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Identification of pteridines in the firebug, *Pyrrhocoris apterus* (L.) (Heteroptera, Pyrrhocoridae) by high-performance liquid chromatography

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Abstract

Pteridines in the wild type of *Pyrrhocoris apterus* were investigated by high-performance liquid chromatography (HPLC). Five different pteridines were identified: neopterin, biopterin, isoxantholumazine (violapterin), isoxanthopterin, and 7-methylxanthopterin. Biopterin and neopterin have been found in *P. apterus* for the first time. No qualitative or quantitative differences were observed between pteridine patterns in males and females. The present paper represents the first identification and quantification of *P. apterus* pteridines using HPLC.

Keywords: *Pyrrhocoris apterus*; Pteridines

1. Introduction

Pteridines form an important group of compounds, many of which are fluorescent and some absorb light in the visible spectrum, acting as pigments in many species [1]. They are widely distributed in micro-organisms, plants and the animal kingdom. They were first isolated by Hopkins (in 1889) from the wings of butterflies of the family Pieridae [2]. In addition to their function as signaling (eg. in butterflies) or screening (eg. insect eyes) pigments, they play several physiologically important roles. They serve as cofactors in hydroxylation reactions [3], among them the conversion of tyrosine into 3,4-dihydroxyphenylalanine (DOPA), a precursor of melanin; they participate in the inactivation of the toxic nitrogen metabolic wastes [4] and play a role in cellular electron transport [5].

Our current knowledge about the nature and significance of insect pteridines has been mainly based on the studies in Endopterygote insects [2,6–9]. However, information is still scarce regarding the comparative distribution of pteridines within heteropteran insects. Only a few species have been studied [10–16]. One of these species, the firebug *Pyrrhocoris apterus* (L.), provides a useful model for biochemical and physiological studies [17], and

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specially for the metabolism of pteridines since several body-colour mutants are available [18].

To date, the reported analyses of pteridines in *P. apterus* have been performed only by paper chromatography and/or TLC [10,12,18,19]. The application of high-performance liquid chromatography (HPLC) analysis with its powerful resolution to this species and its body-colour mutants, will provide a unique opportunity to initiate the complex study on the genetic relationships between pteridine patterns and the biochemical alterations in their metabolic pathway. The present work will set the basis for such studies.

2. Experimental

2.1. Chemicals

Xanthopterin was obtained from Sigma (St. Louis, MO, USA); neopterin, biopterin and isoxanthopterin from Shircks (Jona, Switzerland); isoxantholumazine (formerly called violapterin) [20], and 7-methylxanthopterin were kindly provided by Dr. Wolfgang Pfleiderer. Standards were dissolved in distilled water and their concentration was determined spectrophotometrically [21]. Prior to injection into the HPLC system, the standard solutions were diluted to the concentration of 1 μM.

2.2. Experimental animals

The laboratory culture of the firebug, *Pyrrhocoris apterus*, was reared on linden seeds according to conditions described elsewhere [22,23]. Under these conditions the culture has been maintained in the laboratory for several years. Freshly emerged adults were separated daily from the stock culture and transferred into small petri dishes in groups of 3–5 specimens until the time of utilization.

2.3. Analytical methods

2.3.1. Sample preparation.

Each sample contained the body mass of 15 adult bugs. Males and females were analysed separately. Three-day-old adults were pulverized in liquid nitrogen and then lyophilized; each vial was then filled with nitrogen, closed and the stopper was sealed with paraffin. Samples were stored at −20°C until required.

2.3.2. Extract preparation.

Samples were extracted in 1.5 ml of HPLC buffer. Then they were centrifuged for 10 min at 14 000×g and the supernatants collected, filtered (0.45-μm pore), and stored at −20°C until used.

2.3.3. HPLC analysis.

The chromatographic system consisted of a ConstaMetric 300 isocratic pump from Milton Roy (Riviera Beach, FL, USA), a C_{18} μ-Bondapak column (30×0.9 cm I.D.) from Waters (Barcelona, Spain), a Model F-1050 fluorescence spectrophotometer from Merck-Hitachi (Tokyo, Japan), and a Model SP4290 chromato-integrator from Spectra-Physics (San José, CA, USA).

The mobile phase consisted of 10 mM potassium phosphate buffer, pH 3.2, with 4% methanol. The flow-rate was set at 1.0 ml/min with a pressure of 1500 p.s.i. The spectrofluorimeter monochromators were set at 335 nm [excitation (ex.)] and 440 nm [emission (em.)] for the quantification of neopterin, isoxanthopterin and isoxantholumazine, and at 365 nm (ex.) and 490 nm (em.) for 7-methylxanthopterin.

3. Results and discussion

Pteridines from *P. apterus* were separated using phosphate buffer at pH 3.2. Under these conditions, the retention times for neopterin, isoxantholumazine, biopterin, isoxanthopterin, xanthopterin, and 7-methylxanthopterin, were 5, 7.5, 8.6, 10.6, 10.6, and 15 min, respectively. Different combinations of excitation and emission wavelengths were tested in order to obtain the highest intensity for the peaks corresponding to known pteridines while keeping interfering peaks to a minimum. The combinations that gave the best results were: (a) ex. 335 nm, em. 440 nm, for neopterin, biopterin, isoxanthopterin, and isoxantholumazine (Fig. 1A), and (b) ex. 365 nm, em. 490 nm, for 7-methylxanthopterin (Fig. 1B).

Identification of the peaks was performed by comparison of their retention times and excitation and emission spectra with those obtained for

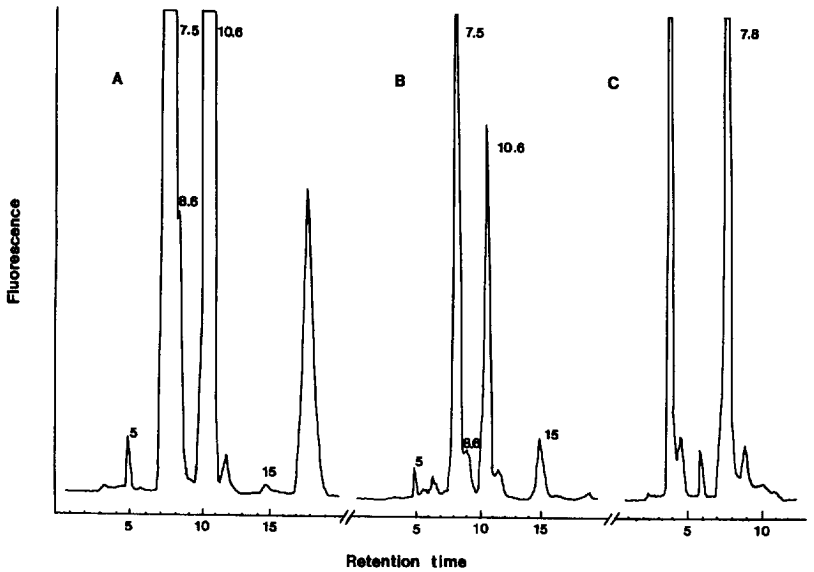

Fig. 1. Chromatograms of an extract of *P. apterus* under different conditions: (A) pH 3.2, ex.=335 nm, em.=440 nm. (B) pH 3.2, ex.=365 nm, em.=490 nm. (C) pH 6.5, ex.=335 nm, em.=440 nm. For chromatograms A and B, 5 μl of sample was injected; for chromatogram C only 2 μl was injected. Peaks eluting at 5, 7.5, 8.6, 10.6, and 15 min in chromatograms A and B corresponded to neopterin, isoxantholumazine, biopterin, isoxanthopterin, and 7-methylxanthopterin, respectively. The peak eluting at 7.8 min in chromatogram C corresponded to isoxanthopterin.

pteridine standards. Further confirmation of the identity was accomplished by joint injections of the samples with the standards.

Fluorescence spectra of the HPLC peaks were obtained by injecting the same sample repeatedly and changing the detector wavelength (either the excitation or the emission) every 10 nm. Plotting the area of the peaks versus the wavelengths produced the spectra shown in Fig. 2. Spectra were obtained for the pteridine standards (neopterin, biopterin, isoxanthopterin, isoxantholumazine, xanthopterin and 7-methylxanthopterin) and for the peaks with similar retention times from extracts of wild-type adults of *P. apterus*. To obtain the spectra of biopterin in *P. apterus*, we used a mutant (*white*) with very low content of isoxantholumazine (data to be published elsewhere), in which these two pteridines elute separately. The spectra of the peak from *P. apterus* with the retention time of 10.6 min were compared with those of isoxanthopterin and xanthopterin, and showed great similarity with the spectra of the isoxanthopterin standard. This suggests that this peak is mainly, if not solely, isoxanthopterin.

Since xanthopterin and isoxanthopterin co-eluted in our HPLC conditions, we chose a different mobile phase (pH 6.5) in which these two compounds eluted separately. Although the separation of the other pteridines was worse at pH 6.5, xanthopterin and

Table 1
Quantification of pteridines in *P. apterus*

Sample	Nep	Ixl[a]	Ixp	7-Mxp
Males	0.08 (0.01)	22	1.6 (0.4)	0.14 (0.02)
Females	0.10 (0.01)	25	1.9 (0.1)	0.13 (0.07)
Mean value	0.09	23.5	1.75	0.135

Values are the mean (S.E.) of two independent replicates and are given as nmoles of pteridine per insect. Nep, neopterin; Ixl, isoxantholumazine; Ixp, isoxanthopterin; 7-Mxp, 7-methylxanthopterin.

[a] Only one replicate was assayed.

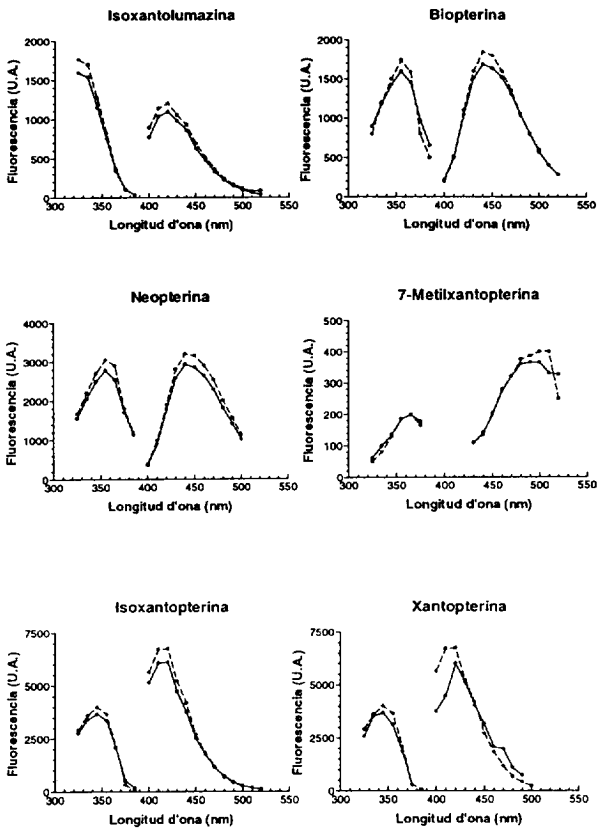

Fig. 2. Excitation and emission spectra of fluorescent compounds from *P. apterus* separated by HPLC (– – –) and of pteridine standards (–)

isoxanthopterin were now well separated, with retention times of 5.6 and 7.8 min, respectively (Fig. 1C). No peak was found at 5.6 min, which indicates that xanthopterin is not found in this species, at least in the conditions of our analyses.

No quantitative differences of pteridines in male and female samples were found (Table 1). The relatively high amount of isoxantholumazine is noticeble (a mean value of 23.5 nmoles/insect), which can suggest an important pterin deaminase activity in this insect [24]. The second most abundant pterin is isoxanthopterin (around 1.75 nmoles/insect), followed by 7-methylxanthopterin and neopterin (around 0.1 nmoles/insect). There is an overall difference of over two orders of magnitude among the four pteridines quantified. Accurate measurement of biopterin turned out to be impossible due to overlapping with neighbouring peaks. For this reason this pteridine was not included in the quantitative study.

The present work represents the first reported study regarding the application of HPLC to the identification and quantification of pteridines in *P. apterus*. Biopterin and neopterin had never been previously reported in Heteroptera. In addition, we have also identified peaks corresponding to isoxanthopterin, 7-methylxanthopterin, and isoxantholumazine. These three pteridines were already known to occur in *P. apterus* [10,12,19]. Xanthopterin, although found in other heteropteran insects [25,26], was not detected in *P. apterus*.

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