Journal of Chromatography, 478 (1989) 231–237
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 632

Note

Chromatographic behaviour and determination of orellanine, a toxin from the mushroom *Cortinarius orellanus*

DANIELLE CANTIN*
Laboratoire de Chimie Analytique, U.F.R. de Pharmacie, Université J. Fourier de Grenoble, Domaine de La Merci, 38 700 La Tronche (France)
JEAN-MICHEL RICHARD
Laboratoire de Toxicologie et Ecotoxicologie, U.F.R. de Pharmacie, Université J. Fourier de Grenoble, B.P. 138, 38 243 Meylan Cedex (France)
and
JOSETTE ALARY
Laboratoire de Chimie Analytique, U.F.R. de Pharmacie, Université J. Fourier de Grenoble, Domaine de La Merci, 38 700 La Tronche (France)
(First received April 4th, 1989; revised manuscript received May 18th, 1989)

Several species of *Cortinarius* mushrooms have been reported to be highly toxic for animals and man[1]. We have shown that orellanine is the true principal toxin of *C. orellanus* and that it causes the same kind of nephrotoxicity as does the whole mushroom[2–4]. This compound was shown[5] to be an hydroxylated and amine oxidized bipyridine which has also been obtained by synthesis[6]. The purity of our samples of orellanine enabled us to confirm its structure by X-ray crystallography as (2,2′-bipyridine)-3,3′,4,4′-tetrol-1,1′-dioxide (see Fig. 1)[7]. The detection, separation and quantitation of orellanine are of great importance with regard to its toxicity. Thin-layer chromatographic methods have been previously reported for quantitation[8,9]. We think that they are not suitable for this purpose. More recently, Holmdahl *et al.*[10] succinctly suggested the use of a reversed-phase ion-pair high-performance liquid chromatograhic (HPLC) system at pH 4.5 with electrochemical detection. Taking into account our knowledge about the physico-chemical behaviour of orellanine, we were not convinced by such a procedure. The chromatographic behaviour of this bipyridine structure bearing six acido-basic functions is extremely complicated. A detailed study of this behaviour appeared necessary. This paper describes the optimization of two rapid and very sensitive HPLC methods for the separation and determination of orellanine, on the basis of its acido-basic properties. The procedure was applied to the quantitation of the toxin in the mushroom, using an UV detector.

EXPERIMENTAL

Apparatus

The liquid chromatograph used was a Shimadzu (Kyoto, Japan) Model LC-6A,

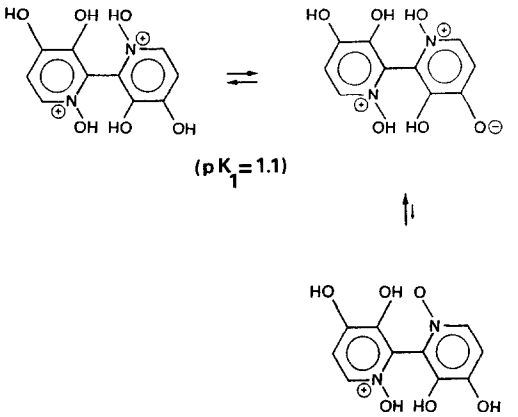

Fig. 1. Ionic forms of orellanine in equilibrium in aqueous solutions below pH 3.

equipped with an UV spectrophotometric detector SPD-6A and a Rheodyne (Cotati, CA, U.S.A.) Model 7 010 injection valve with a 20-μl loop. Retention times and peak areas were measured by a CR-3A Chromatopac (Shimadzu).

Chemicals and reagents

All reagents, solvents and acids used were of analytical reagent grade (SDS, Peypin and Prolabo, Paris, France). Water was deionized and twice distilled in a quartz glass still. Ion-pairing agents (1-hexane- and 1-octanesulphonic acids) were of HPLC reagent grade (Kodak, Rochester, NY, U.S.A.).

Extraction of orellanine

The toxin was extracted from dry powdered carpophores of *C. orellanus* collected locally. Extraction was carried out either at room temperature (method 1) or with a Soxhlet apparatus (method 2). In method 1, fatty material and apolar pigments were removed by successive extractiosns with hexane, chloroform and acetone. Orellanine was extracted with methanol. In method 2, orellanine was extracted according to ref. 11, but that methanolic extraction was carried out with a Soxhlet apparatus for 2 h and repeated ten times.

Purification of orellanine

The toxin was precipitated after standing 48 h at 4°C from an aqueous solution of the dried extract (0.1 g/ml, adjusted pH to 4.5–5). Crude crystals were collected by centrifugation at 2000 *g* for 10 min). After removal of a colloidal layer by aspiration, the crystals were rinsed twice with cooled water. Then, orellanine was suspended in water (0.1 g/ml) and the pH adjusted to 8.5–9 with concentrated ammonia. After centrifugation at 3500 *g* for 10 min to remove insoluble material, the pH was adjusted to 4.5–5 with 3.5 *M* acetic acid. The solution was left to crystallize at 4°C for 48 h. Orellanine was collected by centrifugation. The above recrystallization process was repeated twice. The colourless crystals obtained were rinsed successively with cooled ethanol and cooled diethyl ether and were collected by centrifugation at 3500 *g* for 5 min.

Sample handling

Standard solutions of orellanine. These were prepared by dissolving a known weight in phosphoric acid pH 0, to give a concentration of about $5 \cdot 10^{-4}$ *M* (126 mg/l) checked by UV spectrophotometry after 1/10 dilution (ε = 10 900 l mol^{-1} cm^{-1} at 262 nm and 9100 l mol^{-1} cm^{-1} at 288 nm at pH 1). Stock solutions of this photosensitive and easily oxidable product were stored in the dark at 4°C and used in the following 2 days. They were diluted in a suitable mobile phase, to give a concentration range of 10^{-6}–10^{-4} *M*.

Solutions of mushroom extracts. Dried methanolic extracts (0.5–1.5 mg/ml) were dissolved in phosphoric acid pH 0. Direct assay solutions were made by 1/10 dilution in a suitable mobile phase (pH 1). For the standard addition method, they were added with an equal volume of a solution of orellanine ($5 \cdot 10^{-5}$–$10 \cdot 10^{-5}$ *M*), in a suitable mobile phase at pH 1.

For all assays, 20 µl were injected into the chromatograph.

Chromatographic conditions

Thin-layer chromatography. TLC and HPTLC plates were obtained from Merck (Darmstadt, F.R.G.). Orellanine is easily detected by its absorption at 254 nm or by the blue fluorescence of its spot after 2 min UV irradiation at 366 nm.

High-performance liquid chromatography. Two chromatographic systems were used. The first stationary phase was Rosil CN 5 µm prepacked in a 150 mm × 4.6 mm stainless-steel cartridge from Alltech (Paris, France). The mobile phase consisted of phosphoric acid adjusted to pH 1.0 ± 0.1. Elution was at a flow-rate of 0.5 ml/min. Chromatographic peaks were monitored at 260 or 290 nm, the wavelengths of the UV maxima for orellanine at pH 1.0. The second stationary phase was µBondapak C_{18} 5 µm (150 mm × 3.9 mm) from Waters (Northwich, U.K.). The mobile phase was phosphoric acid adjusted to pH 1.0 ± 0.1–acetonitrile (94:6, v/v) and 1-octanesulphonic acid $2.5 \cdot 10^{-3}$ *M*. It was pumped at a flow-rate of 0.8 ml/min unless stated otherwise. The ion pair was detected at 290 nm.

The solutions were filtered through a 0.5-µm Millipore filter and degassed. All analyses were carried out isocratically at room temperature.

RESULTS AND DISCUSSION

Considerable attention has to be paid to sample preparation. Orellanine has poor solvent solubility and stability. It is better to dissolve the toxin in water. Insoluble between pH 1 and 8.4, it is largely soluble at pH values above 8.4 and below 1. Nevertheless, we noted the low stability of orellanine at alkaline pH. To overcome these solubility and stability problems, we propose a number of precautions. Orellanine should be dissolved at pH 0 and diluted at pH 1. Occasionally, additional spots or peaks were noticed when orellanine solutions were exposed to daylight. To avoid any photolytic decomposition, we recommended that the chromatography be carried out in a fume cupboard.

The choice of a thin chromatographic layer was very difficult. Prast *et al.*[8] recommended a silica gel layer with a mixed polar solvent added with organic acid. We have found that orellanine does not undergo any migration on such a layer. We have investigated many other layers (silica, aluminium oxide, cellulose, silica bonded with

diol, amino, cyano, methylsilane and octylsilane functionalities) and eluents. Most systems tested do not give successful separations. On the generally used polar layers, orellanine does not migrate at all, due to its very high polarity. On the non-polar ones, the toxin migrates with the solvent front or gives a diffuse streak. A cellulose layer allows a separation. Methanolic eluents give hardly reproducible results with diffuse spots. Furthermore, orellanine is of low stability in methanol. Butanolic eluents, added with acetic acid, give successful separations with the water–butanol ratio ranging from 1:3 to saturation. The R_F values obtained with butanol–acetic acid–water (3:1:1 to 4:1:5) eluents are between 0.7 and 0.3 respectively. To transpose the chromatographic system to HPLC, we have tried Si-CN HPTLC plates, the polarity of which is known to be medium. Non-polar solvents give no migration at all while highly polar ones give no compact spots but large diffuse streaks starting from the application point. To obtain suitable spots, the pH has to be lowered to 1–2. Addition of acetonitrile or dioxane reduces the R_F value, for instance, $R_F = 0.5$ with dioxane–phosphoric acid pH 2 (1:1). Decreasing the pH value increases the R_F value.

The pH was shown to be the key to resolving the chromatographic determination. A study of the ionic species of orellanine and the corresponding different pK values was undertaken using electrochemical[12] and spectrometric methods[13]. The first pK value of orellanine is 1.1. At this pH value, contrary to what is observed at pH > 3, only a few ionic forms of the toxin are present in solution (Fig. 1). At lower pH values the fully protonated form is predominant. This bicationic species is hydrophilic though it is not so polar as the other ones present at higher pH. Thus the lowest pH value usable is required to chromatograph that molecule.

For the determination of orellanine, we propose two reversed-phase HPLC systems using columns with different polarities (Si-CN and Si-C_{18}). These bonded phases are known to be stripped by strong acids (pH below 2). Nevertheless, they are usable up to pH 1 if they are rinsed every night with twice distilled water. With these precautions, we used these columns for more than 6 months without any change in the number of theoretical plates. The precision of the HPLC methods employing an UV detector was investigated by calculating the coefficient of variation of the peak area following ten injections of a known quantity onto the column. The chromatographic characteristics are given in Table I.

First system with Si-CN bonded phase

This system allows a rapid, acute, sensitive and economical quantitation of the toxin. With this medium-polar phase, the recommended eluent is phosphoric acid at pH 1. Under these severe conditions, orellanine is swiftly eluted as a very sharp, symmetrical and reproducible peak (retention time, $t_R = 4.4$ min). Addition of a medium-polar solvent (dioxane) from 5 to 50% only slightly reduces the retention time with this reversed-phase system (from 4 to 3.5 min). Increasing the pH results in a lengthening of the retention time ($t_R = 10$ min at pH 2) with a consequent effect on the peak width. The asymmetry of the trailing peaks obtained probably indicates the presence of several ionic forms of orellanine. At pH 1, the peak areas are highly stable during a day and reproducible from day to day. The detector response for orellanine was linear within a wide concentration range with an excellent correlation coefficient, r, and a low detection limit.

TABLE I

HPLC OF ORELLANINE

Systems: 1, Si-CN, eluent H_3PO_4, pH 1, 0.5 ml/min; 2, Si-C_{18}, eluent H_3PO_4, pH 1–CH_3CN (94:6, v/v), $2.5 \cdot 10^{-3}$ *M* 1-octanesulphonic acid, 0.8 ml/min. Other chromatographic conditions as described in Experimental. Detection limit based on a signal-to-noise ratio of 3.

System	*Retention time, t_R (min) ± C.V. (%)*	*Quantitation linearity[a] (calibration range: 5–500 ng)*		*Repeatability, C.V. (%)*	*Reproducibility, C.V. (%)*	*Detection limit (pg)*
		$y = ax + b$	r			
1	4.43 ± 0.09	$y = 2120\,x + 1720$	0.9994	0.61	1.70	40
2	6.58 ± 1.0	$y = 2260\,x - 7080$	0.9994	0.90	0.55	50

[a] y = Peak area; x = amount injected (ng); a = integrator response factor; b = intercept with y axis; r = regression correlation coefficient; C.V. = coefficient of variation.

Second system with Si-C_{18} bonded phase

The same eluent (phosphoric acid, pH 1) permits the separation of orellanine with a substantially longer retention time (8 min at a flow-rate of 0.5 ml/min). Addition of small amounts of medium-polar solvents strongly reduces the retention time (3.1 min with 2% dioxane).

In order to obtain a still longer retention time, one must use paired-ion chromatography which allows strongly ionic compounds, poorly retained in reversed-phase HPLC systems, to be separated. The pH should be adjusted so that the sample is present in its bicationic form and gives an ion-pair complex with a large and strongly ionic organic counter ion added in the mobile phase. This will make the ionized sample behave as a non-ionic species with some non-polar (lipophilic) characteristics. The resulting species can easily be chromatographed by a reversed-phase system.

Holmdahl *et al.*[10] proposed the use of the following mobile phase: 0.05 *M* citrate–phosphate buffer, pH 4.5–15.4% methanol and $5 \cdot 10^{-3}$ *M* 1-hexanesulphonic acid. It should be noted that citrate buffers are known to dissolve the silica in the column because of their chelating properties and again that orellanine is of low stability in methanol. We found that orellanine is not retained with that eluent at pH 4.5. At this pH value, more than seven polar species, non-ionic or bearing positive and/or negative charges, are present in solution[13]. Amphoteric compounds, such as orellanine, are often difficult to chromatograph. Classically, they can readily be chromatographed using either cationic or anionic reagents. The counter ion in the reagent should ion-pair with one functionality of the amphoteric compound while the other one should remain in its electrically neutral form due to the pH of the reagent. That is not the case with orellanine at pH 4.5: non-neutral ion-pair complexes and non-ionic forms of orellanine are present in solution and all behave as non-complexed polar oralline.

Positively charged ion-pairing agents are of no practical interest owing to the low stability of orellanine and of the silica base at the alkaline pH required for their utilization. To have in solution the bicationic form (Fig. 1), able to form a neutral ion pair with negatively charged ion-pairing agents, one must use the lowest pH value possible. We propose phosphoric acid, pH 1. Addition of medium-polar water-

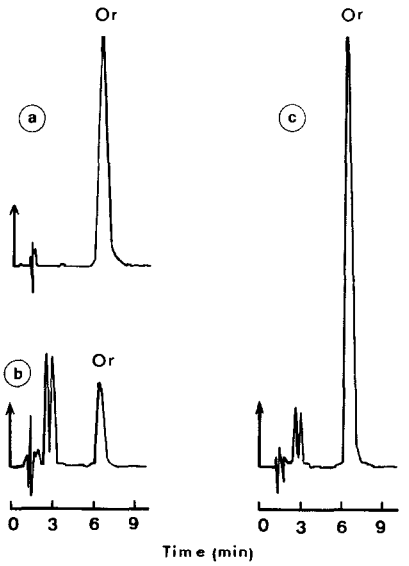

Fig. 2. Typical chromatograms of (a) standard solutions of orellanine (0.25 μg injected), (b) solutions of dried mushroom extract (1.2 μg injected), (c) dried mushroom extract (0.6 μg) spiked with standard orellanine (0.125 μg). Column: Si-C_{18}. Eluent: H_3PO_4 pH 1–CH_3CN (94:6, v/v), $2.5 \cdot 10^{-3}$ *M* 1-octanesulphonic acid; 0.8 ml/min. Other chromatographic conditions as described in Experimental. Or = Orellanine.

miscible solvents in the eluent and variation in the relative size of the lipophilic portion of the counter ion will affect the degree of retention obtained as it is well known for reversed-phase systems. For the evaluation of the method, we used a mobile phase of phosphoric acid pH 1–6% acetonitrile and $2.5 \cdot 10^{-3}$ *M* 1-octanesulphonic acid. The ion pair has maxima at 260 and 290 nm. The latter was chosen as the optimum wavelength permitting both high sensitivity and high selectivity. With a flow-rate of 0.5 ml/min, orellanine is eluted late with a retention time of 11.4 min which greatly lengthens the analysis time. However, certain applications may require the use of this method. With a flow-rate of 0.8 ml/min, calibration graphs were linear over a wide range of standards. Stable and reproducible peak areas and an excellent linearity are obtained, thus allowing the quantitation of the toxin (Fig. 2). With the low detection limit, one can avoid the use of the delicate electrochemical detection which gave Holmdahl *et al.*[10] a ten times higher detection limit (at +900 mV *vs.* Ag/AgCl). Note that, at such an oxidation potential, not only orellanine can be detected, but also every 3,4-dihydroxylated pyridinic compound. Among these products, orellinine and orelline, respectively the monoamine oxidized and the non-amine oxidized compounds, are particularly likely to be present with orellanine as contamination or degradation products.

Quantitation of orellanine in mushroom extracts

The Si-CN chromatographic system does not allow the separation of orellanine from some other constituents of the mushroom extracts. Hence, it cannot be used for the determination of their orellanine contents. We used the Si-C_{18} chromatographic

system to quantitate the toxin in several dried mushroom extracts stored for 1 year (Fig. 2). The same content of 4.0 ± 0.2% (w/w) was assessed either by the direct method or by the standard addition method in these extracts, corresponding to a content of 1.2% (w/w) in the dried powder of *Cortinarius orellanus*. The accuracy of the determination is 1.8%.

CONCLUSION

Two efficient methods of separation and quantitation are now at our disposal to assess the content of orellanine. With the medium-polar or apolar phases chosen, orellanine, or its ion-pair complex respectively, is eluted either rapidly or with retention times which can be lengthened for the purpose in hand. This allows the separation of orellanine from molecules with different polarities in different media with a low detection limit (40 and 50 pg on column).

ACKNOWLEDGEMENT

This work was supported by a grant from the Fondation pour la Recherche Médicale.

REFERENCES

1 T. Schumacher and K. Høiland, *Arch. Toxicol.*, 53 (1983) 87.
2 J.-M. Richard, J. Louis and D. Cantin, *Arch. Toxicol.*, 62 (1988) 242.
3 G. Klein, J.-M. Richard and M. Satre, *Microbiol. Lett., Fed. Eur. Microbiol. Soc.*, 33 (1986) 19.
4 J.-M. Richard, P. Ravanel and D. Cantin, *Toxicon*, 25 (1987) 350.
5 W. Z. Antkowiak and W. P. Gessner, *Tetrahedron Lett.*, 21 (1979) 1931.
6 M. Tiecco,, M. Tingoli, L. Testaferri, D. Chianelli and E. Wenkert, *Tetrahedron*, 42 (1986) 1475.
7 C. Cohen-Addad, J.-M. Richard and J. C. Guitel, *Acta Crystallogr., Sect. C.*, 43 (1987) 504.
8 H. Prast, E. R. Werner, W. Pfaller and M. Moser, *Arch. Toxicol.*, 62 (1988) 81.
9 C. Andary, S. Rapior, A. Fruchier and G. Privat, *Cryptogamie, Mycol.*, 7 (1986) 189.
10 J. Holmdahl, J. Ahlmen, S. Bergek, S. Lundberg and S.-A. Persson, *Toxicon*, 25 (1987) 195.
11 W. Z. Antkowiak and W. P. Gessner, *Bull. Acad. Pol. Sci., Ser. Sci. Chim.*, 23 (1975) 729.
12 D. Cantin, J.-M. Richard, D. Serve and J. Alary, *Electrochim. Acta*, 33 (1988) 1047.
13 J.-M. Richard, D. Cantin and J.-L. Benoit-Guyod, in preparation.