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SPE-HPLC determination of new tetrahydroisoquinoline derivatives in rat plasma

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Abstract

Recently a novel class of non-competitive AMPA receptor (AMPAR) antagonists, such as, *N*-acetyl-1-(*p*-chlorophenyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (PS3Ac) have been developed using molecular modeling studies. In this study we present a validated method for detecting PS3Ac in biological matrices by high performance liquid chromatography with ultraviolet detection. In this study PS3Ac was administered to Wistar rats. After intraperitoneal administration, the plasma concentrations of PS3Ac and its potential metabolic products, i.e., PS3OH, PS3 and PS3OHAc were determined. Serum samples (0.5 ml) were purified by solid-phase extraction of analytes using Oasis cartridges. The chromatographic separation was performed on a LiChrosorb RP-1 at 30 °C. The eluent was made of potassium dihydrogen phosphate/acetonitrile in ratio of 50:50 (v/v); the flow rate was 1 ml/min. The detection was performed at 220 nm. The method exhibited a large linear range from 0.05 to 5 μg/ml for all studied compounds. The intra-assay accuracy ranged from 92% determined at 0.1 μg/ml of PS3OH, to 108% determined at 0.05 μg/ml of PS3OHAc. The average coefficient of variation of inter-assay was 6.27%. The average recovery from plasma was 78.5%. The limits of quantification for all the tetrahydroisoquinoline derivatives was 20 ng. The method proved to be highly sensitive and specific for the determination of the studied compounds in rat plasma and has been successfully applied to the evaluation of the pharmacokinetic profile of the inoculated compound.

Keywords: AMPA receptor antagonist; SPE-HPLC; Pharmacokinetic

1. Introduction

The pathogenesis of different neurological disorders has been linked to excessive activation of excitatory amino acid (EAA) receptors. The development of therapeutically useful *N*-methyl-D-aspartic acid (NMDA) receptor channel blockers and selective non-NMDA receptor antagonists, i.e., 2-amino-3(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) and kainate, may be useful in the treatment of various disorders such as epilepsy, ischemia, and Parkinson's disease [1,2].

It has been reported [3,4] that a selective non-competitive blockade of AMPA receptor (AMPAR) is shown by some 2,3-benzodiazepines, such as the 3-*N*-acetyl-1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-4,5-dihydro-2,3-benzodiazepine, i.e., Talampanel (Fig. 1), which aroused great interest as anticonvulsant agent and whose phase II/III clinical trials are under way [5].

In previous studies, we reported chemical, biological and pharmacokinetic studies of an extensive series of 7,8-dimethoxy-2,3-benzodiazepines, such as compound CFM-2 (Fig. 1), proposed as selective ligand for AMPAR [6–10] and

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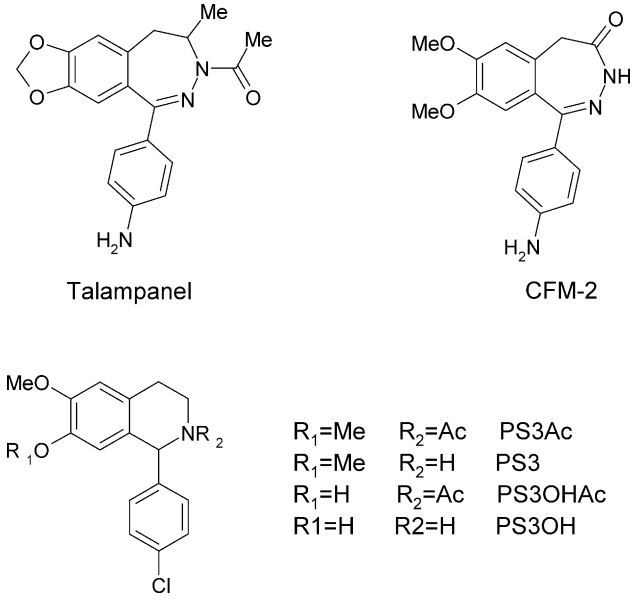

Fig. 1. Structures of Talampanel, CFM-2 and PS3 derivatives.

proved to be more active than other analogue derivatives in various seizure models [11].

In order to obtain new anticonvulsant agents, we have recently proposed a pharmacophore model of negative allosteric modulators of AMPAR [12] which suggested the synthesis of *N*-acetyl-1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolines. Therefore, molecular modeling studies suggested that tetrahydroisoquinolines might satisfy the structural requirements for AMPAR binding.

Indeed, studies in sound induced DBA/2 mouse model of epilepsy, determined that tetrahydroisoquinolines were effective at preventing seizures in this model for generalized epilepsy. In fact, DBA/2 mice represent an excellent animal model for generalised epilepsy for screening new anticonvulsant drugs [13].

Furthermore, subsequent studies determined that the *N*-acetyl-1-(*p*-chlorophenyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (PS3Ac, Fig. 1), is the most active compound in this novel series of anti-epileptic medications which has been demonstrated to possess markedly increased anticonvulsant activity over CFM-2, talampanel and GYKI 52466 [14].

Based on the novel antiseizure activity of PS2Ac, an analytical method for detection of PS3Ac was need to sensitively and accurately determine the concentrations of PS3Ac and its metabolites in biological matrices. Therefore, the goal of this research was to determine if high performance liquid chromatography with ultraviolet detection would be a sensitive and reproducible method for detection of PS3Ac and its metabolites in biological matrices. Once validated we also wanted to determine the pharmacokinetic profile of PS3Ac and its major metabolites in Wistar rats after in vivo administration.

Moreover, in order to investigate the possible biotransformation pathway of this compound, three possible metabolic products were synthesized and added to rat plasma. PS3Ac and its *N*-deacylated and/or *O*-demethylated analogues (PS3OH, PS3 and PS3OHAc) have been successfully isolated from plasma samples by solid phase extraction (SPE) and detected using a reversed-phase chromatographic system. The proposed method has been developed for the simultaneous determination of PS3Ac and its metabolites, and validated according to ICH guidelines [15].

A RP C18 stationary phase to separate these compounds and an isocratic elution profile, consisting of acetonitrile and phosphate buffer, was used. Data supporting the linearity, precision, accuracy, recovery, limit of detection and quantification are presented.

2. Experimental

2.1. Chemicals and standards

Acetonitrile and methanol HPLC grade were purchased from Carlo Erba (Milan, Italy). HPLC-grade water was prepared with Milli-Q water purification system and used to prepare buffers. *N*-acetyl-1-(*p*-chlorophenyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (PS3Ac) and 1-(*p*chlorophenyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (PS3) were synthesized in our laboratories as previously described [14]. A different synthetic procedure was employed to obtain compounds 1-(*p*-chlorophenyl)-7-hydroxy-6-methoxy-1,2,3,4-tetrahydroisoquinoline (PS3OH) and *N*-acetyl-1-(*p*-chlorophenyl)-7-hydroxy-6-methoxy-1,2,3,4-tetrahydroisoquinoline (PS3OHAc) (unpublished data). Stock solutions (1 mg/ml) of all studied compounds were prepared in methanol. Working solutions were made by dilution with methanol and used to prepare aqueous standards and spiked plasma samples on all standard curves.

2.2. Chromatography

The separation was performed using a mobile phase prepared mixing solvent A (10 mM potassium dihydrogen orthophosphate pH 3.45) and solvent B (acetonitrile) in ratio of 50:50 (v/v). It was delivered with a flow rate of 1 ml/min through a column 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 30 °C.

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), utilised as a single wavelength detector at 220 nm. Moreover, a 100 μl loop was used.

Data were processed using Borwin chromatography software (version 1.21) from Jasco (Tokyo, Japan).

2.3. Drug administration

Wistar 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.).

Tetrahydroisoquinoline derivative PS3Ac was dissolved in a solution containing 50% dimethylsulphoxide and 50% sterile saline, and administered intraperitoneally to Wistar rats (20 mg/kg of body weight). Using EDTA in the collection syringes, blood samples were withdrawn from the animals ($n = 3$ each time analysed), by indwelling catheter, after 7.5, 15, 30, 45, 60, 120, 180 and 240 min after administration of tested compound. Blood cells were removed by centrifugation and the separated plasma was stored at −20 °C until assay.

2.4. Extraction

A 500-μl aliquot of plasma samples derived from treated rats and plasma samples enriched with appropriate calibration standards of the inoculated compound and its analogues, i.e., PS3OH, PS3 and PS3OHAc were sampled and refrigerated to –20 °C. The solid-phase extraction (SPE) procedure was applied to buffered plasma samples using a correspondent volumes of 0.1 M phosphate buffer (pH 7.0). Conditioned Oasis SPE cartridge HLB (1 ml, 30 mg) from Waters (Milford, Massachusetts, U.S.A.), provided with a LiChrolut extraction unit (Merck), were chosen to isolate tetrahydroisoquinoline derivatives from biological samples. After cartridges washing with 2 ml of water containing 5% methanol, the analytes were eluted with 2 ml of methanol, twice. For all dry steps the pressure was maintained at 11 in. Hg 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.

2.5. Calibration curve and method validation

The chromatographic identification of the compounds PS3Ac, PS3OH, PS3 and PS3OHAc (50 μl, 10 μg/ml) were realised by their relative retention time. Calibration curves were obtained by plotting the peak-area of the studied substances versus the theoretical concentration of the analyte added to drug-free rat plasma. The curves were constructed from replicate measurements of five concentrations over a range of 0.05–5 μg/ml. The data were subjected to least squares regression analysis (weighted as appropriate), to provided information on linearity of the method.

Plasma samples containing a known amount of PS3Ac, PS3OH, PS3 and PS3OHAc were prepared and stored frozen at −20 °C until use. These samples were utilised as quality control specimens, to compare the measured to the theoretical concentrations of the tetrahydroisoquinoline derivatives.

The coefficients of variation occurring within (intra-) and between (inter-) assay analyses were evaluated to determine the precision of the bioanalytical method.

The intra-assay precision and accuracy were calculated by six replicate analyses, at each chosen concentration (0.05, 0.1, 0.5, 1 and 5 μg/ml). The inter-assay coefficient of variation was determined once a week for 6 weeks, measuring added concentration of 0.1, 0.5 and 1 μg/ml ($n = 6$ at each concentration) to drug free plasma samples.

Chromatograms from blank extracted samples and samples spiked with low and high concentration of the studied compounds were examined.

Moreover, the presence of interfering peaks and changes in retention times were assessed in chromatograms obtained from plasma analysis to verify the specificity of the analytical method.

2.6. Extraction efficiency

The efficiency and reproducibility of the extraction procedure was evaluated by measuring the mean recovery of PS3Ac, PS3OH, PS3 and PS3OHAc from spiked rat plasma.

The determination of the extraction efficiency in all samples was made by adding amounts of 0.1, 0.5 and 1 μg/ml in replicate ($n = 6$). The extraction was conducted as described above. The responses of these standards taken by means of the extraction procedures were compared with those of standard methanol solutions at the same concentrations, injected directly into the liquid chromatographic apparatus. The recovery of tested substances was performed at low, medium and high concentrations on the calibration range.

3. Results and discussion

The HPLC method proposed provides a simple procedure for the determination of tetrahydroisoquinoline derivatives in biological samples.

The ultraviolet spectrum of PS3Ac and PS3OHAc showed two *maxima* (220 and 240 nm). PS3OH and PS3 had a λ_{max} at 220 nm. Indeed, a value of 220 nm was used to detect all substances with a good sensitivity.

PS3Ac is a lipophilic molecule which, by loss of acetyl and/or methyl moieties, could be transformed into the more hydrophilic metabolites PS3OH, PS3, PS3OHAc. Therefore a solid phase extraction, based on a polymeric matrix with a high hydrophilic–lipophilic balance, was chosen. Moreover, it guarantees high and reproducible recoveries for acidic, basic and neutral compounds, even if the cartridge runs dry.

In preliminary studies, the removal of interfering plasma components was attempted by washing the cartridge with eluent at different pH values. 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.

Chromatographic conditions were based on the isocratic separation on a reverse phase column. Preliminary studies with different mobile phase combination of acetonitrile and phosphate buffer were considered. The optimum pH of the mixture was 4.92.

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

3.1. Detection and sensitivity

The chromatographic identification of the compounds were realised by their relative retention time that were 4.08 (±0.01) min for PS3OH, 5.86 (±0.02) min for PS3OHAc, 7.04 (±0.01) min for PS3 and 8.75 (±0.02) min for PS3Ac.

The minimum detection limit (LOD) with a signal-to-noise ratio of 3, was 8 ng/ml for PS3Ac, 10 ng/ml for PS3OH, 15 ng/ml for PS3 and 9 ng/ml for PS3OHAc. The sensitivity of the method allowed an easy quantification (LOQ) of 20 ng of these drugs in rat plasma samples.

3.2. Linearity

A linear response was observed over the examined concentration range (0.05–5 μg/ml). The square of the correlation coefficient was 0.9957 for PS3Ac ($y = 0.6411x + 0.1178$; slope SE = 0.0111, intercept SE = 0.0073; $n = 6$), 0.9867 for PS3OH ($y = 0.6381x + 0.023$; slope SE = 0.0321, intercept SE = 0.0026; $n = 6$), 0.9932 for PS3 ($y = 0.8423x + 0.0869$; slope SE = 0.0326, intercept SE = 0.0621; $n = 6$), 0.9879 ($y = 0.489x + 0.0767$; slope SE = 0.0263, intercept SE = 0.0085; $n = 6$).

3.3. Precision, accuracy and extraction efficiency

Intra-assay and inter-assay precision and accuracy were performed. The results are reported in Tables 1 and 2. For all tetrahydroisoquinoline derivatives, the intra-assay accuracy ranged from 92% determined at 0.1 μg/ml of PS3OH, to 108% determined at 0.05 μg/ml of PS3OHAc. The average coefficient of variation of inter-assay was 6.27%. Acceptable accuracy was achieved for all concentrations investigated, moreover, it was adequate for biological samples.

The recoveries from plasma and aqueous standard of all compounds were evaluated to test the efficiency and reproducibility of the extraction procedure. The compounds were added to drug-free plasma to achieve concentrations of 0.1, 0.5 and 1 μg/ml ($n = 3$ at each concentration). The recovery ranged from 72% for PS3 to 86% for PS3Ac and was independent of the concentration, as reported in Table 3.

All the examined tetrahydroisoquinoline derivatives showed no signs of significant degradation in rat plasma during 10 weeks of storage at −20 °C (data not shown).

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

Table 1
Intra-assay precision of the HPLC assay ($N = 6$)

Nominal concentration (μg/ml)	Measured concentration[a] (μg/ml)	Accuracy (%)	C.V.[b](%)
PS3Ac			
0.05	0.048 ± 0.004	96	8.6
0.1	0.0943 ± 0.005	94.3	5.3
0.5	0.504 ± 0.003	100.8	0.59
1	1.026 ± 0.028	102.6	2.72
5	5.07 ± 0.014	101.4	2.78
PS3OH			
0.05	0.052 ± 0.005	104	9.61
0.1	0.092 ± 0.002	92	2.17
0.5	0.049 ± 0.004	98.9	8.16
1	1.00 ± 0.029	100	2.90
5	5.16 ± 0.008	103.2	1.58
PS3			
0.05	0.047 ± 0.004	94.4	7.67
0.1	0.096 ± 0.007	96.2	7.29
0.5	0.464 ± 0.013	92.9	2.80
1	0.979 ± 0.029	97.9	3.06
5	5.07 ± 0.04	101.2	9.95
PS3OHAc			
0.05	0.054 ± 0.003	108	4.68
0.1	0.103 ± 0.005	103	4.4
0.5	0.53 ± 0.036	105.5	6.8
1	0.98 ± 0.012	97.5	1.32
5	5.18 ± 0.013	103.2	2.45

[a] Mean ± S.D.
[b] C.V.: coefficient of variation.

Table 2
Inter-assay precision of the HPLC assay ($N = 6$)

Nominal concentration (μg/ml)	Measured concentration[a] (μg/ml)	Accuracy (%)	C.V.[b] (%)
PS3Ac			
0.1	0.107 ± 0.011	107	10.2
0.5	0.53 ± 0.036	105.5	6.8
1	0.969 ± 0.048	96.9	5.0
PS3OH			
0.1	0.097 ± 0.005	96.9	5.6
0.5	0.51 ± 0.039	101.8	7.8
1	0.99 ± 0.022	99.01	2.22
PS3			
0.1	0.096 ± 0.007	96.2	6.8
0.5	0.50 ± 0.023	100.9	4.7
1	0.94 ± 0.053	93.7	5.7
PS3OHAc			
0.1	0.098 ± 0.006	97.6	6.4
0.5	0.49 ± 0.058	98.8	11.9
1	0.92 ± 0.02	92.2	2.17

[a] Mean ± S.D.
[b] C.V.: coefficient of variation.

Table 3
Determination of extraction recovery of tetrahydroisoquinoline from plasma ($N = 6$)

Concentration spiked (μg/ml)	Mean recovery (%)	S.D.[a] ±	C.V.[b] (%)
PS3Ac			
0.1	72.7	0.004	4.8
0.5	86.0	0.020	4.7
1	75.7	0.050	6.7
PS3OH			
0.1	76.0	0.002	2.6
0.5	84.7	0.015	3.6
1	79.7	0.002	2.6
PS3			
0.1	72.0	0.007	9.1
0.5	82.7	0.021	5.0
1	75.0	0.060	8.0
PS3OHAc			
0.1	76.0	0.005	6.6
0.5	82.0	0.010	2.4
1	79.0	0.053	6.7

[a] S.D.: standard deviation.
[b] C.V.: coefficient of variation.

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.

Fig. 2B shows chromatograms from drug-free rat plasma spiked with 5 μg of PS3OH (1) ($t = 4.08$), PS3OHAc (2) ($t = 5.86$), PS3 (3) ($t = 7.04$) and PS3Ac (4) ($t = 8.75$).

Chromatograms from rat plasma sample, obtained after i.p. administration of PS3Ac and withdrawn after 45 and 60 min are shown in Fig. 3A and B, respectively. In the chromatograms, the peak referred to the studied compound was not the only peak present. Indeed, it was associated to another two peaks corresponding to the retention time of PS3OH (1) ($t = 4.08$) and PS3 (3) ($t = 7.04$). The determined concentrations of the two metabolites were quite low and this feature suggested that PS3Ac was only partially converted in vivo into deacetyl (PS3) and demethyl–deacetyl (PS3OH) analogues. Moreover, whereas the concentration of PS3 decreases along the time after PS3Ac administration, the concentration of PS3OH detected after 60 min was increased, as shown by the compared chromatograms.

Fig. 4 shows the time-profiles of plasma concentrations of Wistar rats treated with PS3Ac. Three curves could be observed: one curve was due to the inoculated drug, whereas the other curves were due to PS3 and PS3OH.

The C_{max} for PS3Ac was achieved at 60 min after drug administration (5.27 μg/ml), then the plasma concentration decreased significantly.

The concentrations of PS3Ac metabolic products are notably low.

The time course of plasma concentration referred to PS3 showed that the compound is detectable 15 min after the inoculation of the parent compound (0.07 μg/ml), after 45 min reached the concentration of 0.75 μg/ml (C_{max}) whereas it was 0.35 μg/ml at 60 min, than decreased very slowly.

Considering the plasma concentration's profile of PS3OH, it's notable the increase of concentration that followed the decrease of PS concentration; indeed the detection was appreciable 45 min after administration (0.31 μg/ml), increasing at 60 min (0.68 μg/ml) and showing the C_{max} at 120 min

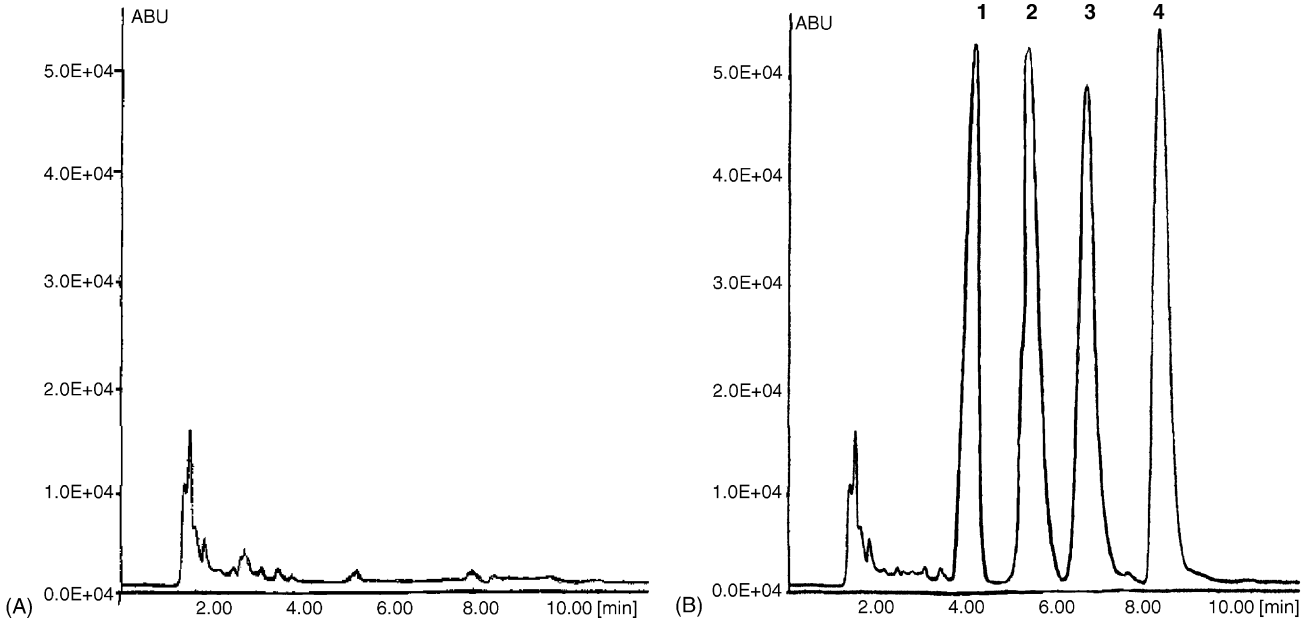

Fig. 2. (A) Chromatogram of extracted drug-free plasma sample. (B) Representative chromatograms of extracted drug-free plasma sample spiked with all compounds used in the analytical procedure, their relative retention times are: $t' = 4.08$ for PS3OH (1); $t' = 5.86$ for PS3OHAc (2); $t' = 7.04$ for PS3 (3) and $t' = 8.75$ for PS3Ac (4).

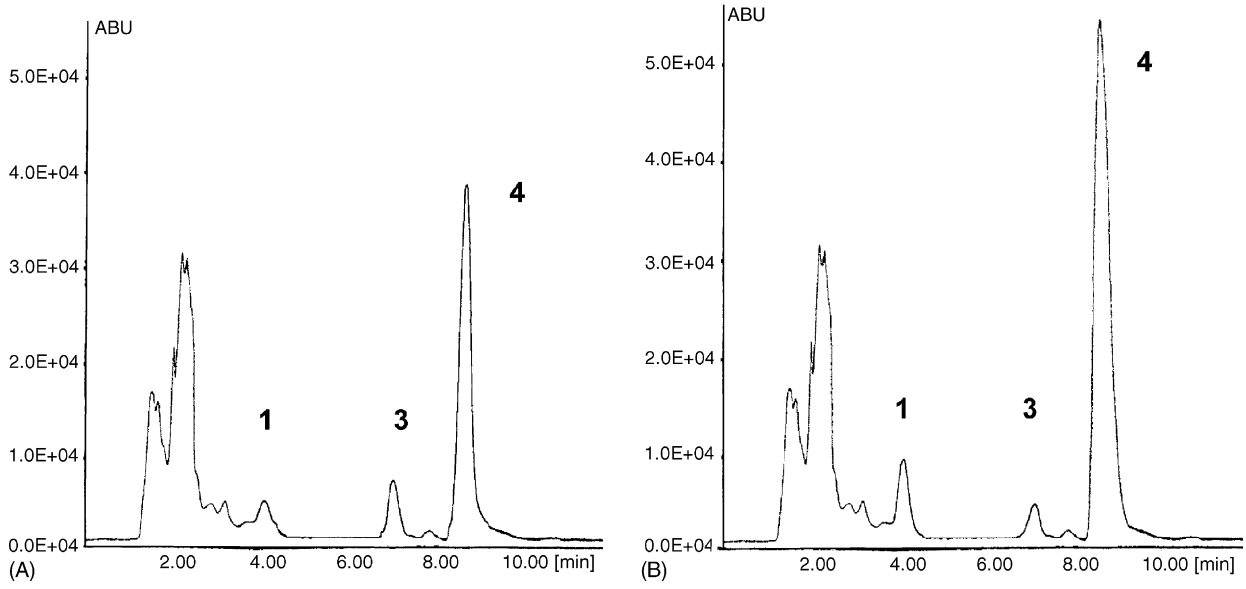

Fig. 3. (A) Chromatograms due to a plasma sample obtained 45 min after administration of PS3Ac; (B) Chromatograms due to a plasma sample obtained 60 min after administration of PS3Ac.

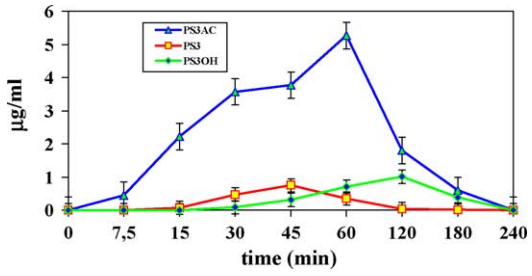

Fig. 4. Time-profile of plasma concentrations of PS3Ac after i.p. administration to rats. Ordinate shows the plasma level; abscissa shows the time after inoculation of the drug ($n = 3$; error bars = S.D.). Concentration data are reported.

(1.01 μg/ml); it was undetectable in plasma 180 min after administration.

4. Conclusion

This report describes the methodology and validation of a high-performance liquid chromatographic assay for the simultaneous determination of PS3Ac and its metabolites in rat plasma. The method appeared rapid and simple.

The extraction procedures from plasma demonstrated excellent efficiency. The chromatography by reversed-phase was selective. The sensitivity of the method allowed the study of the pharmacokinetic profile of *N*-acetyl-1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline derivatives in Wistar rats.

Our study shows that PS3Ac is scarcely bio-transformed into two different metabolites such as *N*-deacetyl and *O*-demethyl derivatives, PS3 and PS3OH respectively. Moreover, the first metabolic event seems to be the *N*-deacetylation followed by the *O*-demethylation.

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

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