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Short Communication

Fast determination of ochratoxin A in serum by liquid chromatography: comparison with enzymic spectrofluorimetric method

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ABSTRACT

A rapid high-performance liquid chromatographic method for the determination of low concentrations of ochratoxin A in serum is described. The extraction procedure was simple and short, and liquid chromatographic analysis was carried out isocratically on a reversed-phase C_{18} column, with methanol–water–acetic acid (30:70:1) as mobile phase and fluorescence detection (excitation at 336 nm, emission at 465 nm). The examined concentration range, 5–50 ng/ml ochratoxin, the recovery method was 87–94%, compared with 62–67% for the enzymic spectrofluorimetric method. The high-performance liquid chromatographic method was faster because the extraction procedure was shorter, and more sensitive so that small sample volumes could be used.

INTRODUCTION

Ochratoxin A (OA) is a nephrotoxic metabolite of several fungal species frequently present in cereals, food and feed. The spontaneous occurrence of OA in cereals [1,2] and its toxic residues in porcine tissue [3] has been demonstrated in several countries.

OA causes porcine nephropathy and has been suggested to be associated with the aetiology of a human kidney disease known as Balkan nephropathy. The highest concentrations of the toxin were found in the serum [4,5] and kidneys [6] and were 3–40 ng/ml and 32–140 ng/g, respectively.

High-performance liquid chromatographic (HPLC) methods are mainly used for the determination of OA in foodstuffs [7,8] and animal feed [9–12]. The aim of

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this study was to select a mobile phase composition providing adequate chromatographic resolution of the serum extract containing OA. The known procedures for the extraction of OA from serum [5,11,14] have been shortened in order to make the method faster, simpler and cheaper.

EXPERIMENTAL

Chemicals

Standards of ochratoxin A and carboxypeptidase A were obtained from Sigma (St. Louis, MO, USA). Chloroform, methanol and acetonitrile were HPLC grade (LiChrosolv, Merck, Darmstadt, Germany). An acidified solution of 0.1 *M* $MgCl_2$ was prepared by mixing 0.2 *M* $MgCl_2$ and 0.1 *M* HCl (1:1). Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) was from Merck.

Apparatus

A Model 250 binary LC pump, an LS-3 fluorescence spectrometer, and a GP 100 graphic printer were all from Perkin-Elmer (Norwalk, CT, USA). An SP-100 spectrofluorimeter was from Pye Unicam (Cambridge, UK) and a reversed-phase C_{18} μBondapak column (10 μm, 30 cm × 0.4 cm I.D.) from Waters, Millipore (Milford, MA, USA). A Model 7125 injection valve (Rheodyne, Cotati, CA, USA) and Epson PCe computer with analytical workstation Omega-2 (Perkin-Elmer-Nelson, Zug, Switzerland) were used.

Chromatographic conditions

The conditions were as follows: mobile phase, redistilled water–methanol (3:7, v/v) containing 1% acetic acid (pH 4); flow-rate, 1 ml/min; pressure, 70 bar; temperature, ambient; detector wavelengths, 336 nm (λ_{ex}) and 465 nm (λ_{em}); injection volume, 20 μl; retention time, *ca.* 6 min.

Sample preparation

For HPLC the sample preparation procedure used for enzymic spectrofluorimetric (ESF) method by Hult and co-workers [5,14] was followed. A 2-ml portion of serum was pipetted into a centrifuge tube: 10 ml of acidified solution containing 0.1 *M* $MgCl_2$ and 5 ml chloroform were added. The tube was rotated for 10 min at 20 turns per min. The mixture was then centrifuged for 10 min at 1600 *g*.

For the HPLC method, 2 ml of the chloroform extract was evaporated under a stream of nitrogen, and the residue was redissolved in 1 ml of methanol. For the ESF method the chloroform extract was purified and prepared as previously described [14].

In two series of experiments, OA in serum was determined in the concentration range 5–50 ng/ml, and the amount of added OA was measured with the HPLC and ESF methods. In one series OA was added to the serum, extracted as described, and quantified by the HPLC system. In the other series, the extract was

divided into two fractions, so that the same sample was analyzed by both methods.

RESULTS AND DISCUSSION

Results of the comparative measurement by the HPLC and ESF method are shown in Table I. The accuracy of the HPLC method, expressed as recovery, was 87–95% in both series of experiments. With the ESF method the recovery was lower, and ranged from 62.5 to 68.7%. In the extract of serum samples containing 5 ng OA/ml, OA could not be detected by the ESF method (Table I).

So, the HPLC method had not only a higher recovery, but also a lower detection limit. The detection response of four OA standard solutions (1, 2, 3 and 4 ng/ml) was linear (correlation coefficient 0.975, and relative standard deviation 5.65%). The minimum detectable amount (sensitivity at 95% confidence) of OA by HPLC was 1 ng of OA per ml of serum.

The reason for better recovery with the HPLC method was the shorter and simpler extraction procedure. Namely, for extraction we used only the first step of the procedures described by Hult and co-workers [5,14] and by Bauer and Garies [11]. OA was extracted from the serum with chloroform in acidic medium with the addition of $MgCl_2$. Further purification of the extract was not considered to be necessary, because the extract of a blank serum sample showed no interfering peaks (Fig. 1).

In our choice of chromatographic conditions we relied on the available literature data. The most frequently used mobile phases contain acetonitrile, methanol or 2-propanol in combination with water, acetic acid, H_3PO_4 or phosphate buffer. We compared the two most commonly used mobile phases: acetonitrile–water acetic acid (57:41:2, pH 3) [11] and methanol water–acetic acid (70:30:1,

TABLE I

COMPARISON OF OCHRATOXIN A RECOVERIES MEASURED IN THE SAME SERUM SAMPLE, BY HPLC AND ESF METHODS

n = 5 for each concentration range.

OA added to serum (ng/ml)	OA recovery				C.V. (%)	
	ng/ml		%		HPLC	ESF
	HPLC	ESF	HPLC	ESF		
5	4.8		95.4	–	2.54	–
10	9.2	6.2	92.0	62.5	7.01	7.47
20	18.0	13.7	90.1	68.7	7.23	3.81
50	43.4	31.2	86.8	62.5	5.96	6.00

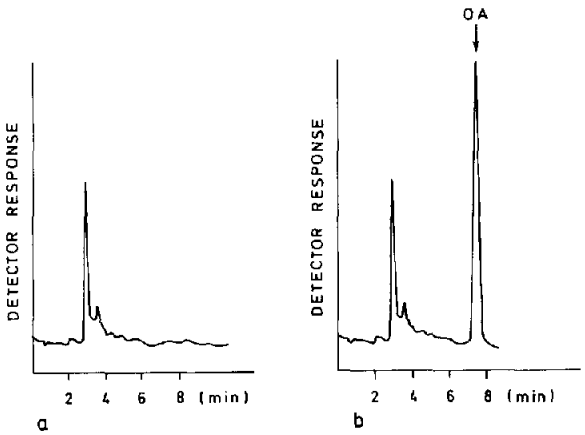

Fig. 1. HPLC separation of ochratoxin A on a μBondapak C_{18} column with methanol–water–acetic acid (70:30:1) as eluent and fluorescence detection: excitation at 336 nm and emission at 465 nm. (a) Blank serum sample; (b) serum sample with ochratoxin A (5 ng/ml).

pH 4) [10]. We found that there was no difference between them, either in the retention time or in the fluorescence spectrum of OA (Fig. 2). Our choice was methanol, which is more stable and cheaper than acetonitrile. Besides, at pH 4 it yielded as good a resolution as acetonitrile did at pH 3, and a higher pH is better for the reversed-phase column. The excitation and emission spectra showed that the respective wavelengths of 336 and 465 nm were suitable for the quantification of OA in this eluent (Fig. 3).

The modified HPLC procedure for OA determination in serum described here offers several advantages over the previously reported HPLC and ESF methods, with respect to sensitivity, recovery and analysis time.

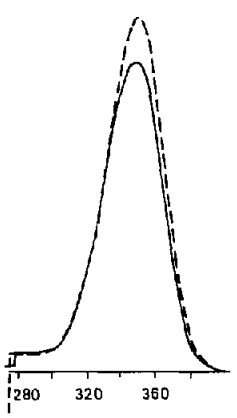

Fig. 2. Comparison of excitation spectra of ochratoxin A in two mobile phases: (——) acetonitrile–water–acetic acid (57:41:2) at pH 3; (---) methanol–water–acetic acid (70:30:1) at pH 4.

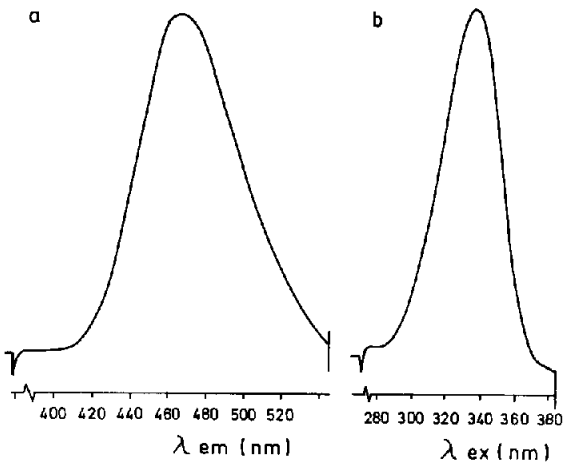

Fig. 3. Fluorescence spectra of ochratoxin A in methanol–water–acetic acid (70:30:1) as mobile phase: (a) emission spectrum with excitation at 336 nm; (b) excitation spectrum with emission at 465 nm.

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