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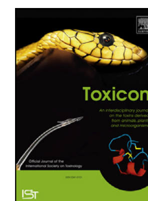

Short communication

Quantification of the environmental neurotoxin annonacin in Rat brain by UPLC-MS/MS

Natacha Bonneau [a], Isabelle Schmitz-Afonso [b, 1], Alain Brunelle [b], David Touboul [b], Pierre Champy [a, *]

[a] Laboratoire de Pharmacognosie, BioCIS, Univ. Paris-Sud, CNRS, Université Paris-Saclay, UFR Pharmacie, 5 rue J.-B. Clément, 92290, Châtenay-Malabry, France

[b] Institut de Chimie des Substances Naturelles, CNRS UPR2301, Université Paris-Saclay, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

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ABSTRACT

The Annonaceous acetogenin annonacin is an environmental neurotoxin identified in the pulp of several fruits of the Annonaceae family, whose consumption was linked to the occurrence of sporadic atypical Parkinsonism with dementia. A method for its quantification in Rat brain homogenates by UPLC-MS/MS in selected reaction monitoring (SRM) mode was developed and validated. This method was applied to the quantitation of annonacin in Rat brain after intravenous (0.5 mg/kg) and oral (10 mg/kg, 100 mg/kg) administration.

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Annonaceous acetogenins (AAGs) are lipophilic polyketides distributed in the Annonaceae tropical plant family. Numerous AAGs have been found in several parts of different species (Bermejo et al., 2005), including worldwide consumed fruits like soursop (*Annona muricata* L.), or leaves used as herbal teas in traditional medicine (Champy et al., 2005). AAGs are potent inhibitors of mitochondrial complex I (NADH ubiquinone oxido–reductase), with IC_{50} ranging from 10 μM to 10^{-4} nM – annonacin, the main AAG in *A. muricata*, reaching about 30 nM in bovine sub-mitochondrial particles (Champy, 2011; Degli Esposti, 1998). These compounds are proposed as pesticidal and antitumoral candidates, among other activities (McLaughlin, 2008) implying non-specific cytotoxicity (Bermejo et al., 2005). Moreover, seeds of species known to contain high amounts of AAGs, like *Annona squamosa* L., have acute toxicity after ingestion and are only used externally in traditional medicine (Champy, 2011). Case-control studies onto unusually high prevalence of atypical parkinsonism in the French West Indies underlined a connexion with dietary and medicinal consumption of soursop and of other Annonaceae (Lannuzel et al., 2007). The disease, named “Guadeloupean parkinsonism”, is a combination of parkinsonian symptoms resistant to dopa-therapy, associated with other motor signs and with frontotemporal dementia. It was thoroughly characterized clinically (Lannuzel et al., 2007). Interestingly, neuronal Tau fibrils accumulation was evidenced at the autopsy of three patients (Caparros-Lefèbvre et al., 2002). Related cases associated with Annonaceae were also reported in New-Caledonia (Angibaud et al., 2004). A remarkably similar Parkinson–Dementia Complex was described in the island of Guam, in which the implication of Annonaceae was also proposed (Caparros-Lefèbvre and Steele, 2005). AAGs share the mechanism of action of rotenone, a formerly widely used natural pesticide, which spreading has been

Abbreviations: AAG, Annonaceous acetogenin; amu, atomic mass unit; CI, confidence interval; EMA, European Medicines Agency; IS, internal standard; i.v., intravenous; LLOQ, lower limit of quantification; LQC, Lower concentration Quality Control; MPTP, 1-methyl-4-phenyl-pyridine; MQC, medium concentration quality control; QC, quality control; RSD, relative standard deviation; s.c., subcutaneous; SRM, selected reaction monitoring; ULOQ, upper limit of quantification; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry.

* Corresponding author.
E-mail address: pierre.champy@u-psud.fr (P. Champy).
[1] Present address: Normandie Univ., COBRA, UMR 6014 and FR 3038; Université; de Rouen; INSA Rouen; CNRS, IRCOF, 1 Rue Tesniere, 76821 Mont-Saint-Aignan Cedex, France.

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forbidden in the European Union since 2008, due to neurotoxicity concern. This molecule is used to elaborate animal models of parkinsonism (Johnson and Bobrovskaya, 2015), like MPTP, another well-known complex I inhibitor. Nevertheless, a complex I dysfunction was observed in idiopathic and atypical parkinsonian patients, bringing consistency to the hypothesis that AAGs could be involved in such neurodegenerative disorders (Schapira, 2010). The hypothetic role of annonacin and of other AAGs in the induction of neurodegeneration and of tauopathies was comforted by *in vitro* (Höllerhage et al., 2009) and *in vivo* experiments. In the Rat, annonacin induced neurodegeneration and neuroinflammation, mostly in the basal ganglia, after subchronic continuous i.v. administration (3.8 and 7.6 mg/kg/day, 28 days) despite any sign of systemic toxicity (Champy et al., 2004). A short term treatment by continuous s.c. administration of annonacin at 6 and 9 mg/kg/day during 3 days increased Tau protein accumulation and phosphorylation in Human tau-transfected mice, evidencing synergistic effects between annonacin and the R406W-tau mutation (Yamada et al., 2014). Similar observations were made in wild type and Human tau-transfected mice fed with *A. muricata* juice containing AAGs, over a 1 year-period (Rottscholl et al., 2011, 2015). Recent methodological developments allowed the detection of AAGs in several Annonaceae derived food products (Le Ven et al., 2012, 2014). Annonacin, the most abundant acetogenin in soursop, was also evidenced in high amounts in the paw paw fruit (*Asimina triloba (L.)* Dunal.) which is grown and consumed in North America (Levine et al., 2015; Potts et al., 2012). The dietary human exposure to AAGs was determined as overcoming the mg level for consumption of a single food product (Champy et al., 2005; Levine et al., 2015; Potts et al., 2012). Nevertheless, dietary supplements aiming at cancer prevention containing Annonaceae are gaining popularity in western countries. Dealing with a worldwide-spread alimentary exposure could give another tone to what was initially thought as a local problem. The French Food Safety Agency expressed its concern towards these issues, prompting at pharmacokinetic investigations (AFSSA, 2010). Regarding the aforementioned neurotoxicity of annonacin, getting an insight of its body distribution, especially in the brain after oral administration, appears then to be relevant. The already published methods for the quantification of AAGs in Rat plasma use single ion monitoring HPLC-ESI-MS for the AAG bullatacin (rolliniastatin-2) (Chen et al., 2012), or selected reaction monitoring for annonacin (Bonneau et al., 2015a). Our latest study put in light a very weak oral bioavailability for annonacin (Bonneau et al., 2015a). We have adapted and validated our UPLC-MS/MS quantitative method to brain homogenate as a different matrix.

Annonacin and annonacinone (internal standard, IS) (purity > 97%) were purified from the seeds of *A. muricata* L., as previously described (Bermejo et al., 2005). Thirty male Wistar rats (300 ± 20 g) were supplied by Charles River (St Quentin-Fallavier, France). Animals were treated according to European Community Council Directive 86/609/EEC. They were fasted with free access to water for 12 h before the experiment. Annonacin was administered by intravenous (i.v.; 0.5 mg/kg, n = 17) or oral route (10 mg/kg, n = 8 or 100 mg/kg, n = 5). For i.v. administration, rats were preimplanted with a catheter in the jugular vein. 300 μL of an annonacin solution prepared in DMSO-ethanol-water (5:5:90, v/v/v) were injected. For oral route, 1 mL of an annonacin solution in DMSO-olive oil (European Pharmacopoeia grade) (5:95, v/v) was delivered by intragastric administration. Eight other rats were used as controls. After administration, animals had free access to food and water. Rats were anesthetized with 56 mg/kg sodium pentobarbital i.p. and sacrificed by transcardial perfusion, 24 or 48 h later. Brains were frozen in dry ice and stored at −20 °C until analysis. Each Rat brain was individually sonicated in methanol (0.2 g/mL), which was selected as the most suitable solvent for quantification in this matrix using LC/MS. An aliquot of each homogenate (1 mL) was transferred in a polypropylene microtube and spiked with 10 μL of IS, then centrifuged at 10,000 rpm for 20 min, and the upper phase was transferred into a 5 mL glass tube for evaporation under reduced pressure at 40 °C with a SpeedVacuum. The residue was reconstituted in 100 μL of acetonitrile by 15 s vortex-mixing, and transferred in a polypropylene microtube for a 5 min centrifugation step at 10,000 rpm. The solution was then transferred in a glass vial, and 5 μL were injected in triplicate for UPLC-MS/MS analysis. Analyses were performed with a Dionex Ultimate 3000 RSLC system equipped with Acquity UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm, Waters, Guyancourt, France) and precolumn (2.1 × 5 mm, 1.7 μm), with an isocratic mobile phase consisting of water–acetonitrile 35:65 during 1 min, then a water–acetonitrile gradient from 35:65 to 15:85 in 5 min, at a flow rate of 0.5 mL/min, at 40 °C. A 2 min isopropanol-acetonitrile 50:50 washout was applied after each run. Mass spectrometry analyses were performed under Selected Reaction Monitoring (SRM) mode using the transitions *m/z* 619.4 → *m/z* 507.4 for annonacin and *m/z* 617.4 → *m/z* 505.4 for IS (Fig. 1A), with a Triple quadrupole TSQ Vantage EMR (Thermo Scientific, Les Ulis, France), equipped with a heated ESI source in the positive ion mode (conditions: see Bonneau et al., 2015b). The method was evaluated for selectivity, carry-over, linearity, accuracy, precision, matrix effect, extraction recovery, and stability, and then validated following the EMA guideline on bioanalytical method validation (EMA, 2011). Full validation of the method is presented in supporting publication (Bonneau et al., 2015b).

A satisfactory linearity ($R^2 > 0.990$) was obtained for the calibration range with weighted (1/x) least square linear regression, *i.e.* from 0.25 to 25 ng/g using 10 calibration concentrations. The typical model equation was $y = 0.201x - 9.836 \times 10^{-3}$ ($R^2 = 0.999$), where y is the peak area ratio of annonacin to IS and x is the concentration of annonacin in the brain (ng/g). Back calculated concentrations of the calibration standards were within ±15% of the nominal value and ±20% for the lower limit of quantification (LLOQ) of annonacin, which was defined as 0.25 ng/g. Quality control (QC) samples at 4 different concentrations, including those corresponding to the LLOQ and the upper LOQ (ULOQ), were prepared at 2 different days, with 3 replicates a day, independently from the calibration standards. Within- and between-run precisions were less than ±15%. Moreover, within- and between-run accuracies were less than ±15% of the nominal concentration (see Table 1 in Bonneau et al., 2015b). A stability study, performed at LLOQ, medium QC (MQC) and ULOQ, showed no loss of annonacin during sample storage and processing (see Table 4 in Bonneau et al., 2015b).

The method was applied to the determination of the ratio of annonacin reaching the Rat brain, after intravenous single dose administration of 0.5 mg/kg, and at 24 h and 48 h, this dose being well-tolerated. A comparison with the oral single high dose of 100 mg/kg after 24 h was performed. The detection being readily possible under these conditions, further experiments were conducted at a lower oral dose (10 mg/kg) after 48 h. The typical chromatograms are shown in Fig. 1B. These doses, in the mg range, are reminiscent of those used in rodent neurotoxicity models (Champy et al., 2004; Rottscholl et al., 2015; Yamada et al., 2014). No sign of acute toxicity was observed. Sacrifice of the animals was performed by transcardial perfusion to ensure blood removal from the brain parenchyma, so as to avoid non-specific detection. The mean concentrations of annonacin in brain, together with the percentages of the administered doses detected in brain are shown in Fig. 2. A very weak proportion of the administered dose was found for each time and route. Only 12.5×10^{-4}% and 8.4×10^{-4}% of

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