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Rapid analysis of ergovaline in ovine plasma using high-performance liquid chromatography with fluorimetric detection

Philippe Jaussaud[a,*], Andrée Durix[a], Bernadette Videmann[a], Alain Vigié[b], Sylvie Bony[a]

[a]*INRA-ENVL Research Laboratory of Comparative Metabolism and Toxicology of Xenobiotics, National School of Veterinary Medicine of Lyon, 1 Avenue Bourgelat, B.P. 83, 69280 Marcy l'Etoile, France*

[b]*LIPHA, Department of Pharmacokinetics, 115 Avenue Lacassagne, 69003 Lyon, France*

Abstract

A rapid high-performance liquid chromatographic method for the determination of the mycotoxin ergovaline in ovine plasma is described here. Ergotamine was used as an internal standard. A simple extraction procedure with diethyloxide was carried out, before chromatography on a C_8 column, with the excitation and emission wavelengths fixed at 250 and 420 nm respectively, on a fluorimetric detector. The method, which was found to be linear between 3.5 and 15 ng/ml, had good specificity, precision and accuracy. The limit of quantification and the limit of detection were 3.5 and 1.2 ng/ml, respectively. A preliminary application of the described assay to a plasma kinetic study, after intravenous administration of a single dose of ergovaline (17 μg/kg body mass) to four sheep, showed a very rapid decrease of the plasma ergovaline levels. The terminal half-life and the total clearance of the mycotoxin were found to be 23.6 min and 0.020 l/min kg^{-1} body mass, respectively.

Keywords: Ergovaline; Mycotoxins; Toxins; Ergotamine

1. Introduction

The use, in grazing land, of tall fescue (*Festuca arundinacea*) infected with the endophytic fungus *Neotyphodium coenophialum* [1] provides agronomic benefits, which include tolerance to both abiotic stresses and pests, as well as a greater persistence from year to year [2,3]. However, together with these desirable properties, the endophyted plant is known to cause severe toxicosis in grazing animals, the symptoms of which are: 'summer syndrome' (stress, photosensitization, excessive salivation, rough hair coats, increased respiratory rates and increased rectal temperature), 'fescue foot' (vasoconstriction in the extremities), mesenteric fat necrosis, reduced reproductive performances, depressed immune response, lower milk production and various endocrine effects [2,4–7]. These disorders have been attributed to the presence in the endophyte-infected plant of two main classes of mycotoxins: loline alkaloids and ergopeptine alkaloids [8,9], these last compounds being the most likely candidates as causative agents of fescue toxicosis [2,6].

As ergovaline (Fig. 1) [10–12] accounts for 85–97% of the total ergopeptine alkaloids which have been identified in endophyted tall fescue [2,13,14], this mycotoxin was submitted to extensive analytical and biological investigations. Some assays, based on HPLC, have already been described for ergovaline determination in fescue forage and seeds [2,5,15–18]. However, to our knowledge, the only publication on the subject of application to bovine serum is a short note [19].

The method described by Savary et al. [19]

*Corresponding author.

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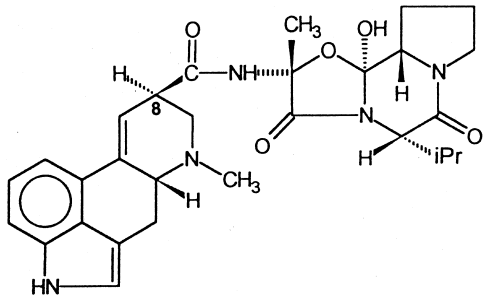

Fig. 1. Structure of ergovaline.

included several successive stages of extraction and purification, and additionally required the use of a gradient chromatographic elution. Moreover, the plasma kinetics of ergovaline in the ovine species, unlike that of other mycotoxins produced by *Neotyphodium* sp., such as paxillin [20], has not yet been studied. In the work presented here, we therefore intended to develop a simple assay, adapted to the ergovaline quantification in ovine plasma. The proposed analytical method was then applied to a plasma kinetic study, following an intravenous injection of a small dose of the pure toxin in sheep.

2. Experimental

2.1. Reagents

2.1.1. General reagents

Solvents used were analytical grade diethyloxide, methanol (Carlo Erba, RS-plus, Milan, Italy) and acetonitrile (far UV for HPLC gradient applications, Fisher Scientific, Loughborough, UK). Ammonium carbonate (Carlo Erba, RPE for analysis), and HPLC grade water prepared using a Milli-Q plus (Millipore, Molsheim, France) system, were used for the HPLC eluent preparation.

2.1.2. Standard preparations

Standard synthetic ergovaline (98% purity) was obtained as a pure crystallized tartaric salt, from Dr. F. Smith (Auburn, USA). An ergovaline primary standard solution (25 μg/ml calculated as free base) was made up in 20 ml methanol, then immediately divided into fifty 400-μl aliquots (each corresponding to 10 μg of ergovaline), which were transferred into 1-ml conical glass tubes. Each fraction was evaporated to dryness at 20°C under a nitrogen stream, before being stored in the dark at −20°C. Prior to each assay, the primary ergovaline solution was obtained by dissolving the ergovaline contained in each conical glass tube with methanol (in order to obtain a final concentration of 1 μg/ml).

Ergotamine (the 5′α-benzyl analogue of ergovaline), used as internal standard, was a commercial product of analytical grade (ergotamine tartrate, E 4768, Sigma, St. Louis, MO, USA). A methanolic solution of ergotamine (320 ng/ml calculated as free base) was prepared to spike plasma pools or samples before they were analysed.

Working standards, containing 750, 600, 450, 300 and 175 ng/ml of ergovaline, were all supplemented with the same amount of ergotamine (320 ng/ml). They were then adequately diluted in methanol, to constitute the methanolic calibration curve (150, 120, 90, 60, 35 ng/ml ergovaline, 64 ng/ml ergotamine).

For the preparation of the sample calibration curve, 20 μl of each working standard solution were added to 1 ml of plasma. The concentrations in plasma were then 15, 12, 9, 6, 3.5 ng/ml for ergovaline, and 6.4 ng/ml for ergotamine. As the extraction procedure resulted in a tenfold concentration, the final levels were 150, 120, 90, 60, 35 ng/ml ergovaline, and 64 ng/ml ergotamine. Three series of spiked samples were prepared, each on a different day.

2.2. Apparatus and chromatographic conditions

An HPLC system (Thermo Separation Product) was used, equipped with a P 2000 pump, a FL 2000 fluorimetric detector, and a Rheodyne injection valve fitted with a 20-μl loop. The control of the HPLC system and the integration of the chromatographic peaks were made using a computer system controller [PC 486 DX2, EGA graphic card, OS2 and PC 1000 (v. 2.5) software].

HPLC separations were performed under the following conditions: an isocratic elution was used, comprised of acetonitrile–2 m*M* ammonium carbonate in water (43:57, v/v) at a flow-rate of 1 ml/min. The HPLC analytical column (150×4.6 mm I.D.), packed with a Zorbax C_8 phase (3.5-μm particle size), was maintained at room temperature. The

fluorimetric detector was set with an excitation wavelength of 250 nm, and an emission wavelength of 420 nm. The total run time of the chromatogram was 15 min.

2.3. Sample extraction

A 1-ml plasma sample was placed in a 10-ml glass tube, then spiked with 20 μl of the standard ergotamine solution (to reach an ergotamine concentration of 6.4 ng/ml). The sample was then adjusted to pH 8 with 100 μl of a 5 m*M* sodium hydroxide solution, and 5 ml of diethyloxide were added. The tube was stoppered, shaken for 15 min on an orbital shaker, and cooled at −20°C for 1 h. The supernatant ether phase was dispensed into a glass vial, and evaporated under a nitrogen stream at 30°C, then the dry residue was redissolved into 100 μl of methanol. A 20-μl aliquot of the final extract was injected into the chromatographic system.

2.4. Validation procedure

2.4.1. Specificity and precision

Six blank plasma samples (1 ml), collected from six different adult sheep, were extracted and analyzed as described previously, in order to test the specificity of the analytical method.

The precision was determined with inter-day and intra-day assays, performed as follows: a pool of blank ovine plasma was prepared by mixing six 60-ml plasma samples, collected from six different adult sheep. A 30-ml aliquot of the pool was then spiked with 300 μl of a 600 ng/ml ergovaline standard solution, and with 300 μl of a 640 ng/ml ergotamine standard solution. Ergovaline and ergotamine concentrations in the pool were therefore 6 and 6.4 ng/ml, respectively. Six 1-ml aliquots were taken, each of them being extracted and chromatographed as described previously. This procedure was repeated three times, on three different days. The variances homogeneity was verified using the Cochran test, and the relative standard deviations were calculated for repeatability ($R.S.D._r$) and reproducibility ($R.S.D._R$) [21].

2.4.2. LOD and LOQ

The noise level was estimated on six 1-ml samples, obtained from the pool of blank ovine plasma, then extracted and chromatographed. The mean value plus three standard deviations was used to estimate the LOD. The LOQ, calculated as the mean noise plus ten standard deviations, was determined chromatographically by measuring the peaks obtained with standard methanolic ergovaline solutions of decreasing concentrations [21–23].

2.4.3. Linearity and accuracy

Five spiked samples (theoretical concentrations: 3.5, 6, 9, 12 and 15 ng/ml) were freshly prepared, by supplementing 1-ml aliquots of the pool of blank ovine plasma with the working standards. Three series of spiked samples were prepared (each one on a different day), then extracted and analyzed as described previously. They were used, as well as the three series of methanolic standard solutions of ergovaline (see Section 2.1.2), for plotting five point calibration curves. On these curves, the peaks–areas ratio of the analyte on the internal standard was represented as a function of ergovaline concentration.

For the study of both linearity and accuracy, the variances homogeneities were verified with the Cochran test. The linear regression equations of spiked samples and standards were compared, and it was verified that the intercepts were not statistically different from zero. Moreover, statistical tests proving existence of a significant slope, and validity of the regression line were performed [21,22].

Recovery of the ergovaline from the extraction procedure was determined by comparing the calibration curve of spiked samples, to that obtained with standards [21].

2.5. Ergovaline administration and plasma sampling

Four castrated male adult Texel sheep, with an average mass of 57 kg, received a dose of 17 μg/kg ergovaline into the right jugular vein. The drug was administered in the form of tartaric salt, in solution with 2 ml of a mixture of methanol–sterile water (1:1, v/v). Blood samples were collected from the left jugular vein into heparinised tubes, at 0, 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 45, 60, 90, 120 and 180 min after drug administration. The samples were

kept in ice and in the dark, then rapidly centrifuged at 4°C for 20 min at 900 *g*. For each sampling time, 1 ml of plasma was extracted and chromatographed as described previously. When the plasma ergovaline level was above the higher concentration of the linearity curve (15 ng/ml), the sample was diluted by a convenient volume of blank plasma, prior to extraction. Blood sampling and plasma analysis were performed on the same day.

2.6. Kinetic data analysis

A kinetic analysis of the concentration profiles, obtained by HPLC following intravenous administration, was performed using a programme for the residuals method. The number of exponents needed for each data set was determined by the application of the SIPHAR (v. 4.0) software [24]. The AUC, which is the total area under the plasma concentration time curve, was calculated by linear trapezoidal rule without extrapolation to infinity. Finally, the total clearance (Cl) was calculated using the formula:

Cl = D/AUC (D: dose administered to the animal)

3. Results and discussion

3.1. Validation of the analytical method

3.1.1. Specificity

Under the selected chromatographic conditions, the capacity factor of ergovaline was 1.6, and that of ergotamine 2.8 (Fig. 2a). The HPLC chromatograms of the six blank extracts (Fig. 2b), compared with that of standard ergovaline (Fig. 2a), exhibited no interfering peaks that could be misleading: the specificity of the tested HPLC assay was thus close to 100%.

The method appeared selective, since the chromatograms obtained with plasma samples, either spiked with ergovaline (Fig. 2c), or collected during the kinetic study (Fig. 2d), exhibited a peak whose capacity factor was 10, and which was due to ergovalinine. This last compound, produced by ergovaline epimerization at the asymmetric center in position 8 [25], has been already identified by HPLC

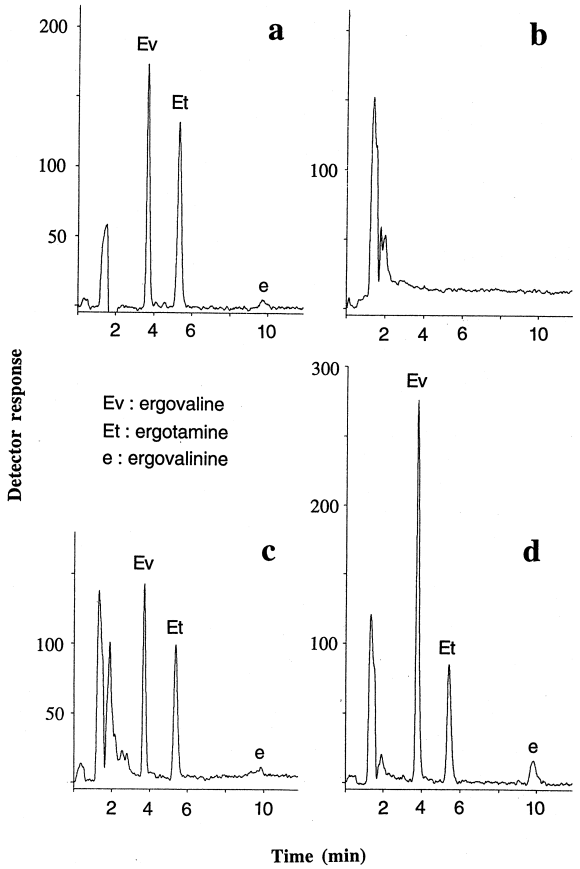

Fig. 2. HPLC chromatograms obtained with (a) a standard solution (60 ng/ml ergovaline, 64 ng/ml ergotamine) and with ovine plasma sample extracts: (b) ovine blank plasma, (c) ovine plasma spiked with 6 ng/ml ergovaline and 6.4 ng/ml ergotamine, and (d) plasma from a sheep which received intravenously a single dose of 17 μg/kg ergovaline (sample collected 10 min post-administration, ergotamine added at the level of 6.4 ng/ml prior to extraction).

in endophyte-infected tall fescue [5] or in bovine serum extracts [19].

3.1.2. LOD, LOQ, linearity and accuracy

The LOD and LOQ were found to be of 1.2 ng/ml and 3.5 ng/ml of plasma, respectively. The LOD was about six times higher than the value given in bovine serum by Savary et al. [19], but as the validation procedure were not fully detailed by these last authors, no valid comparison could be established between the two methods.

For linearity and accuracy, the variances homo-

geneity was found to be verified, using the Cochran test at the limit rate of 5% (tabulated value: 0.68, calculated values: 0.39 for spiked samples, and 0.58 for standards).

The data obtained with the three series of both spiked samples and standards are given in Table 1. They were shown to follow linear models between 3.5 and 15 ng/ml ($r^2 = 0.991$ for the two fitted curves) (Table 1). Moreover, the slopes and origins of the line of the spiked samples were not found to be statistically different from those obtained with standards.

The recovery rate values vary from 90–102.4%, with a mean value of 95.4% (Table 1).

3.1.3. Precision

The variances homogeneity was found to be verified, using the Cochran test at the limit rate of 5% (tabulated value: 0.74, calculated value: 0.64). Since the R.S.D.$_r$ and R.S.D.$_R$ values were found to be 5 and 9.5%, respectively, thus below 10%, the method appeared both repeatable and reproducible [22].

3.2. Application of the method to ergovaline kinetics

Under the described assay conditions, there was no interference with the analysis of ergovaline. The arithmetic plot of the mean plasma mycotoxin concentrations versus time, after intravenous administration, is shown in Fig. 3. A very rapid decrease of the ergovaline plasma levels occurs, the LOQ, being reached 1 h postdosing. This plasma profile is similar to those obtained after intravenous administration of ergotamine or ergosine in calves [26].

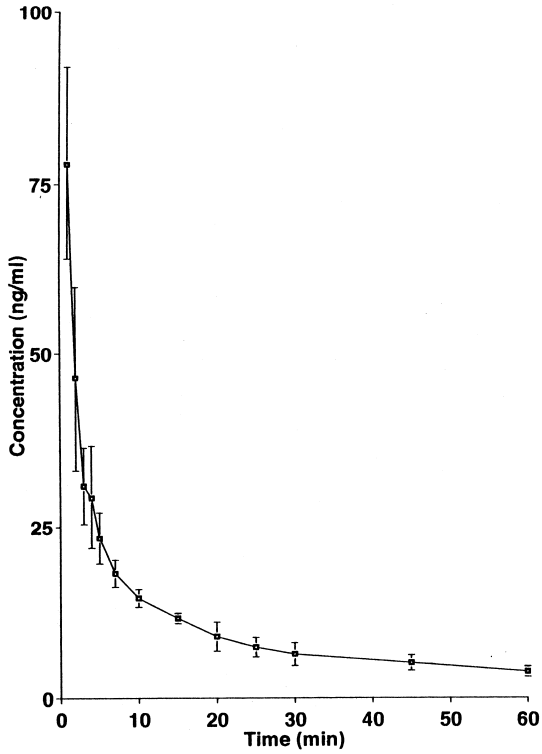

Fig. 3. Mean plasma ergovaline concentration–time profile in four sheep, after intravenous administration of a single dose of 17 μg/kg.

Each individual plasma profile could be fitted to the following biexponential function, characterizing a two-compartment model, and a two-phase kinetic process:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

where A and B are ordinar intercepts, and α and β are slope values. The individual values of different

Table 1
Recovery, accuracy and linearity derived from standard solutions and spiked plasma samples

Concentration of ergovaline in spiked samples (ng/ml)	Observed mean peak-area ratio	Standard mean peak-area ratio	Calculated concentration ergovaline (ng/ml) (percentage of theory)
3.5	0.58	0.60	3.6 (102.37)
6.0	0.92	0.95	5.7 (93.30)
9.0	1.39	1.47	8.7 (95.13)
12.0	1.75	1.91	10.8 (90.00)
15.0	2.35	2.42	14.5 (96.30)

Table 2
Pharmacokinetic parameters describing the disposal kinetics of ergovaline in plasma after an intravenous administration (17 μg/kg) in four sheep

Kinetic parameters (units)	Sheep 1	Sheep 2	Sheep 3	Sheep 4	Mean	S.D.
α (per min)	0.354	0.369	1.15	1.12	0.751	0.389
β (per min)	0.024	0.030	0.031	0.032	0.029	0.003
A (ng/ml)	63.23	107.22	131.01	190.02	122.87	45.76
B (ng/ml)	14.03	14.59	20.98	24.76	18.59	4.48
$t_{1/2}$ β (min)	28.43	22.47	21.98	21.52	23.60	2.81
Cl (l/min kg)	0.022	0.021	0.021	0.017	0.020	0.001
AUC (ng min/ml)	768.0	779.5	786.2	949.9	820.9	74.76
MRT (min)	31.99	21.12	27.21	25.62	26.48	3.88
V_{ss} (l/kg)	0.72	0.47	0.59	0.46	0.56	0.10

A, B = Pre-exponential constants; α, β = exponential constants for biexponential equation; $t_{1/2}$ β = half-life for terminal phase; Cl = body clearance; AUC = area under the plasma concentration curve; MRT = mean residence time calculated until the last sampling time; V_{ss} = volume at steady state.

kinetic parameters for each sheep are presented in Table 2. The plasma clearance and the elimination half-life of ergovaline during the second phase, were found to be 0.02 l/min kg^{-1} body mass and 23.6 min, respectively. These two parameters show a rapid metabolism and/or elimination of the toxin, after intravenous administration.

4. Conclusion

According to its characteristic parameters, the tested bioanalytical method can be considered as acceptable for dosing ergovaline in ovine plasma. Its main advantage is that it uses a very simple extraction procedure, without purification prior to chromatographic analysis. This simplicity makes it possible to perform the assay on a large number of samples, within a short time period.

The preliminary application of the method has provided results of biological importance, since kinetics data on ergovaline have never been obtained up to now. The rapid decrease of the compound in blood, after intravenous administration, is of particularly great interest. It indicates that low ergovaline plasma levels in animals should be expected, after ingestion of tall fescue endophyte-infected with *Neotyphodium coenophialum*.

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