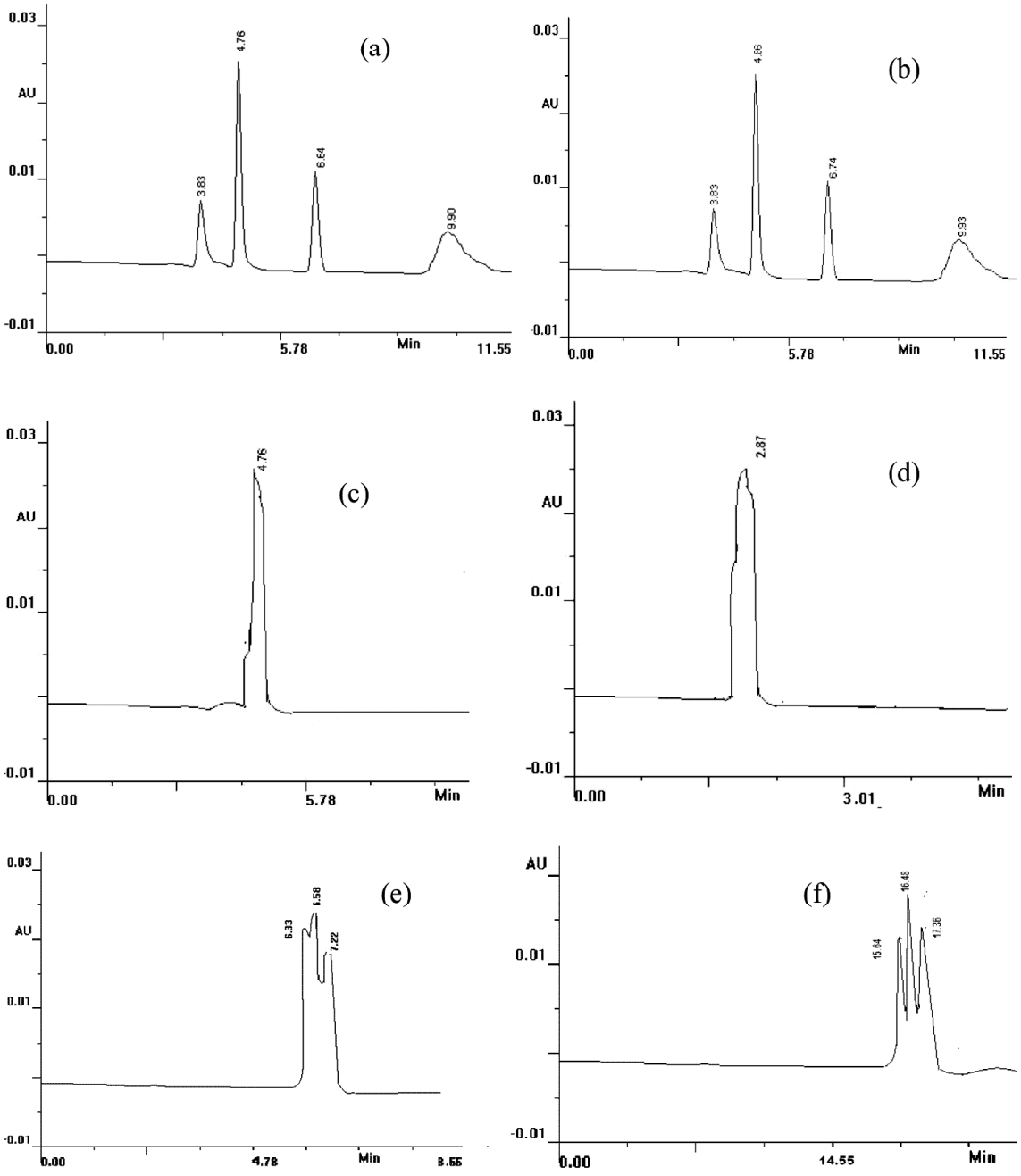

Fig. 1. Chromatograph of standards on mobile phase : acetic acid (50 mM), ammonium acetate (85 mM) and methanol (0.5%) with 3% ACN at flow rate of 1 ml min^{-1} : (a) DNA bases (C, G, T, A), (b) DNA bases with hypoxanthine and xanthine, (c) guanine, hypoxanthine and xanthine in 3% ACN, (d) guanine, hypoxanthine and xanthine in 30% ACN, (e) guanine, hypoxanthine and xanthine with 1% ACN and (f) guanine, hypoxanthine and xanthine with 1% ACN at flow rate of 0.5 ml min^{-1}.

pounds decreases with increasing the ACN percentage in the mobile phase and increased with the decreasing the percentage. As an example, the retention time of G was decreased to 2.87 from 4.76 min in case of 30% ACN.

The technique described in the present paper allows rapid (within 20 min) analysis of all major bases with their damage products in acid hydrolysates of DNA. Peak identification was performed by comparing the retention times of peaks obtained for mangrove leaf samples with those of authentic standards. The identifications and accuracy of the method was assessed by determining the recovery of known quantities of Hx, to the mangrove leaf samples (Fig. 2). The calibration curves exhibited satisfactory linear behavior (r^2 = 0.99) over the wide concentration range, and standard addition of 2, 3 and 4 µM of hypoxanthine in 1 ml of twenty times diluted acid hydrolyzed DNA sample (obtained from 1 g mangrove leaf containing 34 µg DNA) showed the increase of response linearly. Extrapolation of the linear curve enabled the concentration of hypoxanthine in the leaf DNA sample (Fig. 3). The hypoxanthine concentration calculated from the extrapolation in the sample was found to be 1.4 µM.

Experimental

Labboratory analyses :

All bases including Herring sperm DNA were obtained from HIMEDIA. For natural DNA sample, the youngest fully developed two leaf pairs per different mangrove tree (*A. marina*) were collected from an unshaded twig at 10 m height of the canopy (Sundarbans mangrove forest, latitude 20°32′–20°40′ N and longitude 88°05′–89° E) and washed with Mili Q water. 10 g leaf sample without petioles were crushed in liquid nitrogen and homogenized in 50 ml of extraction buffer containing 100 mM Tris-HCl, 10 mM EDTA, and 500 mM NaCl, followed by the addition of 5 ml SDS (20%) with stirring for 15–20 min. The samples were incubated for 10 min at 65 °C. After mixing with 50 mM ammonium acetate samples were incubated at 0 °C. The mixture was centrifuged to remove precipitate of protein[13] and the centrifugate was incubated at –20 °C for more than 1 h after mixing with isopropyl alcohol. The DNA precipitate was separated by centrifugation and washed with 70% ethyl alcohol. DNA was purified by phenol-chloroform

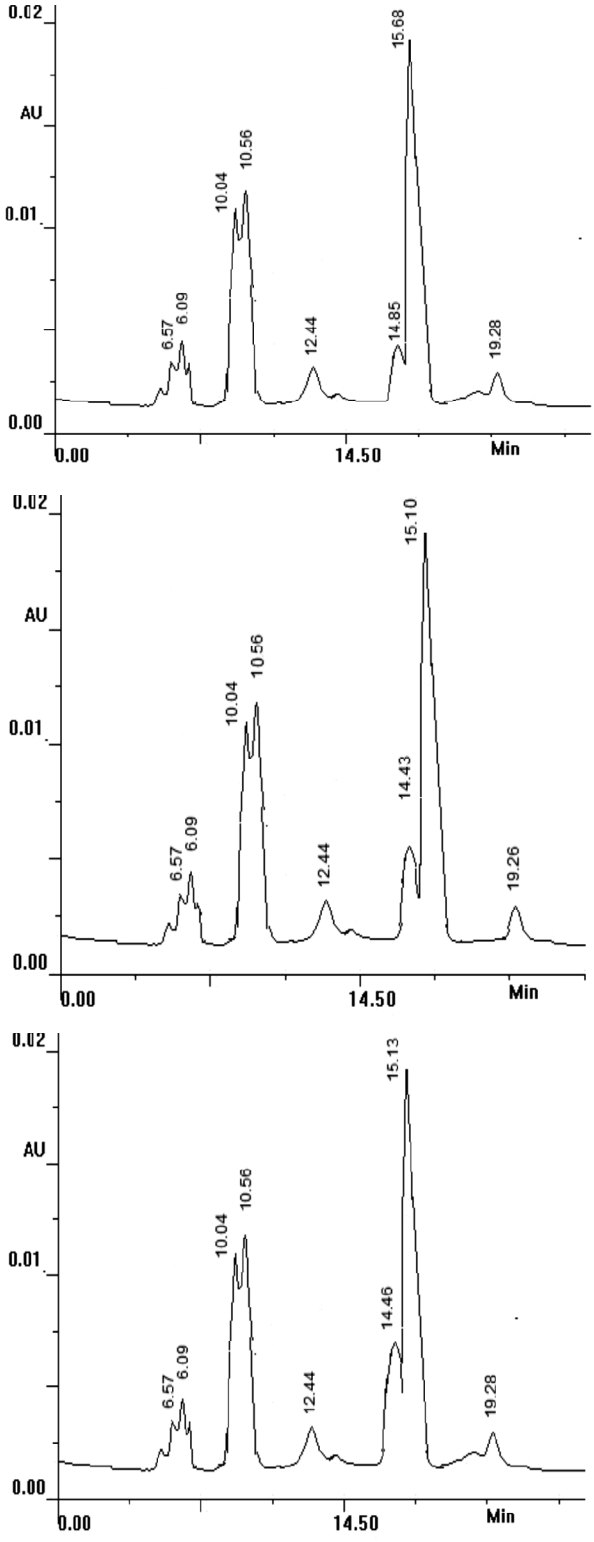

Fig. 2. Chromatograph of mangrove leaf DNA after acid hydrolysis and standard addition of (a) 2 µM, (b) 3 µM, (c) 4 µM hypoxanthine on mobile phase : acetic acid (50 mM), ammonium acetate (85 mM) and methanol (0.5%) with 1% ACN at flow rate of 0.5 ml min^{-1}.

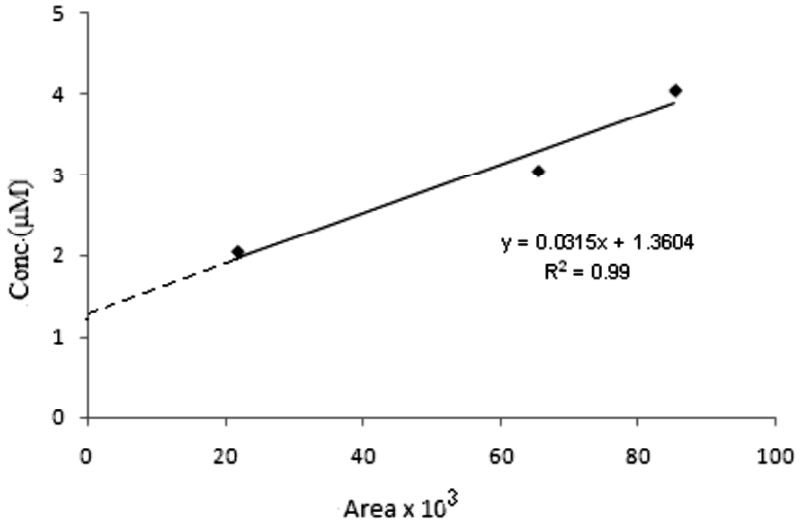

Fig. 3. Extrapolated regression line for calibration after addition of hypoxanthine standard in the mangrove DNA sample after acid hydrolysis.

extraction and ethanol precipitation and was further purified by treatment with LiCl overnight at 0 °C [14]. Deionized water with specific resistance of greater than 18.2 MΩ cm was used.

The HPLC equipment consisted of a JASCO HPLC apparatus (JASCO, Japan) consisting of a pump and controller unit with an injection volume of 20 mL and UV detector. The system was coupled to a ODS Hypersil column (Thermo electron Corporation, Part No. 30109-254630, 4.6 mm × 25 mm, 5 μ particle size) and a guard column (5 mm × 4.6 mm). The column temperature was ambient (22.0 ± 3 °C), and the detector wave length was set at 254 nm.

An accurately known amount of mangrove DNA (500 μg) was hydrolyzed in 1.0 ml of 72% (v/v) perchloric acid (~12 *N*) over a water bath for 2 h in a sealed Teflon bomb. The hydrolysate was diluted to 2.0 ml with water. Then the mixture was neutralized with 5 (*N*) KOH, and centrifuged at (3000 rpm for 30 min). The supernatant was transferred to another tube. The solution was diluted 10 to 100 times with deionized water prior its injection.

Known amount of hypoxanthine standards (2, 3, 4 μM) were added to the natural DNA solution. The area of the peak was plotted as a function of the amount of standard added, and the best straight line was drawn through them. Extrapolation to the horizontal axis gave the equivalent amount of hypoxanthine in the natural DNA sample.

All HPLC buffers and mobile phases were filtered through 47 mm, 0.22 μm micropore filters (Sigma-Aldrich) and sonicated prior to use. Mobile phase was a mixture of acetic acid (50 mM), ammonium acetate (85 mM) and methanol (0.5%) with 30%, 20%, 3% and 1% ACN (acetonitrile) and the column flow rate was varied between 0.5 and 4 ml min^{-1}. Standard solution of 2 μM DNA bases (A, T, G, C) and Hx, Xa were prepared dissolving in the mobile phase.

All bases in plant DNA were identified and quantified by comparing their retention times with those of the individual base solutions and a mixture of four bases from an acedolysed herring sperm standard. Recovery rates obtained by adding pre-determined amounts of individual bases to the diluted hydrolysates of herring sperm DNA were more than 98%. The method described here was found sensitive and applicable to the study of purine and pyrimidine bases in natural DNA.

Conclusion

Quantification of Hx in the sample could be achieve using organic solvent mixture of methanol and ACN to modify the polarity of the the mobile phase. The developed method was successfully applied to natural DNA

and minimum detection limit was > 1 μM for hypoxanthine. This method is simple, rapid and could be employed, as an alternative to existing methods, to determine hypoxanthine occurring in the urine of the patient.

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