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Development and validation of a HPLC method for the quantitation of ochratoxins in plasma and raw milk

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Abstract

A HPLC method with improved sensitivity for the determination of ochratoxins (OT) A, B and α in plasma and milk was developed. Plasma analysis involved a simple liquid–liquid extraction with chloroform; while for milk, an additional immunoaffinity clean-up step was necessary. The method showed a good linearity ($r^2 > 0.999$). The limit of quantification (LOQ) of OTA was 5 and 200 ng/l for milk and plasma, respectively. Average recovery was 89% in both matrices, except for OTα in milk that was only 18% due to poor immunoaffinity binding. OT remained stable in −20 °C stored samples; OTA concentration in plasma and milk did not change after 8 and 18 months of storage, respectively. The developed method has been applied to contaminated plasma and milk samples obtained from dairy ewes fed with ochratoxin-contaminated feed.

Keywords: Ochratoxins; Milk; Plasma

1. Introduction

Ochratoxins, a group of highly toxic metabolites produced by some species of *Aspergillus* and *Penicillium*, are commonly found in food and feeds [1,2]. Ochratoxin A (OTA), the most important toxin of this family, is nephrotoxic, hepatotoxic, teratogenic, and carcinogenic in animals. It was recently classified by the International Agency of Research on Cancer (IARC) as a class 2B, possible human carcinogen. When ingested by ruminants, OTA is mainly metabolized by rumen microorganisms [3,4] into a less toxic metabolite, ochratoxin α (OTα) [5–8]. OTα is excreted in milk and then it might be used as marker of OTA exposure. Fig. 1 shows the chemical structures of OTA, OTB and OTα.

Analysis of OTA typically involves solvent extraction and clean-up by solid phase extraction (silice or immunoaffinity column, IAC), followed by HPLC-fluorescence detection (FD) or HPLC-mass spectrometry (MS). Although several HPLC methods have been described to analyze OTA in plasma [9–13] and milk [9,14–17], they have not been fully validated. Some of these studies did not evaluate the validity of the method in plasma [11,16,18,19] or in milk [20], while other methods have only reported some of the required validation parameters [9,21]. For example, there is scarce information concerning the stability of OTA and its major metabolite OTα in plasma and milk during storage. For OTA, Valenta and Goll [22] reported no decrease in toxin concentration in milk artificially contaminated after a period up to 6 weeks. However, in surveys and experimental studies, it could be useful to store samples for longer periods before they are analyzed. It is therefore important to assess the stability of the analyte during the period of storage. In addition, for intake calculation, pharmacokinetic studies, and milk carry-over studies, a fully validated analytical method is necessary to yield reliable results that can be satisfactory interpreted.

The purpose of this work was to develop a sensitive HPLC method for the determination of ochratoxins in plasma and milk according to the Washington Consensus Conference guidelines on analytical methods validation [23].

2. Experimental

2.1. Preparation of standards and calibration curves

OTA, OTB and the internal standard (piroxicam) were purchased from Sigma (France). OTα was produced according to

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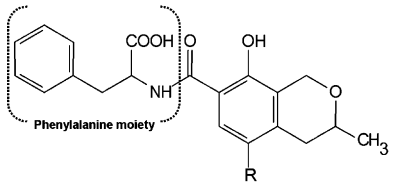
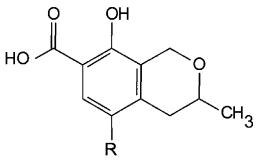
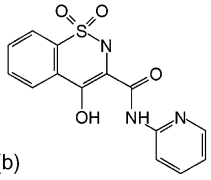




Ochratoxin α

Toxin	R
Ochratoxin A	Cl
Ochratoxin B	H
Ochratoxin α	Cl

(a)



(b)

Fig. 1. Chemical structures of ochratoxins (a) and piroxicam (b) used as internal standard.

Zepnik et al. [13]. Briefly, 0.5 ml of 2 μg/l of OTA solution in phosphate-buffered saline (PBS) (Sigma, France) adjusted to pH 7.6 was mixed with 0.5 ml of carboxypeptidase (5000 units/ml, Sigma, France) and incubated overnight at 37 °C. The completion of the reaction was checked by HPLC. The chromatogram showed a single peak corresponding to OTα. Toxin-free plasma and milk samples were collected locally from healthy dairy ewes. Immunoaffinity column Ochraprep were obtained from R-Biopharm (Lyon, France). All other chemicals were of analytical grade.

Stock solutions of OTA in methanol and OTB and OTα in ethanol were diluted in the same solvent to obtain a concentration of approximately 10 μg/l and calibrated spectrophotometrically at 333, 318, and 335 nm, respectively. The molar absorption coefficients ($M^{-1}\,cm^{-1}$) of 6640 for OTA, 6900 for OTB, and 6200 for OTα were used. Aliquots of 10 μg were transferred into 1 ml conical amber glass, evaporated to dryness at 45 °C under a stream of nitrogen, and stored at −20 °C. Internal standard solution (IS; 100 μg/ml) was prepared monthly in 0.01 M of NaOH, and stored at 4 °C during the validation period.

For plasma determination, calibration curves were prepared with pooled plasma by adding 100 μl of different solutions in 15% methanol to obtain six different concentrations ranging from 0.1 to 10 μg/l. A hundred μl of the IS was added to all calibration and quality control samples (QCS).

For the analysis of milk samples, standard curves were prepared in 60% methanol to obtain working solutions of: 0.25, 0.5, 1, 5, 10 and 20 μg/l OTA and OTα. The concentration of OT is calculated by using the following formula:

$$\text{Concentration (ng/L)} = \frac{M \times V_d}{V \times V_i}$$

where M is the mass (ng) injected into the HPLC system, V_d is the volume (0.2 ml) used to dissolve the dried extract, V is the volume of milk (10 ml) taken for analysis, and V_i is the volume (0.05 ml) injected into the HPLC system.

2.2. Chromatographic conditions

The HPLC system consisted of an AS3000 pump (Thermo Finnigan, France), an automatic sampler (Spectra-physics, France) equipped with a 100 μl loop, and a fluorescence detector (FL-3000, Thermo Finnigan, France). The photomultiplier voltage was set at 1000 V and 800 V for milk and plasma analysis, respectively. Separation of mycotoxins was performed at room temperature on a Nucleodur C_{18} Gravity column (125.0 mm × 4.6 mm, 5 μm, Macherey Nagel, France), using a gradient solvent system (solvent A = 10 ml/l of acetic acid and solvent B = methanol). The solvent program was as follows: the initial percentage of solvent B was 30%, which was raised to 60% in 10 min, then to 90% in 2 min, lowered to 30% in 2 min and held at 10% at a flow rate of 1 ml/min. The mobile phases were daily degassed by passing through a 0.45 μm nylon filter membrane. The column effluent was monitored at 274 nm excitation and 440 nm emission. Acquisition and calculations were made using Thermoquest software (Thermo Finnigan Spectra System, France).

2.3. Sample extraction

2.3.1. Milk samples

To a tube containing 10 ml of milk, it was added 5 ml of phosphoric acid–saline solution (33.7 ml of H_3PO_4 and 18 g of NaCl in 1 l of distilled water) and 10 ml of chloroform. The tubes were mixed in a horizontal shaker for 15 min at 40 rpm, and then centrifuged at 2500 × *g* for 10 min. The top aqueous layer was removed by aspiration. Four ml of PBS, pH 7.6, were added to the organic layer, the mixture was vortexed for 1 min, centrifuged at 2500 × *g* for 10 min and the top aqueous layer was transferred into a clean glass tube. The PBS extraction was repeated once. The recovered aqueous layers were pooled and loaded onto a IAC, which was allowed to drain and was then blow dried. OT were eluted with 3 ml of methanol. The extract was then evaporated at 45 °C under a stream of N_2. The dried residue was redissolved in 0.2 ml of 60% methanol by incubation in an ultrasonic bath for 3 min and 50 μl of this solution were injected into the HPLC system. The IAC were immediately washed after use with 20 ml of PBS, pH 7.4, and stored at +4 °C in 0.02% (w/v) sodium azide in PBS to prevent mold and bacterial growth. The IAC treated in that way could be reused more than three times without any noticeable loss in binding properties. The performance of IAC to bind OT was tested on every batch of columns using different concentrations of OT, alone or in combination, in PBS solution. Elution and analysis of OT were carried out as described above.

2.3.2. Plasma samples

To a tube containing 2 ml of plasma, it was added 10 ml of phosphoric acid–saline solution and 8 ml of chloroform. The tubes were mixed in a horizontal shaker for 15 min at 40 rpm, and

then centrifuged at 2500 × *g* for 10 min. The upper aqueous layer was removed by aspiration, and the organic layer was evaporated to dryness at 45 °C under a stream of N_2. The dried residue was redissolved in 0.2 ml of 60% methanol by incubation in an ultrasonic bath for 3 min and 50 μl of this solution was injected into the HPLC system.

2.4. Confirmation of OTA and OTα by methyl formation

The presence of OTA and OTα in biological samples was confirmed by formation of methyl derivatives. Briefly, the remaining (150 μl) of the milk and plasma extracts were evaporated to dryness and redissolved in a solution of 2.5 ml of methanol and 0.1 ml of concentrated hydrochloride acid. The mixture was incubated overnight at room temperature, evaporated, and the residue was dissolved in 150 μl of 60% methanolic solution before injecting 50 μl into the HPLC apparatus.

2.5. Validation procedure

For each matrix, the linearity, precision, accuracy, as well as the recovery and stability of OTA, OTB and OTα was checked. This validation was performed by analyzing replicate sets of QCS of known concentrations. The QCS were prepared at the beginning of the validation, aliquoted, and stored at −20 °C until analysis.

2.5.1. Linearity and variability of methods

The limit of quantification (LOQ) was determined by the addition of decreasing amounts of ochratoxins to OT-free plasma and milk samples. The intra- and inter-run variability of the method in term of precision and accuracy was performed by analyzing replicate sets of QCS.

2.5.2. Recovery and stability

The stability of OT in plasma and milk following storage at −20 °C was tested. QCS of each matrix were prepared at the beginning of the study by spiking pooled plasma and raw milk samples with the three ochratoxins at different levels. The samples were analyzed before and after different storage times. In addition to long-term storage, the 24 h-stability of QCS reconstituted extract was also investigated. The 24 h-stability was tested because due to sample processing constrains it may be convenient to extract samples 1 day before analysis. Recovery was determined by comparing the peak areas of QCS extracts spiked with known amounts of analyte to those of the same amount of pure OT in the 60% methanol.

2.6. OTA and OTα concentration profiles in contaminated plasma and milk

Blood and milk samples were collected from three dairy ewes after feeding with ochratoxin-contaminated feed. Animals were cared for in accordance with the guidelines for animal research of the French Ministry of Agriculture. Blood samples were obtained by jugular venipuncture and collected into sterile heparinized glass tubes at 0, 1, 2, 3, 6, 9, 12, 24, 30, 36, 48, 72, 96, 120, 150, and 168 h after administration. The plasma was immediately separated by centrifugation at 3500 × *g* for 15 min and stored at −20 °C until analysis. Milk samples were taken daily during 8 days after administration. Sodium azide (1.5 μg/ml) was added as a preservative and samples were stored at −20 °C until analysis.

3. Results and discussion

3.1. Specificity and linearity of the method

The chromatographic conditions were optimized using plasma and milk samples from six healthy dairy ewes that were individually analyzed. Fig. 2 shows representative HPLC chromatograms of extracts from ewe's plasma before and after a single oral administration of 30 and 1.8 μg/kg b.w. of OTA and OTB, respectively. Fig. 3 shows similar HPLC chromatograms of extracts from ewe's milk samples. The chromatographic conditions were the same for both matrices. The IS and the 3 OT (OTα, OTB and OTA) peaks were well separated with retention times of 6.0, 6.7, 10.0, and 12.7 min, respectively. No endogenous co-eluting peaks interfering with OT and IS were detected in all tested plasma and milk samples. As expected, liquid–liquid extraction without IAC purification for the plasma samples resulted in less clean chromatograms than the IAC-treated milk samples. However, the IAC purification of plasma samples was considered unnecessary because the method was sensitive enough to follow OT concentrations up to 3 days after a single oral administration during pharmacokinetics studies. Washing the HPLC column daily with pure methanol helped to maintain a clean chromatogram for the plasma samples. In contrast, OT concentration in milk is several folds lower than that of plasma and the IAC purification step was used to eliminate interfering substances and increased the sensitivity of the

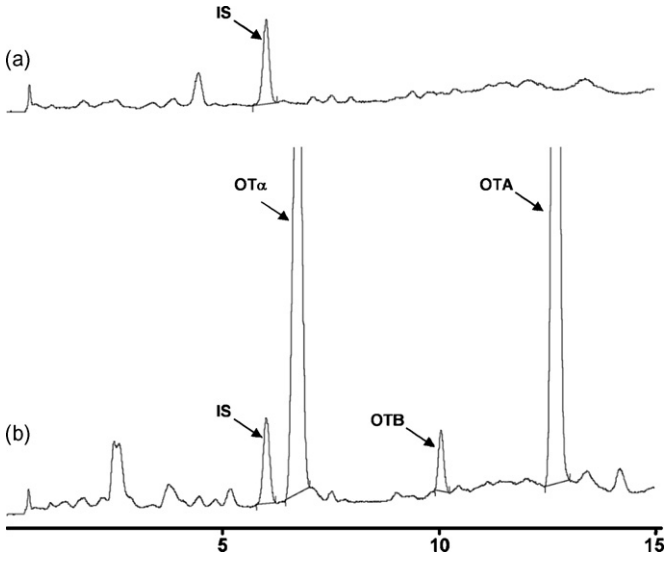


Fig. 2. Typical HPLC chromatogramms of an extract from ewe's plasma before (a), and after (b) oral administration of 30 and 1.8 μg/kg b.w. of OTA and OTB, respectively.

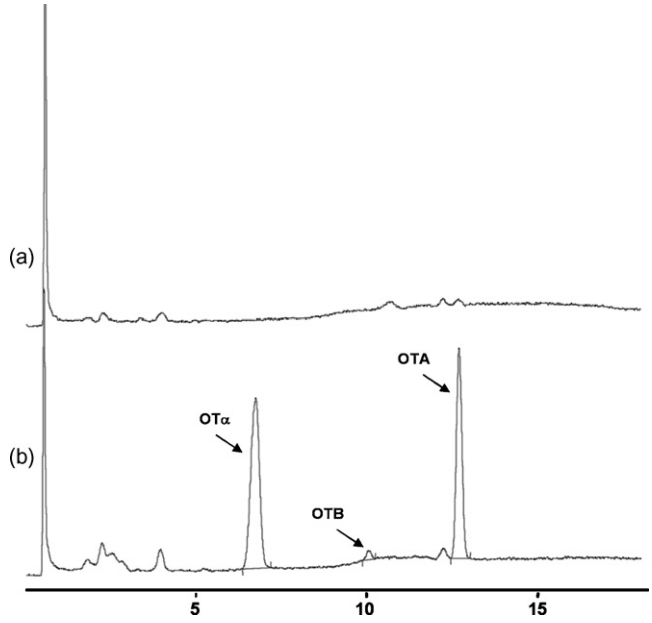


Fig. 3. Typical HPLC chromatograms of an extract from ewe's milk before (a), and (b) after oral administration of 30 and 1.8 μg/kg b.w. of OTA and OTB, respectively.

method. Piroxicam, a non steroidal anti-inflammatory, was satisfactory used as an internal standard for the determination of OT in plasma. Other authors have used OTB as internal standard for OTA determination [24]. However, OTB can also be present in contaminated feeds, a condition that limits its utilization in surveys and natural intoxication cases.

Excellent linearity ($r^2 > 0.999$, $n = 5$) was observed for both matrices. The LOQ was calculated by using a signal-to-noise ratio of 3:1 and it was defined as the lowest concentration measured with satisfactory accuracy and precision. LOQ in plasma was estimated as 200, 500, and 200 ng/l for OTA, OTB, and OTα, respectively. In milk, the LOQ of OTA was 5 ng/l. OTB and OTα were not quantified in milk, because of the low expected concentration in field intoxications of the former, and due to the low recovery by the IAC purification step of the latter (shown below).

3.2. Recovery and stability of OT

Performance criteria of the method for plasma and milk are given in Tables 1 and 2, respectively. For plasma, the mean recovery of OTA, OTB and OTα at the tested concentration averaged 97.0 ± 4.4%, 96 ± 5.8%, and 89.5 ± 5.3%, respectively. The recovery of the IS, at the concentration used, was less efficient but still acceptable (74.9 ± 2.9%). For milk, the mean recovery of OTA and OTB was also good, it averaged 89.4 ± 8.5 and 115 ± 10%, respectively. However, the recovery of OTα in milk was very low (19.8 ± 5.7%). This low recovery was due to inefficient binding by the IAC, result that was confirmed by using pure OTα in PBS solution (Table 3). The lower OTα binding to the IAC could be due to the lack of phenyalanine group

Table 1
Performance criteria of the method for determination of ochratoxins in plasma

Ochratoxins (a)	Concentration (ng/l)	Performance criteria (mean ± SD, $n = 3$)	
		Precision and accuracy	
		Precision (%)	Accuracy (%)
OTA	5	11.5	9.7
	10	12.2	−10.7
	100	9.4	−16.4
OTB	200	10.6	−18.3
	100	9.0	5.5
OTα	100	22.4	−82.0
	1000	35.0	−81.0
		Recovery	
OTA	5	109.7 ± 12.6	
	10	86.3 ± 7.9	
	100	83.2 ± 8.7	
	200	78.6 ± 5.0	
OTB	100	115.7 ± 10.0	
OTα	100	18.0 ± 3.8	
	1000	21.5 ± 7.6	
		Stability	
		After 5 months	After 18 months
OTA	10	8.4 ± 0.1	11.2 ± 3.2
	100	88.2 ± 4.4	82.4 ± 5.9

(a): Ochratoxin A (OTA), Ochratoxin B (OTB), Ochratoxin α (OTα). (b): $n = 3$, except for precision and accuracy of OTA determination where $n = 9$.

Table 2
Performance criteria of the method for determination of ochratoxins in milk

Ochratoxin (a)	Concentration (ng/l)	Performance criteria (mean ± SD) (b)	
		Precision and accuracy	
		Precision (%)	Accuracy (%)
OTA	500	9.6	1.1
	2000	2.9	−1.7
OTα	1500	7.3	1.7
	4000	5.9	−6.5
OTB	1500	6.2	10.1
	4000	3.4	−2.0
		Recovery	
OTA	500	99.4 ± 4.3	
	1000	96.9 ± 6.0	
	2500	94.7 ± 2.8	
OTB	500	94.4 ± 8.2	
	1000	98.6 ± 6.5	
	2500	95.0 ± 2.8	
OTα	1000	82.7 ± 6.1	
	2500	89.8 ± 5.3	
IS		74.9 ± 2.9	
		Stability	
		After 1 month	After 8 months
OTA	500		553.2 ± 45.4
	1000	1134.6 ± 61.1	
	2500	2680.9 ± 90.2	2565.5 ± 264.6
OTB	1000	1281.4 ± 65.2	
	2500	2655.5 ± 332.8	
OTα	1000	1332.0 ± 157.2	
	2500	2779.0 ± 179.7	

(a): Ochratoxin A (OTA), Ochratoxin B (OTB), Ochratoxin α (OTα). (b): $n = 3$, except for precision and accuracy of OTA determination where $n = 9$.

Table 3
Efficiency of immunoaffinity columns (IAC) to bind ochratoxin A, B, and α

Matrix	Ochratoxins	Quantity used (ng)			Bound to IAC (%) (mean ± SD)
Phosphate-buffered saline					
	OTA + OTα	$n = 2$	0.5	OTA	95.3 ± 2.9
				OTα	0.0 ± 0.0
		$n = 2$	5	OTA	95.3 ± 2.9
				OTα	21.9 ± 19.0
	OTA + OTB + OTα	$n = 3$	0.5	OTA	103.9 ± 3.3
				OTB	80.4 ± 4.8
				OTα	0.0 ± 0.0
		$n = 3$	5	OTA	89.8 ± 2.3
				OTB	79.0 ± 8.5
				OTα	6.5 ± 0.6
		$n = 3$	50	OTA	84.2 ± 4.0
				OTB	75.7 ± 1.9
				OTα	6.8 ± 1.5
Milk					
	OTα	$n = 3$	1		18.0 ± 3.8
		$n = 4$	10		21.5 ± 7.6

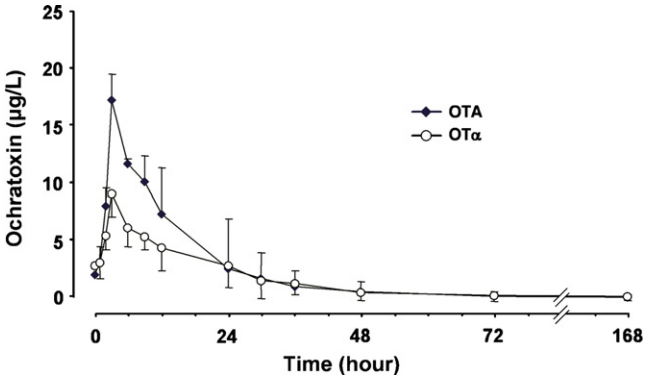


Fig. 4. Concentration-time profile of ochratoxin A and α in plasma after oral administration of contaminated feed. Values are the mean ± standard errors of three dairy ewes that received 30 and 1.8 μg/kg b.w. of OTA and OTB, respectively.

in the OTα structure compared to OTA and OTB. Variability of the method expressed in terms of intra- and inter-run precision and accuracy was less than 18% (Tables 1 and 2). OTA was stable in plasma and milk samples after 8 and 18 months of storage at −20 °C (Tables 1 and 2), and also in reconstituted extracts in 60% methanol over a 24 h period at +4 °C (data not shown).

3.3. Pharmacokinetic profiles

The OTA and OTα profiles in plasma and milk of dairy ewes following oral OT administration are given in Figs. 4 and 5, respectively. The plasma concentrations of OTA and OTα increased progressively to reach a maximal concentration (C_{max}) at 6 h after administration. The C_{max} observed was 17362 ± 476 and 9201 ± 1689 ng/l (mean ± SD, $n = 3$) for OTA and OTα, respectively. The concentration of OTA in milk peaked 1 day after administration. As OTα binds poorly to the IAC, the concentration in milk was corrected using the recovery rate of 19.8% (Table 3). The OTα concentration corrected in that way was at its peak about 7.5 times higher than OTA. A similar OTA:OTα ratio in milk was reported in cow by Ribelin et al. [19]. Therefore, OTα can be considered as a more sensitive marker of OTA exposition than the parent mycotoxin. However, a better purification method should be developed, since the IAC used was not adequate to purify OTα.

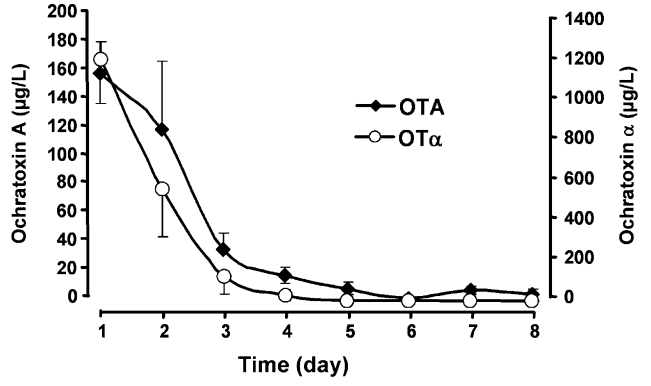


Fig. 5. Concentration-time profile of ochratoxin A and α in milk after oral administration of contaminated feed. Values are the mean ± standard errors of three dairy ewes that received 30 and 1.8 μg/kg b.w. of OTA and OTB, respectively.

4. Conclusion

A validated HPLC method with improved specificity and sensitivity compared to previously published methods was developed for quantification of OT in plasma and milk. Results from kinetic studies indicated that the sensitivity, accuracy, and precision are adequate to monitor OTA and OTα in plasma and milk following administration of contaminated feed at levels that can be found in nature. Unfortunately, the IAC used in this study were not adequate to purify OTα, and this compound can be underestimated in biological samples.

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