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Short communication

Determination of purine bases and nucleosides by conventional and microbore high-performance liquid chromatography and gas chromatography with an ion-trap detector

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Abstract

A reversed-phase high-performance liquid chromatographic method has been developed for the analysis of purine and pyrimidine bases, uric acid and nucleosides largely relating to the purine synthetic and degradation metabolic pathways, with particular attention to the separation of hypoxanthine, xanthine and guanine. Complete separation and quantitation of the purines has been accomplished in the nanogram–microgram scale on conventional 4.6 mm I.D. columns with a standard gradient HPLC instrumentation as well as on 1 mm I.D. microbore columns with a dedicated isocratic micro-HPLC system using a dioxane–sodium acetate buffer. For the definite identification of components in excreta of ticks a GC–MS method has been described involving formation and GC of the trimethysilyl derivatives on a 25-m DB-5 column directly coupled with an ion trap detector. The methods are demonstrated on the analysis of the purine metabolites having an assembly pheromone effect on argasid ticks.

1. Introduction

Blood-feeding arthropods living on high protein diet, such as ticks, excrete relatively high amounts of nitrogenous metabolites into the environment, in particular hematin and purine bases [1–4]. An interest in products of the purine metabolism has recently been stimulated by the finding that certain purine compositions excreted by argasid ticks represent their assembly pheromones which induce ticks' clustering in natural habitat [5–7]. Since ticks obtain food in variable long-term intervals, the concentration of individual purines in physiological fluids, excretory organs and waste material varies with their physiological stage. As a result, assembly efficacy of the pheromone composed of guanine (Gua), hypoxanthine (Hyp) and xanthine (Xan) is also affected and there is a need of reliable monitoring of large-range purine levels in the excretory products of various arthropods by a sophisticated analytical method [6].

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With the advent of high-performance liquid chromatography (HPLC) accurate and sensitive analysis of small nucleic acid constituents in physiological samples has become a routine technique. A number of studies has appeared on this subject including those dealing with the separation of intermediates of purine metabolism (see, e.g. reviews [8,9]). The experimental knowledge accumulated in recent years shows that reversed-phase (RP) HPLC is particularly suited for the separation of purine metabolites. Generally, octadecylsilica packing materials and 0.001–0.1 *M* potassium dihydrogenphosphate or sodium acetate buffers with a methanol gradient as a mobile phase have been most widely used for the analysis of this class of compounds [8–15].

Searching a suitable analytical method for the determination of purines in our samples of tick excretory products we encountered that guanine, xanthine and hypoxanthine are eluted very close together on the RP-HPLC columns [11–14] so that their separation is difficult, particularly if one component predominates. The present investigation was therefore undertaken to develop a RP-HPLC method with emphasis on the separation of the three purines in a large range of concentrations. We also examined 1 mm I.D. microbore columns and a micro-HPLC dedicated instrumentation to this subject. As a complementary method, identity of individual components in the excretory samples was confirmed by gas chromatography (GC)–mass spectrometry (MS) using an ion-trap detector.

2. Experimental

2.1. Chemicals

Purine and pyrimidine bases, uric acid and nucleosides were obtained from: guanine (Loba Chemie, Fischamend, Austria), uric acid, hypoxanthine, adenosine, xanthosine, inosine, guanosine, thymidine, cytidine, uridine, cytosine, thymine, uracil (Merck, Darmstadt, Germany), guanine hydrochloride, xanthine, adenine (Lachema, Brno, Czech Republic). Solvents and other chemicals were purchased from: acetonitrile (Fluka, Buchs, Switzerland), pyridine (Loba Chemie), methanol, dichloromethane, *n*-butanol, *n*-hexane, sodium acetate, phosphoric acid, hydrochloric acid (Lachema), bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Gland, Switzerland). Water, organic solvents and buffer solutions used in HPLC were filtered through a 0.22-μm Durapore membrane (Millipore, Bedford, MA, USA). Pyridine and acetonitrile were dried and distilled before to use.

2.2. Sample treatment

Sample collection and bioassays have been described elsewhere [6]. Collected waste excreta of ticks (about 3 mg) were dissolved in 10% H_3PO_4 and further diluted to the final 1% H_3PO_4. After centrifugation, 10 μl or 0.5 μl (micro-HPLC) aliquots were injected into the HPLC devices. Physiological fluids (coxal fluid, haemolymph) were analysed directly without dissolution in H_3PO_4. In order to study mutual ratios of purines on the surface of spherulic excrements, distilled water and 1% NaCl solution were utilized as extraction reagents instead of H_3PO_4.

2.3. HPLC instrumentation

HPLC system I

Studies with conventional packed columns were carried out on a Varian Vista 5500 apparatus (Varian, Walnut Creek, CA, USA) equipped with a 10-μl injection valve (Rheodyne 7126); a UV-200 variable-wavelength detector set at 254 nm and a DS-604 data station. The column was a Hypersil ODS; 5 μm, 250 mm × 4.6 mm I.D. (Keystone Scientific, Bellefonte, PA, USA); placed in an oven thermostatted to 30°C. Solvent A was 0.004 *M* sodium acetate pH 4.5, solvent B was destilled water, solvent C was acetonitrile–water (80:20). Isocratic elution was with a mixture A–B (10:90) for 10 min, then a 15-min linear gradient to C–B (10:90) was used.

A constant flow of 1.00 ml/min was maintained during the analysis.

HPLC system II

The micro-HPLC system used in this work was a Carlo Erba Instruments System 20 (Carlo Erba, Milan, Italy) consisting of a single microprocessor-controlled syringe pump (Phoenix 20), a 0.5-μl injection valve (Rheodyne 7520); a variable-wavelength UV–visible detector (Micro UVIS 204) set at 254 nm and a SP 4270 integrator (Spectra-Physics, Darmstadt, Germany). An Alltech C_{18} (P.N. 8005) column was used, packed with the Carbosieve C_{18} HS 5V; 5 μm, 250 mm × 1 mm I.D. (Alltech, Deerfield, IL, USA), held at room temperature. The analyses were performed in the isocratic mode using a 0.8% solution of dioxane in 0.5 m*M* sodium acetate, pH 5.5. The flow-rate was 25 μl/min.

2.4. Derivatization procedure for GC–MS analysis

About 0.25 mg of the ticks' excrements were placed in 2-ml Micro reaction vials (Supelco) and dried at 80°C for 1 h. A freshly prepared mixture of BSTFA–pyridine (1:1, v/v) was added (150 μl). The vials were capped with PTFE-lined caps and heated in an oil bath at 150°C for 30 min. After cooling, 1-μl aliquots were analysed.

2.5. GC–MS

GC–MS was carried out on a Varian 3400 gas chromatograph (Varian, USA) directly coupled to an ion-trap detector ITD 800 (Finnigan-MAT, San Jose, CA, USA). Compounds were separated on a 25 m × 0.25 mm I.D. DB-5 fused-silica capillary column (J&W Scientific, Folsom, CA, USA). The operation conditions were: injector temperature 260°C, split 20:1; helium carrier gas velocity about 1 ml/min; column temperature programme, typically: 1 min hold at 90°C, then 10°C/min to 260°C, 15 min hold; transfer line temperature 260°C on average. The ITD 800 was operated under the automated gain control; the mass range 70–650 u was scanned every 1 s.

3. Results and discussion

3.1. HPLC separation of hypoxanthine, xanthine and guanine

The complete separation of the Hyp/Xan/Gua trio was accomplished by the isocratic RP-HPLC system I on a carefully selected 4.6 mm I.D. octadecylsilica column as shown in Fig. 1a and c. Separation of bases together with nucleosides was performed using a methanol–sodium acetate buffer gradient elution (Fig. 2). The column must be thermostatted in order to obtain reproducible retention of components, particularly nucleosides.

Preliminary experiments revealed that resolution of purines is largely influenced by the ionic strength and pH of buffer, organic modifier and also the performance of the manufactured column. The desired resolution of the components was only achieved by means of diluted buffers below the concentration of 5 m*M*. The capacity factors of the purines decrease with increasing pH and, under the condition used, optimal resolution was reached either at pH 5.1 (Fig. 1c) or below pH 4.5 (Fig. 1a).

Having tested various columns in our laboratory, we ascertained that optimization of the ion strength and pH failed in most cases, e.g., also with our microbore column. Fortunately, res-

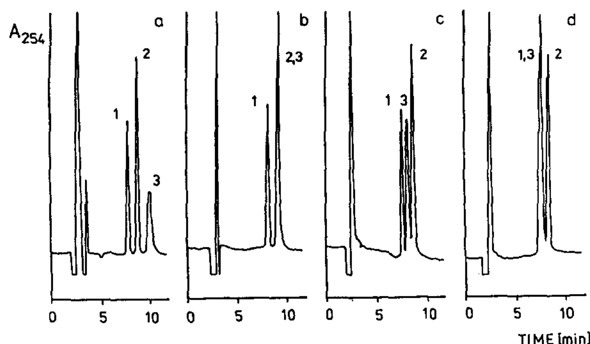

Fig. 1. Elution profile of Hyp (1), Xan (2) and Gua (3). HPLC system I; ODS Hypersil (250 × 4.6 mm I.D., 5 μm) column, isocratic elution with water–4 m*M* sodium acetate (9:1, v/v), pH: (a) 4.2, (b) 4.8, (c) 5.1, (d) 7.4; flow 1.0 ml/min, detection at 254 nm. Amounts injected: 7.8 (1), 18.2 (2) and 24.5 (3) ng.

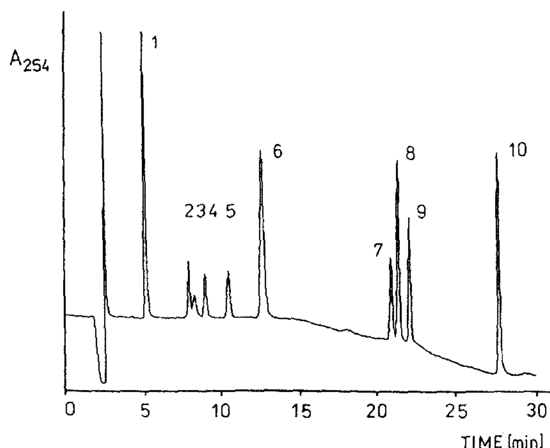

Fig. 2. Elution profile of bases and nucleosides. HPLC system I; ODS Hypersil (250 × 4.6 mm I.D., 5 μm) column, gradient elution, for conditions, see Experimental. Peaks: 1 = uracil; 2 = Hyp; 3 = Gua; 4 = Xan; 5 = uridine; 6 = thymine; 7 = xanthosine; 8 = inosine; 9 = guanosine; 10 = adenosine.

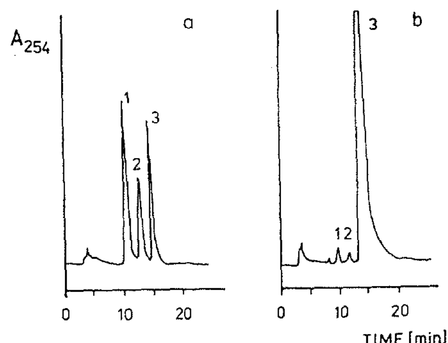

Fig. 3. Separation of the purine triplet Hyp/Xan/Gua on a 1 mm I.D. microbore column. Micro-HPLC system II; Alltech C_{18} (250 × 1 mm I.D., 5 μm) column, isocratic elution with 0.5 m*M* sodium acetate pH 5.5 containing 0.8% (v/v) dioxane; flow 25 μl/min, detection at 254 nm. (a) Standard solution: 1 = Hyp, 2 = Xan, 3 = Gua; amounts injected 3.1, 2.9 and 2.6 ng, respectively. (b) Real sample of tick excreta of *Argas persicus*, Hyp (1), Xan (2), Gua (3).

olution can further be increased by using an organic modifier.

We experimented with the dioxane–sodium acetate mobile phase, since retention of guanine on the reversed-phase packing materials is selectively affected by interaction with this solvent [11]. As an example, the complete separation of the Hyp/Xan/Gua trio using the isocratic sodium acetate–dioxane elution on a 1 mm I.D. octadecylsilica RP-HPLC column (micro-HPLC system II) is shown in Fig. 3a.

Detection limits were estimated to be about 1–2 ng for purine bases with HPLC system I. The use of buffers with low pH values (below pH 4.5) caused the increase of the guanine tailing (Fig. 2) and thus the decrease of its detection limit to 4 ng. The relationship between the concentration and the peak area of these compounds was linear from 10 ng to 100 μg. Detection limits for adenine and nucleosides were about 2 ng and 0.7–1 ng, respectively. With the micro-HPLC system II the detection limits were somewhat lower; about 200 pg for the particular purine ($S/N = 2$). The standard curves were linear over three orders of magnitude from 1 ng to 50 μg of each purine with a correlation coefficient of 0.998.

3.2. Analysis of tick excretory products

Because of the poor solubility of guanine in water, aqueous acids were used for complete solubilization of the excreta. Since uric acid was found in excreta in low concentrations which are readily dissolved in 10% H_3PO_4, its quantitation is also possible. At higher concentrations, uric acid can be determined by a HPLC method using alkaline buffers [16]. Since guanine predominates in the excreta of ticks, the buffer with a pH below 4.2 (Fig. 1a) could only be used for simultaneous quantitation of Xan, Hyp and Gua due to the unfavourable resolution of the components with the increase of its concentration. However, if the dioxane–acetate buffer is employed, the desired resolution of purines can be accomplished at pH 5.5 as documented in Fig. 3b on the analysis of the excrements of the tick *Argas persicus*.

Mutual ratios of the purines in the excreted spherules produced by *Argas persicus* were found to be about 2% for Hyp and 1–10% with respect to the total amount of Gua (as 100%) found as the principle component of the assembly pheromone [5]. The composition of tick excreta varied slightly with their aging; the concentration of xanthine increased particularly

on the surface of excreted spherules, which was accompanied with the decrease of their pheromonal activity. Some species of ticks were found to have similar purine contents in waste excreta (*Ornithodoros moubata, O. tartakovskyi*) or slightly different in concentration of Hyp and Xan (*Argas reflexus, A. polonicus*) [6,7]. Thus, assembly effect of purine pheromones is poorly specific for different tick genera or species. Using the gradient method with HPLC system I, guanosine was found in the excreta of ticks in some cases in the quantity not exceeding about 0.1% with respect to the total amount of Gua. The excretion of energetically rich nucleosides is unusual and, to our knowledge, has not been reported so far.

Identity of the compounds in the tick waste material was further established by a GC–MS method after trimethylsilylation. GC–MS analysis of the standard mixture of nucleic acid constituents of interest and a representative sample of the ticks' waste excreta is shown in Fig. 4 and 5, respectively. All glassware used must be presilylated to avoid adsorption of purines on the surfaces. Since most trimethylsilyl derivatives of purines (with the exception of uric acid) tend to react with active sites in capillary columns, the column must be carefully selected, as in the case of the HPLC method. A short, 10–12-m fused-silica capillary column coated with SE-54 was recommended for analyses of DNA bases [17]. We used a longer, 25-m DB-5 fused-silica column in order to achieve complete separation of the closely eluting uric acid and guanine peaks.

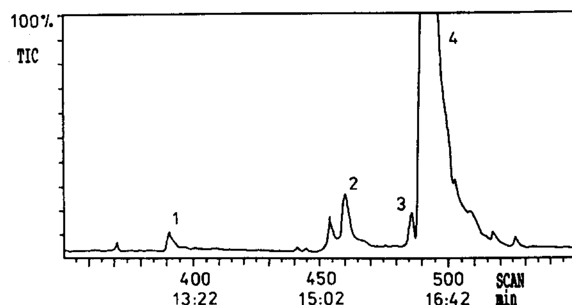

Fig. 5. Representative GC–MS TIC chromatogram obtained from the analysis of the waste excreta of the tick *Argas persicus*. Peaks: 1 = Hyp; 2 = Xan; 3 = uric acid; 4 = Gua. Temperature programme: 90°C, 1 min hold, 10°C/min, 170°C, 6°C/min, 260°C.

Under such conditions some purine exhibited slight tailing. Nevertheless, peak area ratios of the found purines were in fairly good accordance with the results obtained by RP-HPLC. The detection limit for the GC–MS method was estimated to be about 100 pg per each purine injected by measuring the peak area representing the characteristic $M^{+\cdot}$ or $(M-15)^{+}$ ions. Due to the high sensitivity of the ion-trap detector in the full scan mode identification can be easily obtained in the low nanogram range by matching characteristic electron impact mass spectra of the components with the standard mass spectral libraries such as the US National Bureau of Standards library (the ITD 800 standard option). Thus, the presence of Hyp, Xan, Gua and uric acid was unequivocally proved in the waste excreta of argasid ticks [6].

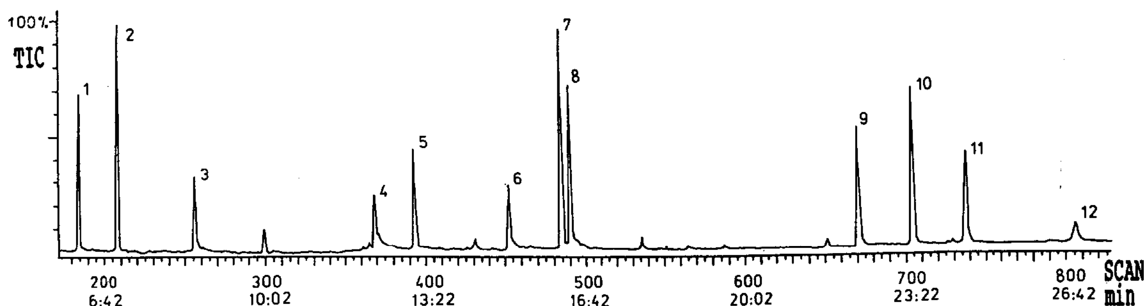

Fig. 4. GC–MS total ion current (TIC) chromatogram of bases, uric acid and nucleosides as trimethylsilyl derivatives. Peaks: 1 = uracil; 2 = thymine; 3 = cytidine; 4 = Hyp; 5 = adenine; 6 = Xan; 7 = uric acid; 8 = Gua; 9 = inosine; 10 = adenosine; 11 = xanthosine; 12 = guanosine. Capillary column DB-5, 25 m. A 20-ng amount of each component was injected. Split injection 20:1.

4. Conclusions

RP-HPLC separation of hypoxanthine, xanthine and guanine is largely dependent on the octadecylsilica sorbent used for the column packing. Using a suitable batch of reversed-phase material, simultaneous separation of the purines and related nucleosides has been accomplished. However, in a large range of concentrations, e.g. if one purine component predominates in sample as it is in the case of the ticks' excretory products, the use of dioxane modifier can further enhance the resolution of the components required for quantitation. The method has been proved valuable in the determination of the purine trio in excretory products showing an assembly pheromone effect on the family of argasid ticks.

Acknowledgements

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