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Analytical Methods

A new, very sensitive method of assessment of ptaquiloside, the major bracken carcinogen in the milk of farm animals

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ABSTRACT

We describe a new method to detect trace levels of ptaquiloside (Pta), a major carcinogen of bracken fern in biological samples such as milk from farm animals. The method involves the absorption of analyte on carbograph followed by elution with solvents mixtures. The unstable analyte is then converted into Br-Pt (II), which is specific for Pta, as it is not a natural decay product of the glycoside in aqueous media. An internal standard, the Br-pterosine-d_2, prepared in our laboratories has been used. Detection and quantification are possible with gas chromatography/mass spectrometry (GC/MS) in single ion monitoring mode (SIM). The detectable amount is in the range of ppb. The method allowed us to detect Pta not only in the milk from bracken fern-poisoned cattle but also, for the first time, in the milk from healthy farm animals such as sheep, goat, horse, and donkey mares.

1. Introduction

Domestic animals grazing on lands rich in bracken fern (*Pteridium* spp.) can be affected by several, well-known acute and chronic syndromes being attributed to ingestion of bracken fern. In horses and pigs the acute manifestation of bracken poisoning is a thiamine deficiency (Smith, 1990). Sheep develops a progressive retinal atrophy responsible for blindness (Watson, Barlow, & Barnett, 1965). In cattle chronic bracken poisoning is associated with epithelial and mesenchymal tumours of the urinary bladder, the most important clinical symptom of which is the presence of blood in the urine characterising a syndrome known as bovine enzootic hematuria (BEH) (Maxie & Newman, 2007). Tumours of the nasopharynx, oesophagus, and the fore stomachs have also been reported (Dobereiner, Tokarnia, & Canella, 1967; Jarrett, McNeil, Grimshaw, Selman, & McIntyre, 1978).

The major bracken carcinogen is a glycoside named ptaquiloside (Pta) (Hirono, 1987), the structure of which was simultaneously elucidated (Niwa et al., 1983; van der Hoeven, Legerweij, Posthumus, van Veldehuizen, & Holterman, 1983). It is possible that not only animals are affected by bracken; it has been suggested that Pta as well as other compounds of bracken may interact with human tissue alone and/or in combination with infectious agents such viruses, particularly papillomaviruses (Campo, 1997). Epidemiological evidence suggests that Pta causes cancer in man (Smith & Seawright, 1995). It has been suggested that the longer the duration of residence of people in areas of dense bracken growth, the greater is the risk of dying from gastric cancer. For people exposed to bracken in childhood the risk of developing gastric malignancies has been shown to be more than twice (OR = 2.34) as compared with non-exposed youngsters (Galpin, Whitaker, Withtaker, & Kassab, 1990). Milk from bracken-eating cows is postulated to be one of the links between the carcinogen effects of bracken and gastric cancer in some geographic areas worldwide (Villalobos Salazar, 1985).

More recently, several studies addressing this problem have been published in this area. The cytogenetic effects of Pta resulting in chromosome aberrations have been studied (Lioi et al., 2004; Moura et al., 1988; Peretti et al., 2007; Santos, Dorea, & Luna, 2006) and detailed hystopathological examinations of bladder

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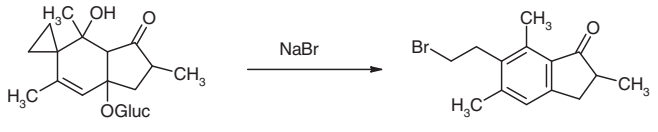
lesions of animals grazing on bracken fern have been reported (Carvalho, Pinto, & Peleteiro, 2006; Roperto et al., 2005, 2010; Sardon et al., 2005). Because of the increased risk for oesophageal and gastric cancer in humans living in areas rich in bracken fern and exposed to foods contaminated by Pta, the need to avoid the distribution of Pta into the food chain has become a crucial topic in the food safety investigations (Alonso-Amelot & Avendano, 2002; Shahin, Smith, & Prakash, 1999).

Inspite of the large interest in the toxicological properties of Pta and the problems arising from the widespread presence of the fern, sensitive methods to detect trace levels of this toxic substance in foods and/or in biological fluids are not available at present. Very recently, a sensitive method for quantifying Pta in soil and groundwater have been published (Jensen, Jacobsen, Hansen, & Juhler, 2008); however, this analytical method, based on liquid chromatography–Tandem mass spectrometry (LC–MS/MS), does not appear suitable to be easily extended to complex matrices, like biological fluids.

The amount of Pta in plants has been evaluated directly by HPLC and/or by converting this substance into Pterosine B (Agnew & Lauren, 1991; Rasmussen, Kroghsbo, Frisvad, & Hansen, 2003; Smith, Seawright, Hertle, Thomson, & Bostock, 1994). It has been suggested that milk from poisoned animals may be an important potential carrier of Pta to humans (Galpin et al., 1990). There is not yet evidence of Pta in milk from apparently healthy farm animals naturally fed on bracken-rich pastures. The milk of cows experimentally fed with known amounts of fern has been analysed but the method requires a large amount of milk (Alonso-Amelot, Castillo, Smith, & Lauren, 1996, 1998).

The key to overcome these difficulties was in the reactivity of Pta towards nucleophiles, which also gives rise to the carcinogenic properties of Pta. The electrophilic character makes it responsible for adenine alkylation and subsequent H-ras activation followed by depurination in the codon 61 of this gene (Prakash, Pereira, Smith, Shaw, & Seawright, 1996).

Recently, we have found that in presence of bromide ions, Pta is converted into bromo-pterosine (Br-Pt) (I) (Bonadies, Borzacchiello, Dezzi, Nicoletti, & Roperto, 2004), which is stable and easy to detect with gas chromatography/mass spectrometry (GC/MS). These findings allow us to design the new methodological approach reported here which requires small amounts of samples.



Ptaquiloside (Pta) (I) Br-Pt

Therefore, the aim of this paper is to outline a sensitive method able to detect ppb of Pta in biological samples such as milk and to have a preliminary test from milk samples collected from poisoned and healthy farm animals feeding on lands rich in bracken fern. Similar procedures, which we hope will be extensively tested and improved, may be applied to the analysis of other biological fluids, such as serum and urine.

2. Material and methods

2.1. Method design

Sample preparations to identify trace substances in biological fluids are generally carried out with the sequence: de-proteinisation with a solvent, fat removal, extraction of analyte. In our case, it would be necessary to introduce a step of transformation of analyte (Pta) into a stable product (Br-Pt): the addition of a salt (NaBr) and the heating of the aqueous alkalinised solution were required.

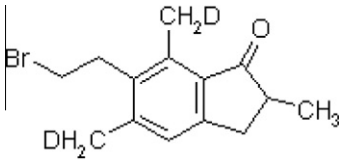
When we tried to develop this standard sequence, we were faced with an unexpected difficulty: the conversion of the analyte after the protein precipitation with acetonitrile induces a new precipitation of small amounts of protein and other purification steps were necessary. These problems suggested the development of unconventional method, i.e., the collection of analyte by filtering the milk through a cartridge of graphitised carbon black. This absorbent has a high affinity towards saccharides: being the Pta a glycoside the absorbance on carbon should be expected. It has also a great advantage to not absorb fat component of milk.

The elution from carbon black can be easily obtained by eluting with organic solvents. This procedure achieved a further goal: the amount of milk may be increased according to the expected concentration of analyte, without the necessity of manipulating large amount of solvents. Of course, there are limits in this procedure: difficulties in processing milk samples with some precipitated proteins, and the necessity to add the reference compound (Br-pterosine-d_2) after the elution of analyte from the carbograph cartridge, because the reference compound, according to its hydrophobic nature, is not sufficiently absorbed by carbograph. Reference samples of Pta used in this work were obtained as mixture of natural substances prepared by the extraction with water of fresh or frozen fern as described elsewhere (Bonadies et al., 2004). The aqueous extract of the milled fresh or frozen plant was stored after lyophilisation. The amount of Pta present in the lyophilised extract was determined in each experiment as described in Section 2.3. This was the result of a choice, our aim being the design of a friendly method which can be applied in laboratories not specialised in analyses of trace pollutants.

2.2. Materials

To carry out trial experiments commercial milk (pasteurised and homogenised) was used. Solvents and reagents were of analytical grade and used as supplied. Cartridges (0.5 cm i.d.) were filled with graphitised carbon black (carbograph 4) provided by LARA, Rome, Italy. Carbograph 4 has a surface area of 200 m^2/g, and it is commercially also referred as CarboprepTM (Resteck, Bellefonte, PA). Frits (20 μm) were provided by SUPELCO (Bellefonte, PA).

Lyophilised extracts of fern obtained according to previous described procedures (Bonadies et al., 2004) were used. The amount of Pta in the plant extracts and in samples was evaluated by adding appropriate amounts of Br-pterosine-d_2 (II), synthesised in our laboratories (Miele, Costi, Bonadies, & Nicoletti, 2008).



(II) Br-Pt-d2

Solutions of deuterated standard (II) were prepared in methanol or ethyl acetate, stored at −20 °C, and were stable for months. Solutions of plant extracts were prepared by dissolving the lyophilised extract and immediately used; in each experiment the calibration of the prepared extract was carried out in parallel.

2.3. Calibration of plant extracts

Two grams of NaBr were added to a known amount of extract in 2 ml of water, 20 μl of a solution containing 20 ng of deuterated