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Determination of Ochratoxin A in small volumes of human blood serum

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Abstract

A new simple and rapid method for analysing Ochratoxin A (OTA) in small volumes of human blood serum using capillary zone electrophoresis coupled to laser-induced fluorescence is described. The clean-up procedure solely consists of a double extraction step. To improve the reproducibility of migration times and quantification, two internal standards were used. The limit of detection was 0.55 ng/ml, with a linear range of 1–100 ng/ml of OTA in spiked human blood serum. The method is used to rapidly screen suspected patients.

Keywords: Ochratoxin A

1. Introduction

Ochratoxin A (OTA) is a naturally occurring mycotoxin which is produced by several species of Aspergillus (e.g. *A. ochraceus*) and Penicillium (e.g. *P. verrucosum*). OTA is found mainly in food derived from plants, such as cereals [1], coffee [2,3], nuts [4], dried fruits, spices [5] and wine and grapes [6,7]. The intake of OTA through contaminated feed may lead to its occurrence in the blood, kidneys and liver of pigs and poultry [8]. The consumption of several foods, including cereal products, wine, beer and pork, can also cause high plasma levels of OTA in human blood [9]. OTA has been reported to have nephrotoxic [10], carcinogenic [11], genotoxic [12] and immunotoxic effects [13,14]. Moreover, OTA is suspected of causing Balkan Endemic Nephropathy, a kidney disease in south-eastern Europe [15].

Several methods have been developed to determine OTA in various matrices, like milk [16], certain tissues [17], animal feeds [18], urine [19] and human blood serum [20].

Because of the low concentrations of OTA usually found in human blood serum, its analytical determination calls for sensitive techniques. The methods currently used are often based on enzyme-linked immunosorbent assay (ELISA) [21] or reversed-phase high-performance liquid chromatography (RP-HPLC) [9,16].

To analyse OTA in human blood serum with RP-HPLC, various sample clean-up procedures have been developed. In most studies OTA was extracted from blood serum or plasma by chloroform after acidification to a pH below 2.5 [16]. Recently developed methods using immunoaffinity columns have been developed for the determination of OTA in blood and tissues [22]. Depending on sample clean-up and instrumentation, limits of detection (LOD) between 5 and 50 pg/ml have been achieved.

In addition to these methods, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has been employed. Corneli and co-workers developed a method for the determination of OTA in coffee, corn and sorghum using CE-LIF. Due to the good naturally fluorescence property of OTA a LOD of 0.2 ng/g has been achieved with this method [23,24]. However, capillary electrophoresis has the disadvantage that the injection volumes are very small. To overcome this, several injection techniques have been developed [25–28]. One technique to enhance the sensitivity of detection is on-line sample concentration by sample stacking with reversed polarity [29–31]. Sample stacking occurs at the interface between the low conductivity sample and the high conductivity separation zone. Sample ions experience a low electrical field when they enter the separation buffer. This causes an abrupt decrease in their electrophoretic velocities, focusing the sample ions within a thin zone. To enhance the performance of sample stacking for negatively charged ions, a polarity-switching step is necessary to min-

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imise dispersion caused by the mismatch of different local electroosmotic flow velocities. Due to reversed polarity, the sample buffer is pushed towards the column head by the electroosmotic flow [32,33]. When the sample matrix is removed from the column, the polarity is switched back and analysis subsequently starts.

Biomonitoring is absolutely essential in order to assess the existing risk in indoor environments containing mould. Therefore the aim of this study was to develop a method for the determination of OTA in human blood serum with CE-LIF starting from a 50 μl sample volume. A method with a small sample volume had to be developed since no more sample serum was available. The majority of the above-mentioned methods work with 2 ml blood serum. Therefore the LOD and the limits of quantification (LOQ) are expected to be much higher than in other published methods. However, due to the low sample volume, sample clean-up ought to be simplified. In most of the published methods, the extraction of OTA followed by a clean-up protocol with immunoaffinity columns is performed. However, avoiding this clean-up step is desirable because the extract has a lower concentration of matrix components.

2. Experimental

2.1. Material and reagents

Ochratoxin A, dansylphenylalanine (DNS-Phe) and coumarin-3-carboxylic acid (C3A) were purchased from Sigma–Aldrich (Germany). Acetonitrile (Suprasolv grade), dichloromethane (Lichrosolv grade), sodium chloride and phosphoric acid (80%) were obtained from Merck (Darmstadt, Germany). Di-sodium hydrogen phosphate-7-hydrate was purchased from Riedel-de-Haen.

2.2. Solutions

DNS-Phe stock solution was prepared by dissolving 2 mg DNS-Phe in 500 μl acetonitrile. Ten microlitres of this solution was added to 10 ml distilled water. The final concentration of this solution was 4 μg/ml. C3A stock solution was prepared by dissolving 2.9 mg C3A in 1 ml acetonitrile. All stock solutions were stored at −20 °C. The solutions remained stable at this temperature for 2 months. Solution A was prepared by dissolving 1.2 g sodium chloride and 1.3 ml 85% phosphoric acid in 10 ml distilled water.

The following solutions were prepared daily prior to use. Solution B was prepared by adding 125 μl of DNS-Phe stock solution to 1 ml distilled water. The injection solution was composed of 770 μl distilled water and 330 μl of C3A stock solution. Before use all solutions were filtered using a filter with a pore size of 0.45 μm.

A 30 mM phosphate buffer (pH 7) was prepared by dissolving 4.02 mg di-sodium hydrogen phosphate-7-hydrate in 400 ml bidistilled water. The solution was adjusted with 20% phosphoric acid until the required pH was reached. The solution was then made up to 500 ml.

2.3. Sample clean-up

Twenty microlitres 0.2 M sodium hydrogenhydrocarbonate solution was added to 50 μl serum and extracted twice with 100 μl dichloromethane. The organic layer was discharged. Subsequently 100 μl solution A, 20 μl solution B and 100 μl dichloromethane were added to the water phase, agitated in a vortex mixer for 5 min at room temperature, and then the mixture was centrifuged at 14,000 × *g* for 2.5 min. Three phases were obtained: aqueous (upper), white ring (middle) and dichloromethane (lower). The lower phase was transferred into a vial fitted with conical inset. The aqueous layer and the white precipitate were extracted with 100 μl dichloromethane again. The combined dichloromethane fractions were evaporated to dryness and reconstituted in 20 μl of the injection solution.

The recovery was estimated by adding 1 μl OTA in methanol (500 ng/ml) to 50 μl blank serum sample (final concentration 10 ng/ml) and analysing the samples described above.

2.4. CE-LIF

An HP3D CE system (Agilent, Waldbronn, Germany) equipped with a laser-induced fluorescence detection system with a 15 mW helium–cadmium ion laser with an excitation wavelength of 325 nm (Picometrics, Toulouse, France) was used. Separation was performed on a fused silica capillary (60 cm × 50 μm; 45 cm to detector). The capillary was thermostated at 20 °C by air. Samples kept in the autosampler were also thermostated at 20 °C.

Prior to first use, the capillaries were conditioned by rinsing for 15 min with methanol, 5 min with water, 20 min with 1N NaOH, 10 min with 0.1N NaOH and finally 20 min with separation buffer. The capillaries were prepared for daily use by rinsing for 10 min with water, 10 min with 1N NaOH, 10 min with 0.1N NaOH and 15 min with separation buffer. Before each analysis the capillary was flushed for 1 min with 0.1N NaOH and 2 min separation buffer.

A 30 mM phosphate buffer pH 7.0 was used as separation buffer. Injection was performed hydrodynamically with a pressure of 50 mbar for 60 s. Subsequently a voltage of −20 kV was applied to the column. After 30 s the voltage was switched to +20 kV. The run time of the analysis was 12 min.

3. Results and discussion

3.1. Reproducibility of migration times

In CE migration times can vary from run to run. These drifts in migration times make proper peak identification

difficult. Several groups have proposed methods to improve migration time reproducibility. Bocek and co-workers proposed using the migration times of two standards and an unknown to determine the electrophoretic mobility of the unknown [34]. Jumppanen and Riekkola developed a similar method using up to four markers of known electrophoretic mobilities [35]. However, these methods entail knowing the electrophoretic mobilities of the markers. Two other methods for migration time correction using one or two internal standards as marker were developed by Li et al. [36]. To define standard conditions, for both methods a reference electropherogram was defined. The other electropherograms were normalised to the standard electropherogram. The single-marker method only reduces the variation of the electroosmotic flow, whereas the two-marker method also reduces differences in the electrophoretic mobility of the analytes. Single-marker correction and two-marker correction were performed according to Eqs. (1)–(3), where $t_{m_1,s}$, $t_{m_2,s}$ are the migration times of DNSPHE and C3A in the standard electropherogram, and t_x, t_{m_1}, t_{m_2} are the migration times of OTA, DNSPHE and C3A under non-standard conditions. The derivation of these equations can be found in Li et al. [36].

$$t_{\text{corrected,x}} = \left[\frac{1}{t_x} - \left(\frac{1}{t_{m_1}} - \frac{1}{t_{m_1,s}}\right)\right]^{-1} \quad (1)$$

$$t_{\text{corrected,x}} = \left[\frac{1}{t_{m_1,s}} - \frac{1}{\gamma}\left(\frac{1}{t_{m_1}} - \frac{1}{t_x}\right)\right]^{-1} \quad (2)$$

$$\gamma = \frac{(1/t_{m_1}) - (1/t_{m_2})}{(1/t_{m_1,s}) - (1/t_{m_2,s})} \quad (3)$$

Fig. 1 plots the migration times of 51 subsequent runs within 6 days. The jump in migration times after the first day was caused by changing the separation capillary. The decrease in the migration times within each day arises from the depletion of the separation buffer. The relative standard deviation (R.S.D.%) for the migration times within a day was 2.56% and between days 15.81% (Table 1). Nevertheless, these fluctuations in the migration times were reduced after correction with the single-marker method. The R.S.D.% of the migration times within a day was 0.84% and the day-to-day variation was 1.15%. Using the two-marker correction method did not further improve reproducibility. Therefore, it can be concluded that mainly the electroosmotic flow is responsible for the variation in migration times.

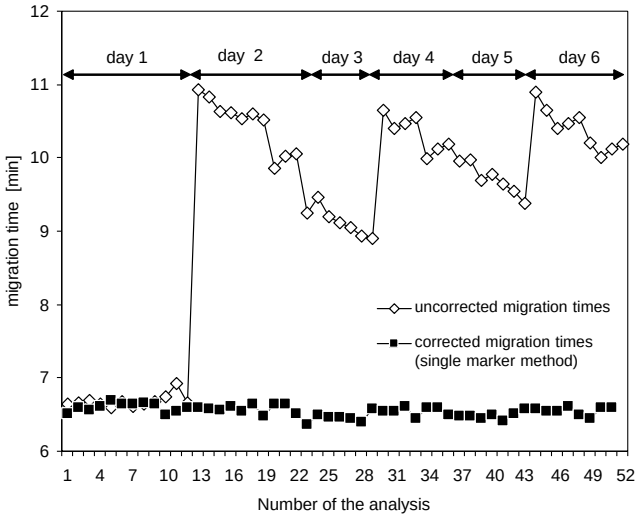

Fig. 1. Migration times of OTA within 6 days.

Table 1
R.S.D.% of migration times of OTA

	Intraday (%)	Interday (%)
Uncorrected	2.56	15.81
Correction with single-marker method[a]	0.84	1.15
Correction with two-marker method[a]	0.9	1.39

[a] According to Li et al. [36].

3.2. Internal standards

The use of internal standards improves the quantitative performance of capillary electrophoresis methods in terms of precision and linearity [37,38].

This method was developed using two internal standards, DNSPHE and C3A. These compounds have good fluorescent properties and are commercially available in sufficient purity. The migration time of OTA lies within a time window marked by DNSPHE and C3A. DNSPHE was used as recovery standard. It was added to the serum before starting the clean-up. C3A was used as injection standard. This standard enhances the precision of injection and the subsequent sample stacking with reversed polarity. Table 2 summarises the effect of the internal standards on quantification. The quantification of OTA with no internal standard yielded a R.S.D.% of 14%. Using only one of the two internal standards, the R.S.D.% dropped to 9.6% for C3A and 7.4% for DNSPHE, respectively. When quantifying OTA using both internal standards, C3A and DNSPHE, a R.S.D.% of 5.5% was achieved. The linearity of the calibration curve, represented by the correlation coefficient, is improved by taking two internal standards into account.

To correct the migration time both internal standards are used (see above).

3.3. Validation of the method

Fig. 2 shows a calibration curve between 0.75 and 100 ng/ml. The LOD and LOQ were estimated according

Table 2
Precision and linearity with and without different internal standards

	Precision ($n = 5$) (%)	Linear correlation
No internal standard	14.1	0.9810
Only C3A	9.6	0.9990
Only DNSPHE	7.4	0.9880
DNSPHE and C3A	5.5	0.9998

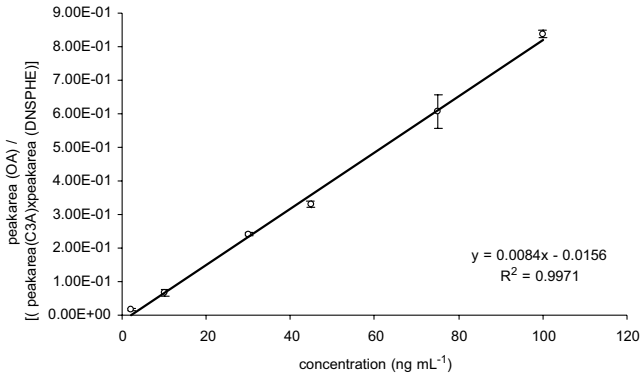

Fig. 2. Calibration curve of OTA between 0.75 and 100 ng/ml.

Table 3
Recovery, LOD and LOQ of the method

Recovery (%)	95 (±6.3%)
LOD (ng/ml)	0.5
LOQ (ng/ml)	1.0 (±9.3%)

to Thompson [39]. Table 3 lists the analytical parameters of this method. Due to the reduced sample volume (50 μl), the LOD (0.55 ng/ml) and LOQ (1.0 ng/ml) are higher than in other published methods.

A recovery of 85% was achieved with the extraction procedure described in Section 2. The samples are extracted twice. The first extraction step was carried out at pH 9 in order to remove matrix components. At this pH, OTA and DNSPHE are charged and therefore these compounds remained in the water phase. After acidification below pH 2, OTA and DNSPHE are extracted by dichloromethane. Slightly higher extraction yields (92%) were achieved without the first extraction step at pH 9. However, as the matrix components disrupted analysis, the first extraction step was inserted and a lower extraction yield accepted.

Fig. 3 shows electropherograms of blank serum sample and spiked serum sample. The method is selective and no interfering peaks were detected.

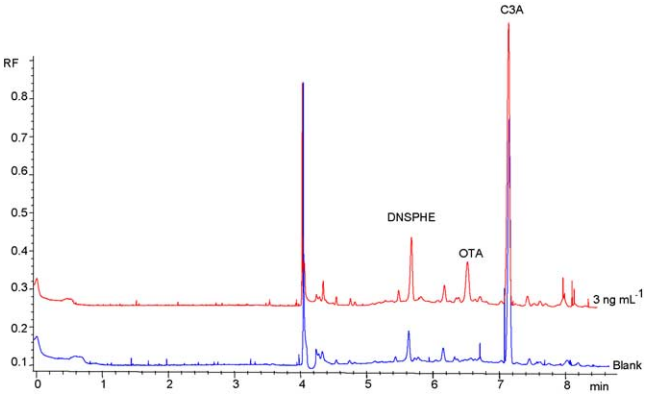

Fig. 3. Electropherograms of a blank and a spiked serum sample.

4. Conclusion

A method for the determination of OTA in small volumes of human blood serum with CE-LIF was developed. Due to the fluctuations of the migration times, the migration times needed to be corrected for proper peak identification. For this method the correction of migration times with a single-marker approach reduced the variations in the migration times to 0.84% (intraday) and 1.15% (interday).

The precision of quantification was reduced to 5.5% when two internal standards were used. As expected, the LOD and LOQ were higher than in other published methods (Zimmerli and Dick [16]: 5 pg/g; Thuvander et al. [9]: 10 pg/ml). This method was developed for the rapid screening of patients exposed to a mould-contaminated indoor environment, which may present a health risk. The lack of any solid-phase extraction columns or immunoaffinity columns in the sample clean-up step makes it faster and less expensive than other methods.

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