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Short Communication

Determination of ptaquiloside in bracken fern (*Pteridium esculentum*)

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ABSTRACT

A high-performance liquid chromatography method is described for the analysis of the potent carcinogen, ptaquiloside in bracken fern (*Pteridium esculentum*). The method involves aqueous extraction at room temperature followed by clean-up through a polyamide resin column. An aliquot of the cleaned extract may be analysed directly for ptaquiloside by reversed-phase high-performance liquid chromatography. An alternative analysis is also described using base–acid conversion of ptaquiloside to pterosin B with subsequent high-performance liquid chromatography analysis. The mean recovery of standards through the method was 95% when measured as ptaquiloside, and 89% when measured as pterosin B. Detection limits for the two methods were 30 and 5 mg ptaquiloside/g bracken, respectively. The method has also been used to identify, for the first time, the presence of ptaquiloside in rock fern (*Cheilanthes sieberi*).

INTRODUCTION

Bracken fern (*Pteridium esculentum*) has been linked with several animal health problems world-wide [1–5]. In 1983 workers in Japan [1] and in The Netherlands [3] independently isolated and characterised a carcinogenic norsesquiterpene glucoside, ptaquiloside (Pta, Fig. 1a). Pta is unstable under both acidic and basic conditions. In weak acid Pta is converted to a mixture of compounds such as pterosin B (PtB, Fig. 1b), while in aqueous base, Pta is converted to the conjugated dienone (Fig. 1c) which may then be converted to PtB by weak acid [1]. Analysis is difficult due to the extremely unstable nature of the compound [1–3,6–8].

There was the need to develop an analytical method to quantitate the level of Pta in bracken fern to assist in surveying the toxic potential of bracken from different areas of New Zealand [4]. Published methods for large-scale isolation of Pta have used hot water [1,2] or methanol [3] for extraction followed by multi-step clean-up procedures. These methods gave low recoveries and were unsuitable for analysis. More recently, better recoveries have been reported using water extraction at room temperature [6–8]. However, attempts at high-performance liquid chromatographic (HPLC) analysis

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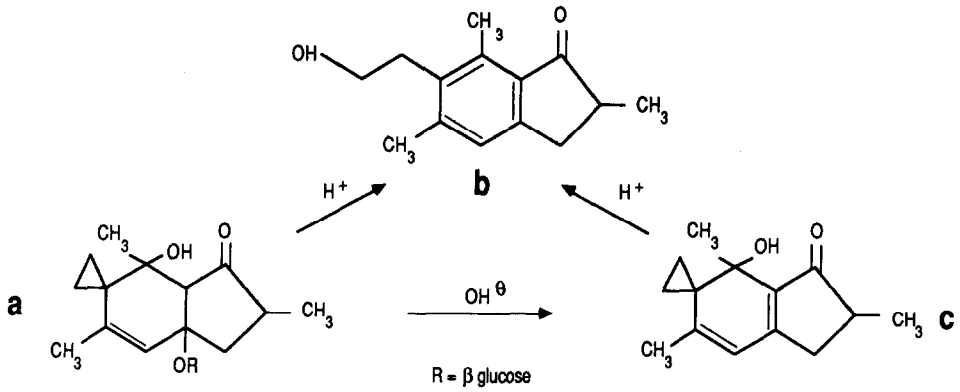

Fig. 1. Structures of ptaquiloside (a), pterosin B (b) and the unstable conjugated dienone (c), and the route by which ptaquiloside is converted to them.

were abandoned due to problems with work-up and low recoveries, although a thin-layer chromatographic assay was devised [8]. When developing our method we decided to try extraction of dried and ground bracken by water at ambient temperature, followed by minimal clean-up prior to analysis by HPLC.

This paper describes a rapid and simple method for the analysis of Pta in bracken fern. It may also be applied for the analysis of other plant materials. The method consists of aqueous extraction followed by clean-up through a polyamide resin column. The sample is then analysed by HPLC with UV detection. The sample may also be reacted to form PtB, then analysed again for confirmation.

EXPERIMENTAL

Materials

Pta was supplied by Professor Kiyoyuki Yamada (Nagoya University, Nagoya, Japan) and stored over silica gel at −20°C. PtB was obtained by reaction of Pta with base, followed by acid, in water. HPLC mobile phases were mixtures of purified water (Millipore Milli-Q) and HPLC-grade methanol (BHD, Poole, U.K.). Polyamide 6 S resin was purchased from Riedel-de Haen (Seelze, Germany).

Preparation of primary standards

Stock solutions of Pta were made in methanol at about 0.1 mg/ml. Working standards (10 μg/ml) were obtained by dilution with water. Both stock and working solutions were stored at −20°C.

For PtB, a solution of Pta in water (0.1 mg/ml) was treated with 75 μl/ml of 1 *M* NaOH and allowed to react for 1 h at 40°C, when 75 μl/ml of 5 *M* HCl was added. This solution was diluted to final volume for working standards. The concentration was calculated on the basis of 100% conversion of Pta to PtB and expressed as Pta equivalents.

Instrumentation and analytical conditions

The HPLC system used was a Spectra-Physics 740B binary pumping system with

either a Rheodyne 7120 manual injector or a Micromeritics 725 autosampler, each fitted with a 50-μl sample loop, a Micromeritics 731 column oven, a Shimadzu SPD-2A UV detector and a Spectra-Physics SP4170 integrator. The analytical column (either Zorbax ODS 5 μm or Chrompak CPSpher C_8; 25 cm × 4.6 mm I.D.) was preceded by a 2-μm in-line filter (Rheodyne) and an MPLC RP-8 guard column (Brownlee Labs, Santa Clara, CA, U.S.A.). All these items were maintained at 35°C in the column oven. The mobile phase was water–methanol (60:40) for Pta or water–methanol (40:60) for PtB. The flow-rate was 1 ml/min. The mobile phases were selected to give retention times of around 10–12 min for both Pta and PtB. Detection was at 220 nm. Concentration calculations were based on both area and height measurements relative to external standards of concentrations in the range 5–15 μg/ml.

Extraction and clean-up

Bracken fern was dried in an air oven (50°C for 5 days) and then ground to pass a 0.6-mm mesh screen. The ground sample (2 g) was shaken with water (100 ml) in a large boiling tube on a flat-bed orbital shaker (350 rpm) for 1 h at room temperature. The extract was filtered through Whatman No. 1 filter paper, and the clear amber filtrate retained.

An aliquot (20 ml) of the filtrate was added to a glass column (25 cm × 12 mm I.D.) dry packed with polyamide 6 S resin (2.5 g). The total eluate (*ca.* 12 ml) was collected and mixed. An aliquot was removed and used for direct determination of Pta. A further aliquot (1 ml) was converted to PtB by the method used for the standard and was then analysed for PtB without further dilution. Concentrations of PtB were expressed as Pta equivalents using a dilution factor of 1.15.

RESULTS AND DISCUSSION

The first extraction solvent tried was methanol. This required evaporation of the extract and dissolving in water prior to clean-up which was time consuming for large numbers of samples. There was also the added risk of degradation of Pta. The proposed method using aqueous extraction and minimal clean-up overcomes these problems. It has given repeatably high recoveries of Pta from bracken over several seasons.

Shaking with water for 1 h at room temperature followed by clean-up through polyamide 6 S resin was found to be suitable (see Table I). In fact, prolonged extraction gave poor recovery of Pta due to breakdown to PtB in aqueous solution. The clean-up column removed all PtB present in the sample extract and thus allowed the development of a confirmatory test based on the conversion of Pta to PtB. As the UV response of PtB was 5–6 times greater than that of Pta, this alternative method was more suitable for determining low levels of Pta. The levels of Pta found in samples ranged from less than 30 μg/g up to 4000 μg/g dry weight, equivalent to from 0.6 μg/ml up to 80 μg/ml in the original extract solutions. The limit of detection was 30 μg/g for Pta and 5 μg/g Pta equivalents after hydrolysis to PtB.

Standards of both Pta and PtB were analysed in the range 0.1–200 μg/ml. Both compounds showed linear response in the range tested. The regression equations for the log-transformed data are for Pta, $\log_{10}$(peak height) = 2.692 + 1.002 $\log_{10}$ (concentration), correlation coefficient 1.000; and for PtB, $\log_{10}$(peak height) = 3.467 + 0.975 $\log_{10}$(concentration), correlation coefficient 0.999.

TABLE I

EFFECT OF EXTRACTION TIME ON Pta AND PtB CONCENTRATIONS IN AQUEOUS EXTRACTS OF BRACKEN

Time shaking (h)	Clean-up	Pta (μg/g)	PtB[a] (μg/g)
1	None	2300	780
1	Polyamide	1590	0
2	Polyamide	1500	0
4	Polyamide	1120	0
24	None	545	>1400

[a] Expressed as Pta equivalents; PtB occurring naturally or from degradation of Pta during extraction is removed by the polyamide clean-up.

The mean recovery of standards in the range 2–35 μg/ml put through the clean-up method was 95% (range 90–100%) when measured as Pta and 89% (range 85–94%) when measured at PtB. Coefficients of variation for five replicate 2- and 10-μg/ml standards put through the clean-up were respectively 2.7% and 3.2% for estimations as Pta and 2.9% and 2.2% for estimations as PtB. The procedure of collecting an aliquot of the eluent from the clean-up column for analysis was shown to be satisfactory by collecting and analysing the eluate from a 10-μg/ml standard in 2-ml

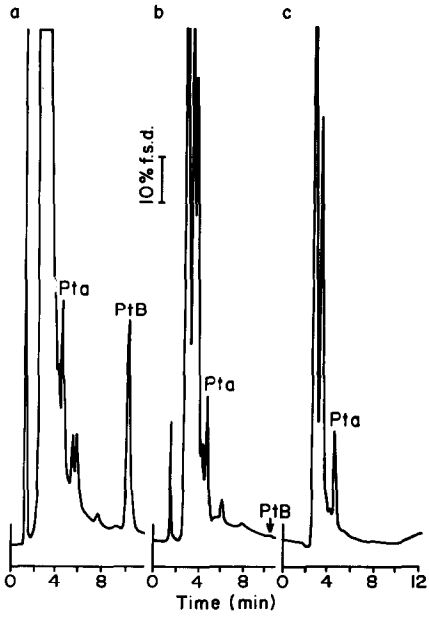

Fig. 2. Chromatograms of crude bracken extract (0.02 g/ml). (a) Without clean-up; (b) after 1-g polyamide 6 S clean-up column; (c) after 2.5-g polyamide 6 S clean-up column. Analysis conditions as in text except for mobile phase, water–methanol (30:70); column temperature, 22°C; UV detection at 220 nm and 0.04 a.u.f.s. Peaks: Pta = ptaquiloside; PtB = pterosin B.

fractions. The first 2-ml fraction showed 6.8 μg/ml while the next five 2-ml fractions showed from 8.9 to 10.2 μg/ml. This result showed that Pta is almost non-retained on the polyamide clean-up medium.

A crude bracken extract was analysed without clean-up and following passage through either a 1-g polyamide column or a 2.5-g polyamide column (see Fig. 2). Comparison of Fig. 2a and Fig. 2c shows complete removal of any PtB naturally present in the sample. In this experiment the estimated amount of Pta after the 1-g and 2.5-g polyamide columns had reduced to 77% and 71%, respectively, of the estimate for the crude extract. An accurate estimate on the crude material was difficult, however, due to co-extractive interferences. The 2.5-g polyamide column (Fig. 2c) gave superior clean-up and was used in the final method. Collection and analysis of the column eluent in 2-ml fractions gave a similar pattern to that seen with standards. Reproducibility of the method was tested on both replicates ($n = 6$) of a bulk extract and on replicate extractions ($n = 5$), in each case analysed both as Pta and PtB. The coefficients of variation were all in the range of 0.8–2.7%.

Fig. 3 shows the results of both immature and mature bracken fronds analysed using the method. Figs. 3a and b show analysis of Pta directly, and Figs. 3c and d show the analysis of the same extracts after base–acid conversion of Pta to BtB.

The reaction conditions required to convert Pta to PtB were studied. Pta may be converted to PtB in either acidic or basic conditions [1]. It was found that reaction of Pta with acid gave two or more products with ratios varying according to solvent composition. Table II shows the results of Pta breakdown under acid conditions in

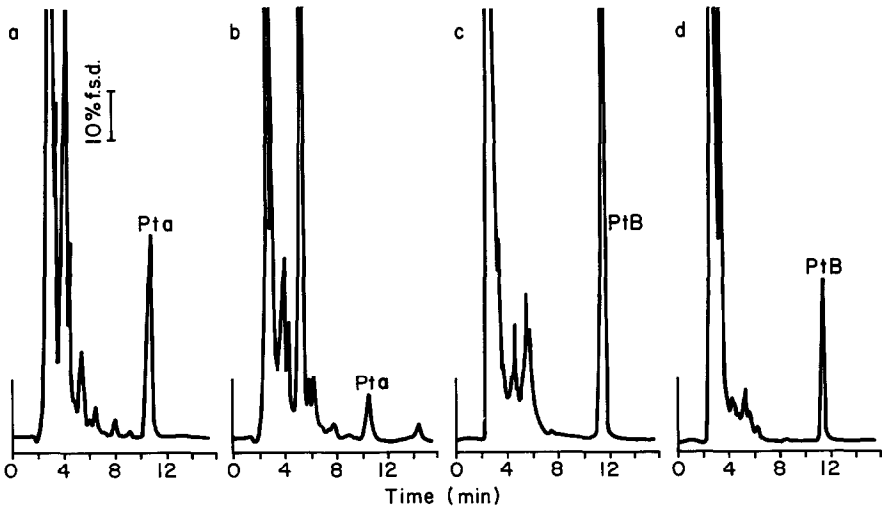

Fig. 3. Chromatograms of (a) extract of immature bracken fronds (0.02 g/ml); shows 28.6 μg/ml Pta, equal to 1430 μg/g in the bracken sample; (b) extract of mature bracken fronds; shows 6.65 μg/ml Pta, equal to 332 μg/g; (c) extract as in (a) after base–acid treatment; shows 26.8 μg/ml Pta equivalents as PtB, equal to 1610 μg/g in the bracken sample; and (d) extract as in (b) after base–acid treatment; shows 7.5 μg/ml Pta equivalents as PtB, equal to 450 μg/g. Analysis conditions as in text with mobile phase water–methanol (60:40) and detector attentuation 0.08 a.u.f.s. for (a) and (b), and mobile phase water–methanol (40:60) and detector attenuation 0.16 a.u.f.s. for (c) and (d).

TABLE II

REACTION OF PTAQUILOSIDE AT pH 1.5

Reaction of Pta standard (100 μg/ml) in water–methanol (1:1) at 24°C. Analysis conditions were as in text except for mobile phase water–methanol (30:70) with a 20-μl sample loop.

Time (min)	f.s.d. at 0.16 a.u.f.s. (%)		
	Ptaquiloside	Pterosin B	Pterosin O
0	37	1	0
15	26	30	38
30	14	46.5	58
45	8	56	70
60	5.5	61.5	77.5
75	3.5	63	79.5

water–methanol (1:1). Even using 100% water as the reaction solvent, a mixture of PtB, a product presumed to be the methoxy derivative pterosin O (PtO) [1], and a third unidentified product resulted. On the other hand, initial reaction with base to convert Pta to the conjugated dienone (Fig. 1c) followed by acid treatment to produce PtB [1] was tried and yielded a single product (HPLC) with reproducible results. This reaction proved to be consistent from one experiment to the next, and was used to prepare a stock solution of PtB. As HPLC of the solution did not reveal any peaks other than PtB the conversion was assumed quantitative. The estimates of Pta measured by direct

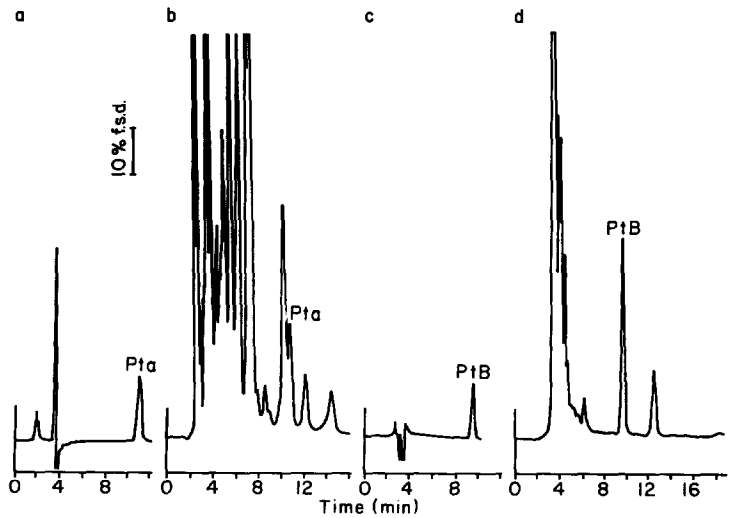

Fig. 4. Chromatograms of (a) ptaquiloside (Pta) standard, 8.25 μg/ml; (b) extract of Australian rock fern (0.02 g/ml); (c) pterosin B (PtB) standard, 4.8 μg/ml; and (d) extract of Australian rock fern (0.02 g/ml) as in (a) after base–acid treatment. Analysis conditions as for Fig. 3 except for detector attenuation for (c) and (d) of 0.32 a.u.f.s.

analysis as Pta or by indirect analysis as PtB generally agreed within 15%. This suggests that the two methods are comparable.

The method has also been used to analyse other species of fern for Pta. Fig. 4 shows results from analysis of *Cheilanthes sieberi* (rock fern). In this species, compounds interfering in the determination of Pta are still present after clean-up (Fig. 4b), although full separation can be achieved by changing the HPLC mobile phase. However, after conversion to PtB (Fig. 4d), no interference remains. This shows a further use for the indirect analysis method as the principal method for difficult samples.

For rock fern the estimates of PtB as Pta equivalents were generally much higher than the direct estimates of Pta. This suggests that some of the interferences for the direct analysis could be compounds similar to Pta which are also able to produce PtB after base–acid treatment. Such compounds may also be expected to be toxic [7,8].

Finally, as shown in Fig. 2a, PtB occurs naturally in samples of bracken containing Pta. In some instances it may be required to measure this naturally occurring PtB. The PtB is removed from the extract by the polyamide clean-up, but may be eluted from the polyamide column with methanol (2 × 5 ml) after first washing excess extract from the column with water. Crude bracken extracts should not be analysed directly as they will quickly destroy the HPLC column.

CONCLUSION

The methods described for analysis of Pta and its principal degradation product PtB are rapid and easy to use. They allow large numbers of bracken samples to be processed with minimum delay between extraction and analysis. The indirect estimate by analysis of PtB produced by base–acid treatment of the cleaned-up extract allows confirmation of the direct analysis results as well as offering an alternative analytical approach for difficult samples and for Pta precursers or conjugates.

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