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Determination of a potent non-competitive AMPA receptor antagonist in rat brain

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Abstract

N-acetyl-1-(*p*-chlorophenyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline derivative (PS3Ac) has been determined in brain tissues by high performance liquid chromatography (HPLC) coupled with a diode array detection. In a previous paper we presented a validation method for detecting PS3Ac and its metabolites in plasma samples after intraperitoneal administration to Wistar rats. In the present paper, we report the results of the determination of PS3Ac and its *N*-deacetyl (PS3) and *O*-demethyl (PS3OH) metabolites, in the brain after extraction based on a polymeric matrix with a high hydrophilic–lipophilic balance, using Oasis cartridges. The chromatographic separation was performed in an octadecylsilica stationary phase at 25 °C using a mixture of 10 mM potassium dihydrogen orthophosphate (pH 2.24) and acetonitrile in ratio of 30:70 (v/v) as mobile phase, with a flow rate of 0.8 ml/min. The method exhibited a large linear range from 0.05 to 2 μg/ml for all studied compounds ($n = 6$). In the within-day assay ($n = 4$), the accuracy ranged from 87.5% determined with 0.05 μg/ml of PS3 to 110.1% determined with 0.2 μg/ml of PS3OH. In the between-day assay the coefficient of variation ranged from 2.4 determined with 0.05 μg/ml of PS3 to 9.7 determined with 0.2 μg/ml of PS3OH. The extraction efficiency ranged from 77.8% for PS3OH at 0.2 μg/ml to 94.3 for PS3Ac at 0.5 μg/ml. The limit of detection for all the tetrahydroisoquinoline derivatives ranged around 50 ng/ml. The method proved to be highly sensitive and specific to determinate PS3Ac and its metabolites and has been successfully applied to value their concentrations in brain matrix over the time.
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Keywords: AMPA receptor antagonist; SPE-HPLC; Tetrahydroisoquinoline; Brain

1. Introduction

Selective non-NMDA (*N*-methyl-D-aspartic acid) receptor antagonists, *i.e.*, 2-amino-3(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) and kainate have been proposed as a useful treatment of various disorders such as epilepsy, ischaemia, and Parkinson's disease [1,2]. Indeed, different neurological disorders have been linked to excessive activation of excitatory amino acid (EAA) receptors.

The 2,3-benzodiazepines (2,3-BZs) represent an important class of selective AMPA/kainate receptor antagonists [3,4].

GYKI 52466 (1-(4′-aminophenyl)-4-methyl-7,8-methylenedioxy-2,3-benzodiazepine), at first, was pharmacologically differentiated from classical 1,4 and 1,5-benzodiazepines for its muscle relaxant and anticonvulsant properties, acting as a highly selective non-competitive antagonist at AMPA/kainate receptor site and showing no affinity for the benzodiazepine receptors (BZRs) at GABA (γ-aminobutyric acid) complex [5,6].

Successively other 2,3-BZs, chemically similar to GYKI 52466 (Fig. 1), were synthesised in our laboratories and proved to possess anticonvulsant activity in various experimental models of seizures [7,8]. It has been demonstrated that these 2,3-benzodiazepine derivatives are non-competitive antagonists at the AMPA/kainate receptor and do not affect NMDA and GABA receptor-mediated responses [8,9].

Particularly, we studied an extensive series of 7,8-dimethoxy-2,3-benzodiazepines, such as [1-(4-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiazepin-4-one] (CFM-2, Fig. 1), demonstrating a marked anticonvulsant activity higher than that of GYKI 52466 [10] and comparable to that of a 3-*N*-acetyl-1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-4,5-dihydro-2,3-benzodiazepine, *i.e.*, Talampanel (Fig. 1),

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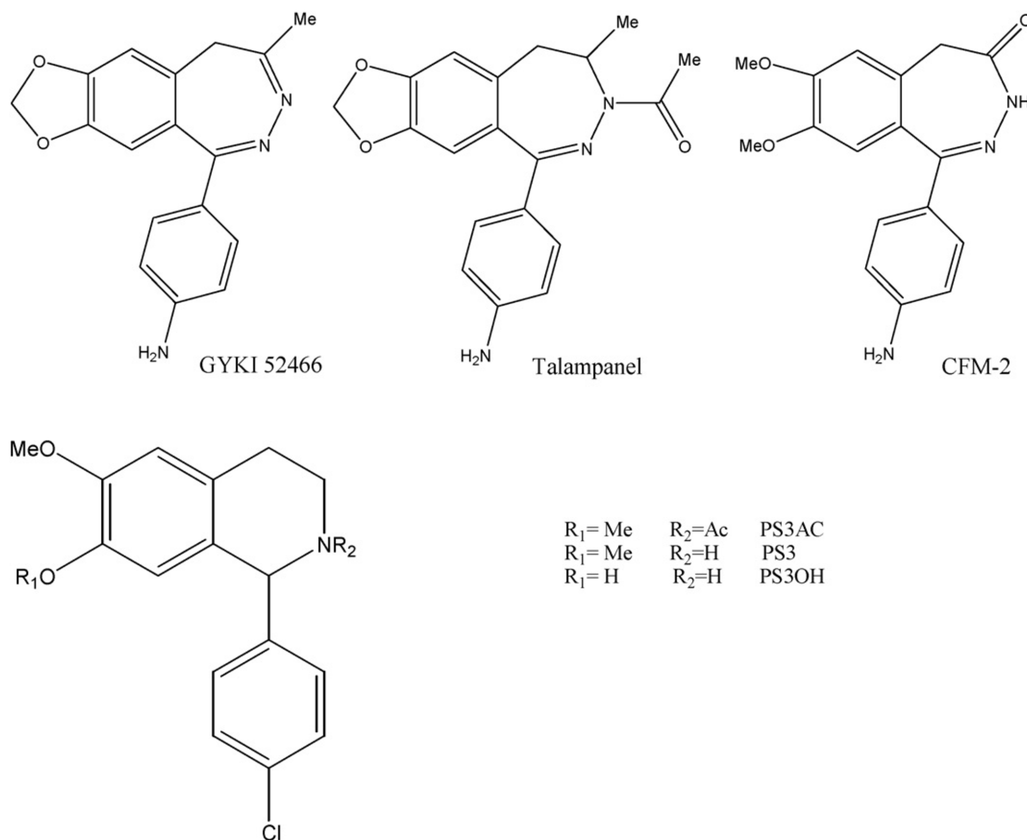

Fig. 1. Structures of GYKI 52466, Talampanel, CFM-2 and tetrahidroisoquinoline derivatives.

which aroused great interest as anticonvulsant agent and whose phase II/III clinical trials are under way [13].

In previous studies, we also reported a pharmacokinetic study of an extensive series of 7,8-dimethoxy-2,3-benzodiazepines, such as compound CFM-2, proposed as selective ligands for AMPA receptor (AMPAR) [11,12].

More recently, by using molecular modelling approaches, we have proposed a pharmacophore model of negative allosteric modulators of AMPAR which suggested us the synthesis of *N*-acetyl-1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolines which might satisfy the structural requirements for AMPAR binding [14]. Indeed, *N*-acetyl-1-(*p*-chlorophenyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (PS3Ac, Fig. 1), was the most active compound in this novel series of anticonvulsant agents which has been demonstrated to possess markedly increased activity over CFM-2, Talampanel and GYKI 52466 [15].

Recently, we reported an analytical study of the pharmacokinetic profile of *N*-acetyl-1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline derivative in Wistar rats, showing that PS3Ac is scarcely bio-transformed into two different metabolites such as *N*-deacetyl and *O*-demethyl derivatives, PS3 and PS3OH, respectively (Fig. 1). All the metabolites are more polar than the parent compound and the study suggests that at least for the analysed time point, metabolism had a limited effect.

Moreover, those results have been cited recently by some of us and confirmed in a study where PS3Ac has been labelled with carbonium-11 and tritium and evaluated as a potential ligand for *in vivo* imaging of AMPA receptor using positron emission tomography (PET) [16].

The goal of the present study was the assessment of the metabolic process in the brain, as determined in plasma, to confirm that the observed pharmacological anticonvulsant activity could be attributed exclusively to the inoculated drug PS3Ac.

With this aim, we have developed an assay suitable for simultaneous determination of tetrahydroisoquinoline derivatives in rat brain by using high performance liquid chromatography (HPLC) with diode array (DAD) detection.

The studied compounds have been isolated from brain samples by solid-phase extraction (SPE). The proposed method has been validated according to International Conference on Harmonisation (ICH) guidelines [17].

2. Experimental

2.1. Chemicals and standards

Tetrahydroisoquinoline derivatives PS3Ac and PS3 were synthesised in our laboratories as previously described [14]. A different synthetic procedure was employed to obtain PS3OH, *i.e.*, 1-(*p*-chlorophenyl)-7-hydroxy-6-methoxy-1,2,3,4-tetrahydroisoquinoline [16]. Stock solutions (1 mg/ml) of all studied compounds were prepared in methanol. Working solutions were made by dilution with methanol and were used to prepare aque-

ous standards and spiked brain samples on all standard curves. The internal standard (I.S.), 3,5-dihydro-7,8-dimethoxy-3-methyl-1-phenyl-4H-2,3-benzodiazepin-4-one, was synthesised in our laboratories and prepared in methanolic solution at a concentration of 50 μg/ml [18].

Acetonitrile and methanol HPLC grade were purchased from Carlo Erba (Milan, Italy). HPLC-grade water was prepared with Milli-Q water purification system and was used to prepare buffers.

2.2. Chromatography

A Jasco PU 1580 pump and LG 1580-02 ternary unit (Tokyo, Japan) with a 100 μl loop injection valve was used. The chromatographic system was associated to a diode-array Jasco MD-1510 detector (Tokyo, Japan), set to determine the maximum wavelength of absorption of the compounds that was 220 nm. Data were processed using Borwin chromatography software (version 1.21) from Jasco (Tokyo, Japan).

A mixture of 10 mM potassium dihydrogen orthophosphate (pH 2.24) and acetonitrile in ratio of 30:70 (v/v) was used as mobile phase. It was delivered at a flow rate of 0.8 ml/min through an octadecylsilica stationary phase Lichrosorb RP18 Hibar (25 cm × 4.6 cm, 7 μm) reverse phase column (Merck, Darmstadt, Germany), with a relative guard column (4.5 cm × 0.46 cm). A block heater Gastorr GF 103 (Jones Chromatography, Colorado, U.S.A.) was utilised to maintain the analytical column at 25 °C.

2.3. Drug administration

PS3AC was dissolved in a solution containing 50% dimethylsulfoxide and 50% sterile saline, and administered intraperitoneally (i.p.) to Wistar rats (20 mg/kg of body weight). Brains were withdrawn from the animals 15, 30, 60, 120 and 240 min after i.p. administration of the drugs ($n = 3$ each time analysed). The entire brains were weighed and refrigerated at −20 °C until analysis.

Rats were housed three per cage in stable conditions of humidity (60 ± 5%) and temperature (22 ± 2 °C), and allowed free access to food and water until the time of the experiments. The animals were maintained on 12-h light/12-h dark cycle (lights on 7.00 a.m.–7.00 p.m., off 7.00 p.m.–7.00 a.m.).

2.4. Extraction

Each brain, derived from treated rats, was accurately weighted and placed in homogenisation; phosphate buffer (0.01 M, pH 7.0) was added to the brain in ratio of 1 ml for 1 mg of biomass, and the process was carried out in bath of ice for 3′. The homogenate underwent refrigerated centrifugation (6000 × g for 5 min); supernatant was withdrawn, divided in Eppendorf test tubes, containing each 1 ml of extracting phase, and then centrifuged at 5000 × g for 5 min. All samples (1 ml) were added with 50 μl of a methanolic solution of internal standard, prior to undergoing the solid-phase extraction (SPE) procedure. Conditioned cartridge, based on a polymeric matrix with a high hydrophilic–lipophilic balance HLB (Oasis SPE 1 ml, 30 mg; Waters-Milford, MA, U.S.A.), provided with a LiChrolut extraction unit (Merck), was chosen to isolate tetrahydroisoquinoline derivatives from biological samples.

After washing samples with 2 ml of water containing 5% methanol, the analytes were eluted twice with 2 ml of methanol. For all dry steps the pressure was maintained at 39.9 kPa for 3 min. The eluate was dried under a nitrogen stream at 45 °C and the residue was dissolved in 300 μl of mobile phase and 100 μl of this extract underwent the chromatographic separation.

Brains derived from untreated rats underwent the same extraction procedures after addition of 50 μl of a I.S. solution and appropriate standard solutions of the studied compounds. The spiked brain homogenate samples were used to build standard curves and were refrigerated to −20 °C until assay.

2.5. Calibration curve and method validation

Methanolic solutions of PS3Ac (2 μg/ml), PS3 (1 μg/ml), PS3OH (1 μg/ml) and I.S. (4 μg/ml) were injected into the column and identified by their relative retention times.

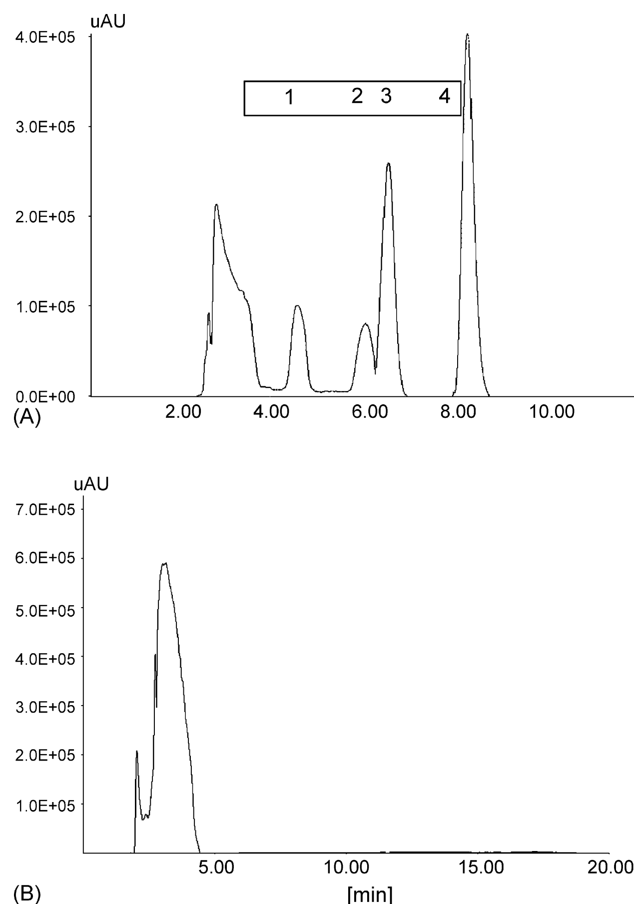

Fig. 2. (A) Chromatograms of methanolic solution of studied compounds showing their relative retention times are: $t' = 4.48$ for PS3OH (**1**); $t' = 5.98$ for PS3 (**2**); $t' = 6.41$ for PS3Ac (**3**), $t' = 8.17$ for I.S. (**4**). (B) Representative chromatogram of extracted drug-free brain homogenate sample.

Calibration curves were obtained by plotting the peak area ratio of the drugs to the I.S., versus the known concentration of each reference substance added to drug-free rat brain homogenate. The curves were constructed from four replicate measurements of six concentrations of each compound over a range of 0.05–2 μg/ml (0.05–0.1–0.25–0.5–1–2) and analysed one curve a week for a month. Brain samples containing a known amount of all studied compounds and I.S. were prepared and stored frozen at −20 °C until assay. These samples were utilised as quality control specimens. The within-day and between-day precision and accuracy were calculated by four replicate analyses at each chosen concentration (0.05–0.2–0.5–1–2 μg/ml) and analysed over a period of 4 weeks.

2.6. Extraction efficiency

The recovery was calculated from spiked brain homogenates and aqueous standards of all compounds and I.S. were evaluated to test the efficiency and reproducibility of the extraction procedure. The determination of the extraction efficiency in rat brain was made by adding amounts of 0.5–1 and 2 μg/ml in replicate ($n = 4$). The extraction was conducted as described above and 50 μl of internal standard working solution (50 μg/ml) was added prior to the extraction. The responses of these standards taken by means of the extraction procedures have been compared with those of standard solution at the same concentration injected directly into the liquid chromatographic apparatus. The

Table 1
Within-day precision of the HPLC assay ($n = 4$)

	PS3Ac (μg/ml)				
	0.05	0.2	0.5	1	2
1	0.057	0.179	0.473	1.024	2.032
2	0.053	0.164	0.443	0.817	1.851
3	0.045	0.172	0.487	1.127	1.897
4	0.048	0.215	0.518	0.779	1.846
Mean	0.051	0.183	0.480	0.937	1.907
S.D.	0.005	0.023	0.031	0.166	0.087
CV%	10.4	12.3	6.4	17.7	4.5
RE%	1.5	−8.7	−3.9	−6.3	−4.6
Accuracy	101.5	91.2	96.0	93.6	95.3
	PS3 (μg/ml)				
	0.05	0.2	0.5	1	2
1	0.043	0.140	0.520	1.094	2.044
2	0.042	0.165	0.563	0.966	1.910
3	0.044	0.231	0.510	1.147	1.827
4	0.046	0.191	0.488	1.079	1.810
Mean	0.044	0.182	0.520	1.072	1.898
S.D.	0.002	0.039	0.031	0.076	0.107
CV%	3.9	21.3	6	7.1	5.6
RE%	−12.5	−9.1	4	7.1	−5.1
Accuracy	87.5	90.8	104	107.1	94.8
	PS3OH (μg/ml)				
	0.05	0.2	0.5	1	2
1	0.043	0.169	0.410	0.940	2.033
2	0.046	0.277	0.432	1.098	1.820
3	0.045	0.184	0.456	1.111	1.827
4	0.042	0.251	0.455	1.078	1.806
Mean	0.044	0.220	0.438	1.057	1.872
S.D.	0.002	0.052	0.022	0.079	0.108
CV%	4.1	23.6	4.9	7.4	5.7
RE%	−12	10.1	−12.3	5.6	−6.4
Accuracy	88	110.1	87.6	105.6	93.5

S.D.: Standard deviation; CV: coefficient of variation; RE: relative error.

peak–area ratios were compared to the ratio of the standard aqueous samples without extraction. Four replicate samples were determined at each point and performed once a day for 4 days.

3. Results and discussion

PS3Ac is a lipophilic molecule which, by loss of acetyl and/or methyl moieties, could be transformed into the more hydrophilic metabolites PS3OH and PS3.

The presented analytical study was designed to illustrate the metabolic pathway of the inoculated compound, PS3Ac, in brain matrix. Therefore a solid-phase extraction, based on a polymeric matrix with a high hydrophilic–lipophilic balance HLB, that, in our experience, guarantees high and reproducible recoveries for acidic, basic and neutral compounds, was chosen to isolate the studied compound and its metabolites from the brain homogenate. The largest recoveries and the cleanest extraction procedure was achieved by washing the polymeric matrix with water containing 5% methanol, with a dry step of 3 min.

In a preliminary study, different kinds of buffer with a acid, neutral and basic characteristic have been added to the brain in variable ratios over the biomass, to determine the best condition to treat the brain during the homogenisation. Acid or basic buffers appeared to have adverse effects on the ionisation of the studied compounds, with a drastic reduction of their detections; moreover, other components of the homogenate gave important interfering peaks in the chromatograms of blank spiked extracts. Phosphate buffer (0.01 M, pH 7.0) was chosen because

Table 2
Between-day precision of the HPLC assay ($N = 4$)

	PS3Ac (μg/ml)				
	0.05	0.2	0.5	1	2
1	0.048	0.181	0.461	1.094	1.847
2	0.052	0.209	0.466	0.922	1.867
3	0.049	0.190	0.450	0.942	2.056
4	0.051	0.202	0.419	0.979	1.768
Mean	0.050	0.196	0.449	0.984	1.885
S.D.	0.002	0.012	0.021	0.077	0.122
CV%	3.6	6.3	4.6	7.8	6.4
RE%	0.0	−2.2	−10.2	−1.5	−5.7
Accuracy	100	97.7	89.8	98.4	94.2
	PS3 (μg/ml)				
	0.05	0.2	0.5	1	2
1	0.052	0.181	0.513	0.940	1.766
2	0.051	0.175	0.436	0.860	1.630
3	0.053	0.207	0.437	0.865	1.661
4	0.054	0.184	0.473	0.749	1.686
Mean	0.053	0.187	0.465	0.854	1.686
S.D.	0.001	0.014	0.036	0.079	0.058
CV%	2.4	7.5	7.8	9.2	3.4
RE%	5	−6.6	−7	−14.6	−15.7
Accuracy	105	93.3	92.9	85.3	84.2
	PS3OH (μg/ml)				
	0.05	0.2	0.5	1	2
1	0.042	0.149	0.438	0.910	1.850
2	0.035	0.177	0.473	0.902	1.930
3	0.041	0.183	0.460	0.860	1.770
4	0.044	0.186	0.477	0.820	1.765
Mean	0.041	0.174	0.462	0.873	1.829
S.D.	0.004	0.017	0.018	0.042	0.078
CV%	9.5	9.7	3.8	4.7	4.2
RE%	−19	−13.1	−7.6	−12.7	−8.5
Accuracy	81	86.8	92.4	87.3	91.4

S.D.: Standard deviation; CV: coefficient of variation; RE: relative error.

it gave the best results in the extraction procedures of the tested compound over the other interfering components of the brain matrix. The best isocratic separation was obtained maintaining a reverse phase column at 25 °C and delivering the mobile phase at 0.8 ml/min. The more performing ratio of organic and buffered solution was 70:30. The ultraviolet spectrum of PS3Ac showed two *maxima*, *i.e.*, 220 and 240 nm. PS3OH and PS3 had a λ_{max} at 220 nm. Indeed, a diode array detector was used to detect all substances at maximum of the absorption.

The method was validated with regard to limit of detection and quantification, sensitivity, linearity, precision, accuracy and extraction efficiency.

3.1. Detection and sensitivity

Fig. 2A shows the retention time of the tested compounds. The drugs and the internal standards were detected at maximum of absorption and the retention times were $t' = 4.93$ (±0.018) for PS3OH; $t' = 5.52$ (±0.025) for PS3; $t' = 6.14$ (±0.015) for PS3Ac, $t' = 8.28$ (±0.021) for I.S.

The lower limit of detection (LOD), with a signal-to-noise ratio of 3, was 20 ng/ml for PS3Ac, 32 ng/ml for PS3OH and 48 ng/ml for PS3. The sensitivity of the method allowed a quantification of 50 ng/ml (LOQ) of these drugs in brain samples.

3.2. Linearity

A linear response was observed over the examined concentration range (0.05–2 μg/ml). The curves were constructed from replicate measurements of six concentrations (0.05–0.1–0.25–0.5–1–2). The square of the correlation coefficient was 0.98997 for PS3Ac ($y = 0.7481x + 0.0147$; slope S.E. = 0.0270, intercept S.E. = 0.0045; $n = 6$), 0.99867 for PS3OH ($y = 0.8359x + 0.0245$; slope S.E. = 0.0425, intercept S.E. = 0.0056; $n = 6$), 0.99469 for PS3 ($y = 0.8739x + 0.0193$; slope S.E. = 0.0471, intercept S.E. = 0.0083; $n = 6$).

3.3. Precision, accuracy and stability

Precision and accuracy for within-day and between-day assay of the studied compounds are shown in Tables 1 and 2. In the within-day assay, the accuracy ranged from 87.5% determined with 0.05 μg/ml of PS3 to 110.1% determined with 0.2 μg/ml of PS3OH. Acceptable precision was achieved for all concentration investigated. In the between-day assay the coefficient of variation ranged from 2.4 determined with 0.05 μg/ml of PS3 to 9.7 determined with 0.2 μg/ml of PS3OH.

The extraction efficiency, expressed as recoveries from brain homogenates and aqueous standards, of all compounds were evaluated to test the efficiency and reproducibility of the extraction procedure. It ranged from 77.8% for PS3OH at 0.2 μg/ml to 94.3 for PS3Ac at 0.5 μg/ml, as reported in Table 3.

The compounds studied showed no signals of significant degradation during 10 weeks of storage at −20 °C. All of them appeared to be stable in rat brain homogenate at ambient temperature (*ca.* 22 °C) for up to 2 h (data not shown).

Table 3
Determination of extraction recovery of tetrahydroisoquinolines from brain ($N = 4$)

Concentration spiked (μg/ml)	Mean concentration found (μg/ml)	S.D.	CV (%)	Recovery (%)
PS3AC				
0.2	0.172	0.008	4.8	86.2
0.5	0.471	0.052	10.9	94.3
1	0.894	0.022	2.4	89.4
2	1.843	0.024	1.3	92.2
PS3				
0.2	0.166	0.008	5	82.8
0.5	0.451	0.022	4.8	90.3
1	0.884	0.012	1.4	88.4
2	1.855	0.005	0.2	92.7
PS3OH				
0.2	0.156	0.008	5.3	77.8
0.5	0.413	0.013	3.1	82.6
1	0.807	0.070	8.7	80.7
2	1.717	0.015	0.8	85.8

S.D.: Standard deviation; CV: coefficient of variation.

In extracts of three separate batches of drug-free rat brain homogenate, there were no interfering peaks present in chromatograms corresponding to the retention times of the studied compounds (Fig. 2B), which could have affected the precision and accuracy of measurements, specially at the lowest calibration standard.

3.4. Application to pharmacokinetic study

The present method has been applied to the pharmacokinetic study of PS3Ac after i.p. administration to Wistar rats.

Chromatograms from rat sample, obtained after i.p. administration of PS3Ac and withdrawn after 60 and 120 min are shown in Fig. 3A and B, respectively. In the chromatograms, the peak referred to the inoculated compound was not the only peak present.

Indeed, Fig. 3A shows a chromatogram, related to a brain withdrawn after 60 min, where the peak of PS3Ac was associated with another peak corresponding to PS3 (**1**) ($t = 5.52$). Fig. 3B shows a chromatogram, related to a brain withdrawn after 120 min, where the peak of PS3Ac was associated to a peak corresponding to PS3OH (**1**) ($t = 4.93$).

Moreover, we observed a difference between the two metabolic compounds (PS3 and PS3OH) in reaching T_{max}, that could be related, at least, to two different factors: their different lipophilicity and different metabolic pathway. Indeed, the absence of the methyl group on the 7-methoxy function of isoquinoline system, inducing reduction of lipophilicity, could be responsible of a systemic distribution of PS3OH and then also of the concentration detected in the brain, is not exclusively derived from a central metabolic event. This result confirm, as observed in plasma, that the metabolic pathway seems to be the *N*-deacetylation followed by the *O*-demethylation.

Moreover, whereas the concentration of PS3 decreases along the time and it is not more detectable at 120 min after PS3Ac

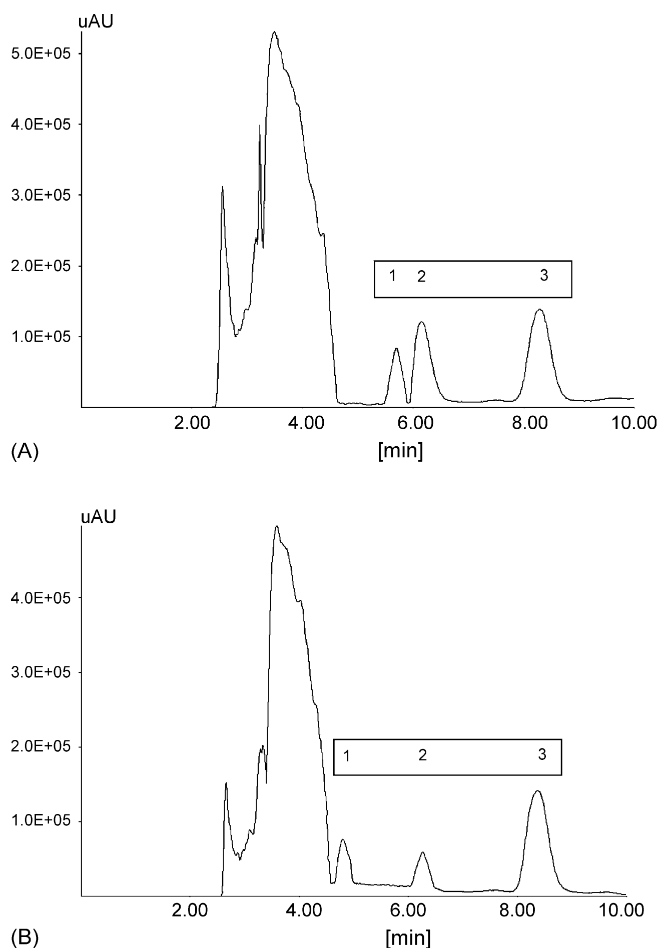

Fig. 3. (A) Chromatograms due to a brain sample obtained 60 min after administration of PS3Ac; $t' = 5.52$ for PS3 (**1**); $t' = 6.14$ for PS3Ac (**2**), $t' = 8.28$ for I.S. (**3**). (B) Chromatograms due to a plasma sample obtained 120 min after administration of PS3Ac; $t' = 4.93$ for PS3OH (**1**); $t' = 6.14$ for PS3Ac (**2**), $t' = 8.28$ for I.S. (**3**).

Table 4
Concentration values expressed as μg/ml of PS3Ac and metabolic products over the time (min)

	0	15	30	60	120	240
PS3Ac	0.0	0.396	0.760	1.35	0.549	0.0
PS3	0.0	0.0	0.296	0.697	0.0	0.0
PS3OH	0.0	0.0	0.0	0.00	0.515	0.0

administration, the PS3OH was detected only at 120 min, as shown by the compared chromatograms.

The determined concentrations of this metabolite were very low and this feature suggested that PS3Ac was only partially converted *in brain* into the *N*-deacetyl derivative (PS3).

Table 4 shows the time-profiles of brain concentrations of Wistar rats treated with PS3Ac. The C_{max} for PS3Ac was achieved at 60 min after drug administration (1.35 μg/ml), then the brain concentration decreased significantly.

4. Conclusion

This report describes the methodology and validation of a HPLC assay to determine tetrahydroisoquinoline derivatives in rat brain matrix. The method has been supplied to study the concentration-profile of the compound PS3Ac in Wistar rats.

The sensibility of the method allows us to determine very low concentration of two different metabolites derived by *N*-deacetylation and *O*-demethylation process thus demonstrating that PS3Ac is only scarcely bio-transformed.

In conclusion, the results of the analytical study suggested that PS3Ac is principally responsible of the anticonvulsant effects.

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