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Determination of fumonisin B_1 in plasma and urine by high-performance liquid chromatography

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ABSTRACT

Fumonisin B_1 (FB_1), the major compound of the newly described fumonisin mycotoxins, has been shown to be the causative agent of the animal diseases leukoencephalomalacia in horses and pulmonary oedema in pigs. Whereas previous analytical methods have dealt with the determination of FB_1 in feed and foodstuffs, this report for the first time details methods for FB_1 determination in the physiological fluids, plasma and urine. The methods involve solid-phase anion-exchange clean-up, precolumn derivatisation with *o*-phthaldialdehyde and reversed-phase high-performance liquid chromatography with fluorescence detection. These methods were shown to be sensitive (detection limit around 50 ng ml^{-1}), reproducible (relative standard deviation on six replicates less than 5%) and accurate (recoveries on spiked blank samples above 85%).

INTRODUCTION

The recently described fumonisin mycotoxins [1–3] are a group of structurally related secondary metabolites produced by the fungus *Fusarium moniliforme* Sheldon, a common contaminant of corn world-wide [4]. Of the fumonisins currently identified, fumonisin B_1 (FB_1) is the major form produced in culture and found in contaminated feed and foodstuffs [5–7]. The chemical structure of FB_1 has been shown to be the diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane in which the C-14 and C-15 hydroxy groups are esterified with the terminal carboxy group of propane-1,2,3-tricarboxylic acid [1] (Fig. 1).

FB_1 has been shown to be the causative agent of the animal diseases equine leukoencephalomalacia (ELEM) and porcine pulmonary oedema (PPE) [8,9]. It also possesses hepatocarcinogenic and hepatotoxic properties in rats [2,10] and has been statistically associated with an increased risk of oesophageal cancer in man [11,12]. In view of these pathological effects in animals and its

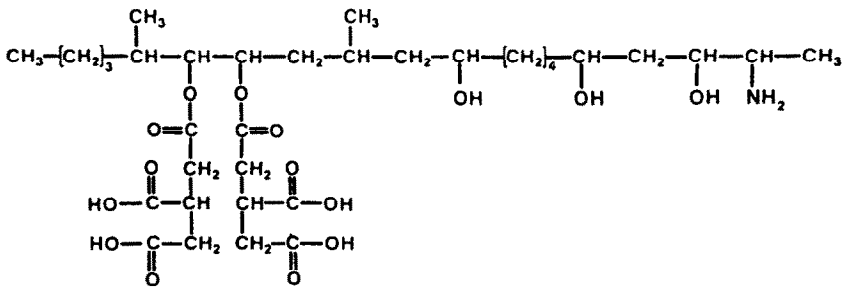

Fig. 1. Structure of fumonisin B_1 (FB_1).

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possible importance in human health [13], the development of analytical methodology for toxicological and toxicokinetic investigations of FB_1 is of some importance.

This publication reports, for the first time, analytical methodology for the quantitative reversed-phase high-performance liquid chromatographic (HPLC) determination of FB_1 in plasma and urine of laboratory rats using precolumn *o*-phthaldialdehyde (OPA) derivatisation with fluorescence detection. Prior clean-up of these physiological fluids was achieved on strong anion-exchange (SAX) solid-phase extraction cartridges.

EXPERIMENTAL

Reagents

Methanol, sodium dihydrogenphosphate, orthophosphoric acid, glacial acetic acid, disodium tetraborate, 2-mercaptoethanol and OPA were analytical-grade reagents from Merck (Darmstadt, Germany). Bond-Elut SAX cartridges (3 ml capacity containing 500 mg sorbent) were purchased from Analytichem International (Harbor City, CA, USA). FB_1 was isolated from *Fusarium moniliforme* MRC 826 cultures at the Research Institute for Nutritional Diseases (Tygerberg, South Africa) as described elsewhere [2,3].

Animals and sample collection

Male BD IX rats were bred at the Research Institute for Nutritional Diseases and, at the time of the experiment, weighed around 150 g. In order to collect urine samples for method development, rats were confined in plastic metabolic cages and given free access to feed and water. In this manner, sufficient toxin-free rat urine was collected for experimental use. To obtain urine samples naturally containing FB_1, four rats in a metabolic cage were each given an intraperitoneal injection of FB_1 in 0.9% saline at a dose of 7.5 mg FB_1 per kg body weight. Urine was collected for 24 h after which the experiment was terminated.

Rat blood was obtained by exsanguination of anaesthetised rats. Blood was collected from the abdominal aorta by needle and syringe and transferred to blood collection tubes containing tripotassium ethylenediaminetetra acetic acid as anticoagulant. Plasma was obtained by centrifugation at 1200 *g* for 10 min at 4°C. Plasma naturally containing FB_1 was obtained by blood collection 20 min after the intraperitoneal injection of FB_1 at a dose of 7.5 mg FB_1 per kg body weight.

Determination of FB_1 in urine

A 250-μl aliquot of rat urine was diluted with 750 μl of distilled water and 3 ml of methanol. This sixteen-fold diluted urine sample was applied to a Bond-Elut SAX solid-phase extraction cartridge which had been conditioned with 5 ml of methanol and 5 ml of methanol–water (3:1, v/v). The urine sample container was rinsed with 1 ml of methanol–water (3:1, v/v) and the rinse was applied to the SAX cartridge, which was then successively washed with 5 ml of methanol–water (3:1, v/v) and 5 ml of methanol. The toxin was then eluted from the sorbent with 10 ml of 5% acetic acid in methanol. The flow-rate through the SAX cartridge for both the application and elution of FB_1 was around 1 ml min^{-1} and at no stage was the cartridge allowed to run dry. The eluate was dried down at 60°C under a stream of nitrogen. The residue was redissolved in 200 μl of methanol prior to derivatisation and HPLC analysis.

Determination of FB_1 in plasma

A 500-μl aliquot of rat plasma was deproteinised by the addition of 2.5 ml of methanol. The protein precipitate was centrifuged down at 1200 *g* for 10 min at 10°C. A 2-ml aliquot of the sample supernatant was applied at a flow-rate of around 1.0–1.5 ml min^{-1} to a SAX cartridge conditioned as in the urine analysis. Immediately after this application, the sorbent was washed and eluted as for the urinary determination of FB_1. The eluate was dried down at 60°C under a stream of nitrogen and the residue was redissolved in 200 μl of 0.1 *M* sodium borate immediately prior to derivatisation and HPLC analysis.

Chromatographic analysis

FB_1 in the sample residues after SAX clean-up was quantified by reversed-phase HPLC of preformed OPA derivatives. The OPA reagent was prepared by dissolving 40 mg of OPA in 1 ml of methanol followed by addition of 5 ml of 0.1 *M*

sodium borate and 50 μl of 2-mercaptoethanol. The reagent was stored in the dark at room temperature for up to a week without deterioration. Derivatives were prepared by mixing a 50-μl aliquot of the redissolved cleaned-up sample extract with 200 μl of OPA reagent. The OPA derivative of FB_1 was found to be stable for 4 min, after which a decrease in response occurs. Hence each aliquot of sample must be derivatised individually prior to HPLC injection, which can be manual or automatic, preferably at a reproducible time within 4 min of derivatisation.

The HPLC system consisted of a Waters Assoc. (Milford, MA, USA) M-45 pump and U6K injector. The eluent was methanol–0.1 *M* sodium dihydrogenphosphate (66:34, v/v) adjusted to pH 3.4 with orthophosphoric acid and pumped at a flow-rate of 1 ml min^{-1}. The injection volume varied between 5 and 25 μl of derivatised sample. The analytical column was a reversed-phase Merck Hibar column (12.5 cm × 4.0 mm I.D.) prepacked with LiChrosorb RP-8 packing of 5 μm particle size. The fluorimetric detector was a Perkin Elmer (Norwalk, CT, USA) Model 650S fitted with a 18-μl flow cell. Excitation and emission wavelengths were 335 and 440 nm, respectively. Quantification was achieved by peak-area measurement using a Waters 745 data module based on the response of a similarly derivatised FB_1 standard.

RESULTS AND DISCUSSION

Analytical methodology for the determination of FB_1 has, to date, been restricted to its quantification in culture material and in feed and foodstuffs [2,5,7,13–15]. The first analytical determination of FB_1 was in corn cultures [2] utilizing HPLC with ultraviolet detection of the maleyl derivative of FB_1 with a detection limit of the order of 10 μg g^{-1} [16]. The lower levels of FB_1 associated with the natural contamination of feed and foodstuffs necessitated the development of more sensitive methods. Corn samples associated with field outbreaks of ELEM and PPE have been analysed for FB_1 using either gas chromatography–mass spectrometry (GC–MS) of the hydrolysis product (aminopentol) of FB_1 or HPLC with fluorescence detection of the fluorescamine derivative of FB_1 [15]. Of these techniques, GC–MS requires the availability of sophisticated and expensive analytical equipment, while the precolumn fluorescamine method is unsatisfactory as FB_1 yields two derivatives which separate on reversed-phase HPLC [15,16]. Precolumn OPA derivatisation of FB_1 coupled with reversed-phase HPLC and fluorescence detection has been used to determine FB_1 reproducibly in corn-based feed and foodstuffs, with a detection limit of 50 ng g^{-1} [5,7]. Hence, the use of OPA was investigated in order to develop analytical methods to quantify FB_1 in physiological samples such as urine and plasma. In order to obtain urine and plasma samples naturally contaminated with the toxin, laboratory rats were confined in suitable metabolic cages and dosed with FB_1 by intraperitoneal injection as described in the Experimental section. Hence all toxin levels determined represent naturally occurring levels in dosed laboratory rats.

Prior to the HPLC quantification of FB_1 in rat urine, sample aliquots (pH approximately 6) were diluted and subjected to a single step SAX clean-up using a modified silica sorbent. Selective elution of the toxin from the sorbent yielded a sample residue suitable for derivatisation and HPLC analysis. Fig. 2a shows the chromatogram of such a purified urine sample containing 2.5 μg ml^{-1} FB_1. The OPA derivative of FB_1 is well separated from other components which mainly elute in the front-end of the chromatogram. The identity of the FB_1 peak was confirmed by spiking the sample with authentic FB_1 standard (Fig. 2b). The absence of co-eluting interfering compounds is shown by the chromatogram obtained on analysis of a blank urine sample collected from rats not injected with FB_1 (Fig. 2c).

The rat urine collected in the metabolic cages was a relatively concentrated medium. The effect of this high solute concentration on the performance of the ion-exchange clean-up was investigated by diluting various urine volumes up to 1 ml with water prior to the addition of 3 ml of methanol (Table I). Whereas the 100- and 250-μl samples (40- and 16-fold total dilution, respectively) yielded similar analytical results for the toxin concentration, there was a decrease in recovery for the 500-μl sample (dilution 8-fold).

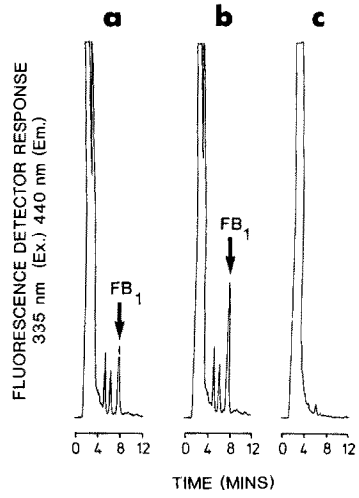

Fig. 2. Chromatograms of OPA-derivatised rat urine samples. (a) Urine sample containing 2.5 μg ml^{-1} FB_1 (amount FB_1 injected was 8 ng). (b) The same urine sample spiked with FB_1 standard. (c) A urine sample free of FB_1 contamination.

For the 1-ml sample (dilution 4-fold) the analytical recovery was less than 50% compared to the 100- and 250-μl aliquot analyses. That this effect was not due to saturation of the ion-exchange capacity of the sorbent, was shown by increasing the total dilution of the 500- and 1000-μl aliquots to 12-fold or above while maintaining the same sample load on the clean-up cartridge. The analytical results then fell in line with those achieved with the smaller aliquots of urine. It is thus important to ensure that the ionic strength of the sample applied to the sorbent is sufficiently low for retention of FB_1.

This analytical method for the determination of FB_1 in urine was validated with respect to precision and accuracy at two separate concentration levels. Six replicate determinations of FB_1 at a low level (0.90 μg ml^{-1}) gave a reproducibility of 4.8% relative standard deviation while six replicates at a higher level (11.0 μg ml^{-1}) gave a reproducibility of 1.3% relative standard deviation. The accuracy of the method was assessed by spiking six blank urine samples with FB_1 standard at a level of 1.0 μg ml^{-1} and a further six blank samples at 12 μg ml^{-1}. Determination of FB_1 in these spiked samples gave mean recoveries of 94 ± 4.5 and 91 ± 1.3% relative standard deviation for the two levels, respectively. The OPA derivatisation method was previously found to yield a linear detector response for up to 100 ng injected toxin [5]. The detection limit of this method was found to be around 50 ng ml^{-1} (signal-to-noise ratio = 4:1) determined by spiking a blank urine sample at this low level. All analytical results were based on an external standard method using a similarly derivatised FB_1 standard. In view of the good recoveries and reproducibilities achieved, the use of an internal standard was considered unnecessary.

This assay for the determination of FB_1 in urine was slightly modified for plasma samples. Prior to sample clean-up, the plasma proteins were precipitated by the addition of methanol. After centrifugation, the supernatant was applied without further treatment to the preconditioned SAX cartridge. As the pH of this supernatant is slightly alkaline (around 8.7), the flow-rate through the cartridge was maintained at around 1.0–1.5 ml min^{-1}. Very low flow-rates were observed to yield low and variable recoveries. Fig.

TABLE I

EFFECT OF SAMPLE DILUTION ON DETERMINATION OF FB_1 IN URINE

Volume urine analysed (ml)	Volume water added (ml)	Volume methanol added (ml)	Total dilution (fold)	Concentration FB_1 (μg ml^{-1})
0.10	0.90	3.00	40	39.7
0.25	0.75	3.00	16	38.8
0.50	0.50	3.00	8	33.8
1.00	–	3.00	4	18.5
0.50	1.00	4.50	12	37.7
1.00	4.00	15.00	20	38.7

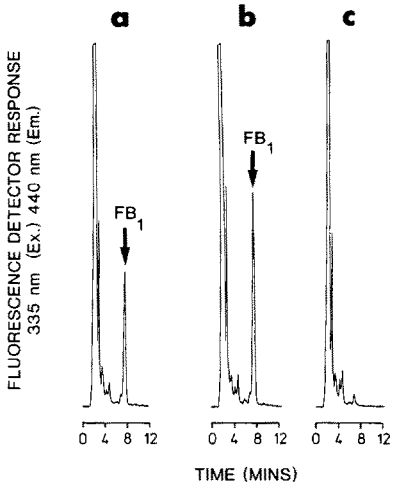

Fig. 3. Chromatograms of OPA-derivatised rat plasma samples. (a) Plasma sample containing 1.0 μg ml^{-1} FB_1 (amount FB_1 injected was 25 ng). (b) The same plasma sample spiked with FB_1 standard. (c) A plasma sample free of FB_1 contamination.

3a shows a chromatogram of a plasma sample containing 1 μg ml^{-1} FB_1. The majority of the impurities elute at the front-end of the chromatogram. A small impurity elutes just prior to FB_1, but is sufficiently separated from the toxin peak for accurate quantification. The identity of the FB_1 peak was again confirmed by spiking the sample with authentic standard (Fig. 3b), while the absence of co-eluting impurities was demonstrated by the analysis of a blank plasma sample (Fig. 3c).

The precision of this assay for determining FB_1 in plasma was assessed by analysis of six replicate samples at each of two levels. The reproducibility for the samples containing 1.39 μg ml^{-1} was 4.5% relative standard deviation and for the samples containing 8.78 μg ml^{-1}, it was 3.5%. The accuracy of the analytical method was also assessed by analysis of a spiked blank plasma at levels of 1.4 and 6.2 μg ml^{-1}. Six replicate analyses at each of these two levels gave mean recoveries of 86 $\pm$ 5.0 and 87 $\pm$ 3.3% relative standard deviation, respectively. The detection limit of this assay was found to be similar to that for the analysis of FB_1 in urine.

Both these new methods for FB_1 determination in physiological samples obtained from rats dosed with FB_1 are based on a rapid, single-step anion-exchange clean-up which yields sample residues sufficiently clean for subsequent reversed-phase chromatography of their OPA derivatives. These assays are sensitive with good recoveries and reproducibilities within the range of FB_1 levels that are shown here to occur during the dosing of the toxin to laboratory rats. Hence the new techniques reported here for the first time will enable detailed toxicokinetic studies to be undertaken. Such studies will enable the absorption, distribution, metabolism and excretion of FB_1 to be determined in animals and will hence indicate the feasibility of screening animal or human populations for fumonisin exposure.

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