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Analysis of new potential anticonvulsant compounds in mice brain tissue by SPE/HPLC/DAD

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ABSTRACT

This paper describes a novel reversed-phase high performance liquid chromatography (RP-HPLC) with photo-diode array detection (DAD) method for the determination of three new derivatives of 4-alkyl-5-(3-chlorophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione with different antiepileptic activity in the brains of mice treated with the doses of 300 mg kg^{-1} of body weight. Samples were prepared by solid-phase extraction (SPE) method using BAKERBOND^{TM} spe Octadecyl (C_{18}) and analyzed by the use of an isocratic elution mode over an Zorbax Extend-C18 column (150 mm × 4.6 mm I.D., 5-μm, Agilent Technologies). The mobile phase consisted of 80% methanol (for compound TP-315) and 85% acetonitrile (for compound TP-321) for 80% 2-propanol (for TP-323) at a flow rate of 1.0 mL min^{-1} and 0.5 mL min^{-1} in the last case. Gradient elution mode was also proposed for all examined analytes in mixture with common antiepileptic drugs: carbamazepine, phenobarbital and phenytoin in view of possible synergistic activity. Photodiode-array investigations of the peaks after degradation studies indicate the stability of the compounds under conditions proposed for sample preparation procedure. Linear coefficients of correlation (r^2) were >0.995 for all analytes. The proposed strategy gives extraction yields higher than 95% with the intra- and inter-day relative standard deviation lower than 3% and 5%, respectively. This method was applied to the analysis of brain tissue of mice treated with investigated compounds. Obtained results enable to explain the differences in their pharmacological activity.

1. Introduction

Epilepsy is one of the most common neurological disorders. Carbamazepine, phenobarbital and phenytoin are regarded as the antiepileptic drugs of first choice in its treatment. Pharmacological activity of drugs is associated with a reduction in the number and severity of tonic–clonic seizures in patients [1]. Some of the studies demonstrate the usefulness of these drugs in the treatment of other diseases such as bipolar disorder [2], anxiety disorder, alcoholism or mood disorder [3]. The most common methods for their monitoring in biological fluids are chromatography and immunoassay [4–6]. However, chromatographic methods require application of sample pretreatment before the final analysis such as: protein precipitation, solid-phase or liquid–liquid extraction to elongate the life-time of HPLC columns [7,8]. Among chromatographic techniques only micellar liquid chromatography (MLC) offers the possibility of determining drugs in biological fluids without the need for previous sample clean-up steps [4].

Standard anticonvulsant drugs such as carbamazepine, phenobarbital, phenytoin, primidone, valproic acid, and ethosuximide are characterized by complex pharmacokinetics. They cause the occurrence of side effects due to induction or inhibition of hepatic enzymes. This mechanism is the cause of drug interaction and finally may result in serious problems in combination therapy. In turn, the new antiepileptic drugs (gabapentin, lamotrigine, topiramate, tiagabine, oxcarbazepine, levetiracetam and zonisamide) introduced recently to the treatment are characterized by smaller impact on the CYP450 enzyme system and other metabolic pathways. However according to American Academy of Neurology and American Epilepsy Society, some of them induce severe allergic, psychotic symptoms or depression [9].

In our previous work [10] we described synthesis method of new derivatives of 4-alkyl-5-(3-chlorophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione with a potential neurological activity. Three of the most promising ones (TP-315, TP-321, TP-323) have been investigated according to their antiepileptic activity. It has been

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found that investigated compounds differ in their activity. While the 5-(3-chlorophenyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione derivative (TP-315) showed the strongest antiepileptic activity, even several times higher than that of valproate, the remaining two derivatives (those with longer alkyl chains, namely decyl and dodecyl) were almost inactive. The question is whether the lack of activity of these two derivatives resulted from the lack of interaction with the central nervous system receptors (the substance penetrates the brain but demonstrates no affinity to the receptor) or from the inability to penetrate the blood–brain barrier (BBB).

The aim of this research was to determine the chromatographic behavior of these new compounds to develop procedure for their determination in mice brain tissue samples.

2. Experimental

2.1. Chemicals

The compounds: derivatives of 4-alkyl-5-(3-chlorophenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione investigated in the following study (Fig. 1) were synthesized in Department of Organic Chemistry, Medical University of Lublin. The synthesis procedure of this novel bioactive set of compounds was described in the Polish Patents [11]. The structures of all the investigated solutes were confirmed by GC–MS, ^{1}H-NMR, ^{13}C-NMR and IR analysis. Their purity was assessed by elemental analysis. Carbamazepine, phenobarbital and phenytoin were purchased from Sigma (St. Louis, MO, USA).

HPLC grade methanol (MeOH), acetonitrile (ACN), 2-propanol and chromatographic reagents were obtained from E. Merck (Darmstadt, Germany). Perchloric acid (70%, w/v) was purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Chromatographic conditions

Experiments were performed using a LaChrom HPLC Merck Hitachi (E. Merck, Darmstadt, Germany) model equipped with diode array detector, column oven L-7350 and solvent degasser L-7612. The column (150 mm × 4.6 mm I.D.) was packed with 5-μm Zorbax Extend-C18 (pore size: 80 Å, surface area: 180 m^2/g) Agilent Technologies (Santa Clara, CA, USA); its void volume was determined to be 1.31 mL by the injection of thiourea in acetonitrile–water (50:50) eluent system. The column was thermostated at 20 °C ± 0.1.

The mobile phase was a mixture of acetonitrile, methanol or 2-propanol in water. The mobile phase was filtered through a Nylon 66 membrane filter (0.45 μm) Whatman (Maidstone, England) by the use of a filtration apparatus.

Retention data were recorded at a flow-rate of 1 mL min^{-1} for 80% methanol and 85% acetonitrile and 0.5 mL min^{-1} for 80% 2-propanol used as a mobile phase.

The detection of the compounds was set at appropriate wavelength chosen accordingly with the recorded spectra. Typical injection volumes were 20 μL.

2.3. Preparation of stock solutions and calibration curve standards

The stock solutions of the analytes at concentration of 300 μg mL^{-1} were prepared in methanol, acetonitrile or 2-propanol for first, second and third compound, respectively. The stock solutions were stored in darkness at 4 °C in glass vials. The working-standard solutions were prepared daily by diluting the stock solutions by the use of appropriate organic solvent. The solutions were determined to be stable for up to one week. During this period no degradation products were detected.

2.4. Sample collection

Three 1,2,4-triazole derivatives: TP-315, TP-321, TP-323 were suspended in a 1% aqueous solution of Tween 80 (Sigma, St. Louis, MO, USA) and administered i.p. (in a dose of 300 mg kg^{-1}) in a volume of 5 mL kg^{-1} body weight. The control animals received adequate amounts of the vehicle. Mice pretreated with given compounds (or with the vehicle only) were decapitated at time reflecting the peak of maximum anticonvulsant effect for the TP-315 in the maximal electroshock seizure test. The whole brains of mice were removed from skulls, weighed, harvested and homogenized using Abbott buffer (1:2, w/v; Abbott Laboratories, North Chicago, IL, USA) in an Ultra-Turrax T8 homogenizer. Procedures involving animals were conducted in accordance with current European Community and Polish legislation on animal experimentation. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.5. Sample preparation

Blank sample of the first compound (TP-315) was prepared as follows: 200 μL of the brain tissue homogenate was mixed with 50 μL of methanolic standard solution. Obtained mixture was incubated in 37.1 °C for 60 min. After that 1 mL of methanol and 250 μL of 3% $HClO_4$ in water were added and sample was heated to 60 °C for 30 min. The mixture was centrifuged at 9000 × *g* for 15 min. An aliquot of the supernatant was further analyzed by SPE procedure.

The brain homogenate of the mouse treated by the dose of 300 mg kg^{-1} was weighted and mixed with 4 mL of methanol and 1 mL of 3% $HClO_4$ (the ratio of methanol to perchloric acid should be 4:1 to receive the best extraction efficiency). Further procedure was carried out as for blank sample.

The preparation of samples of the second compound (TP-321) required application of acetonitrile instead of methanol, the ratio of organic solvent to 3% perchloric acid should be 2:1 and temperature of deproteinization was 70 °C.

In case of the last compound (TP-323) in described procedure of sample preparation the following changes were made: 2-propanol was used as organic solvent, the ratio of organic solvent to 3% perchloric acid was 1:1 and temperature of protein precipitation was set at 80 °C.

2.6. SPE procedure

Solid-phase extraction (SPE) was carried out using BAKERBOND™ spe Octadecyl (C_{18}) J.T. Baker cartridges (200 mg, 3 mL and 500 mg, 6 mL) on a Baker spe-12G apparatus. The C_{18} cartridges were activated and conditioned with 2× 1 mL of methanol and 2× 1 mL of water respectively and dried applying full vacuum for 1 min. Aliquots of the supernatant were loaded onto the conditioned cartridge. Then they were washed with 2× 1 mL of water in case of the first compound and additionally by the use of 1 mL of 50% methanol in case of second compound or 1 mL of 50% acetonitrile in case of the third one. Analyte elution was carried out with 2 mL of methanol, acetonitrile or 2-propanole in the case of the first, the second and the third compound respectively, applying full vacuum for 1 min (−30 kPa). 20 μL of the eluate was injected directly into the HPLC column.

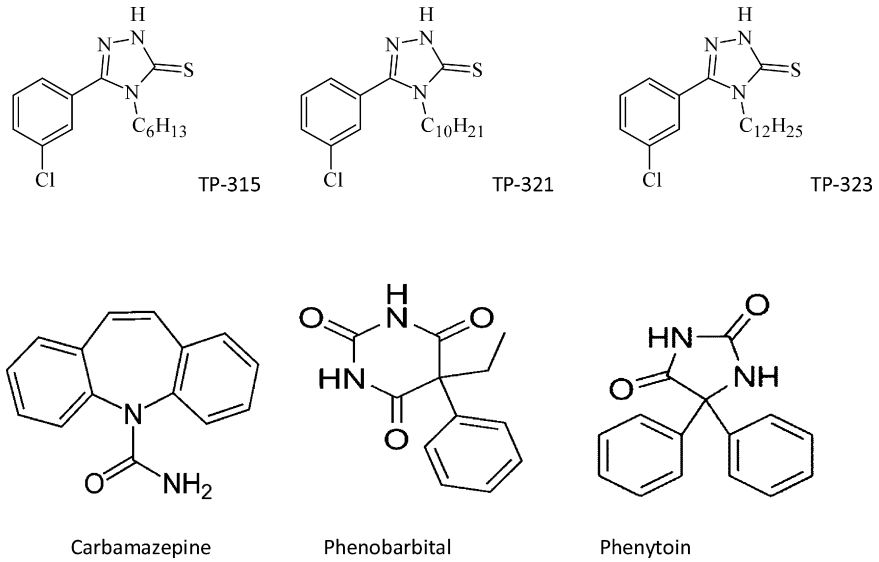

Fig. 1. Investigated compounds.

2.7. Method validation

The analytical-method validation was carried out according to the ICH Q2 (R1) method-validation guidelines [12]. The following validation parameters were established: selectivity, precision, linearity, limit of detection (LOD), limit of quantification (LOQ) and degradation of the tested compounds under chosen stress conditions.

2.7.1. Linearity

Analyte standard solutions at six different concentrations were added to 200 μL of blank sample, subjected to the SPE and HPLC procedure. The analyte peak area was plotted against the corresponding concentrations and the calibration curves were set up by means of the least-squares method. Limit of quantification (LOQ) and limit of detection values were determined as the analyte concentrations which give rise to peaks whose height is 10 and 3 times that of the baseline noise, respectively.

2.7.2. Extraction yield and precision

Spiked blank samples were prepared as follows: 50 μL of standard solution of different concentration were added to 200 μL of the brain tissue homogenate and incubated at 37.1 °C for 30 min. At least three concentrations were prepared and analyzed for each compound, corresponding to the lower, middle and upper limit of the respective linearity curve. Spiked blank samples were prepared and analyzed according to described procedure. The determined analyte peak areas were substituted into calibration curve equation and the percentage extraction yield was calculated according to the following formula $100 \times [(\text{after spiking} - \text{before spiking})/\text{added}]$. The analysis was repeated six times giving intraday precision values and six times in another day giving intermediate precision values both expressed as percentage relative standard deviation value (RSD%).

3. Results and discussion

3.1. Choice of chromatographic conditions (selectivity)

In Table 1 the pK_a's (dissociation constants) and log $P_{o/w}$ (octanol/water partitioning coefficients) values of the common antiepileptic drugs like carbamazepine, phenobarbital, phenytoin together with new synthesized compounds are collected. Fig. S1 (the supplementary materials), presents relationships between log D (octanol–water distribution coefficient in its logarithmic form) values and pH for each of them. As it can be seen, all compounds exist in neutral forms in the examined conditions (organic–aqueous mobile phase) exhibiting simultaneously the greatest lipophilicity.

According to spectra elaborated on for examined compounds in the range from 220 nm to 400 nm, the maximum wavelengths were also established. Further optimization experiments were performed at chosen analytical length of waves.

Taking into account differences in lipophilicity to analyze whole group of antiepileptics in one run in reversed-phase system, gradient elution mode should be applied. The use of 2-propanol, as an organic modifier, was most advantageous for new synthesized compounds (TP-315, TP-321, TP-323) but only under isocratic elution conditions. Changing concentration of this solvent in the mobile phase in gradient elution method resulted in the peaks splitting effect. In turn, for antiepileptic drugs (carbamazepine, phenobarbital, phenytoin) methanol caused overlapping of the peaks whereas acetonitrile appeared to be the most advantageous taking into account separation selectivity. Considering all previous experiments we propose gradient program starting from acetonitrile gradient and ending with gradient of 2-propanol. In this way the contact of new compounds with

Table 1
pK_a and log $P_{o/w}$ values for the antiepileptic drugs and investigated compounds.

Compound	pK_a	log $P_{o/w}$	λ_{max}
Carbamazepine	7.0	2.45[a]	280 nm
Phenobarbital	7.4	1.47[a]	240 nm
Phenytoin	8.3	2.47[a]	240 nm
TP-315	–	4.43[b]	254 nm
TP-321	–	6.09[b]	254 nm
TP-323	–	6.81[b]	260 nm

(–) A lack of ionizable basic or acidic functional groups.
[a] [4].
[b] log *P* predicted by Prolog D (module of the Pallas system).

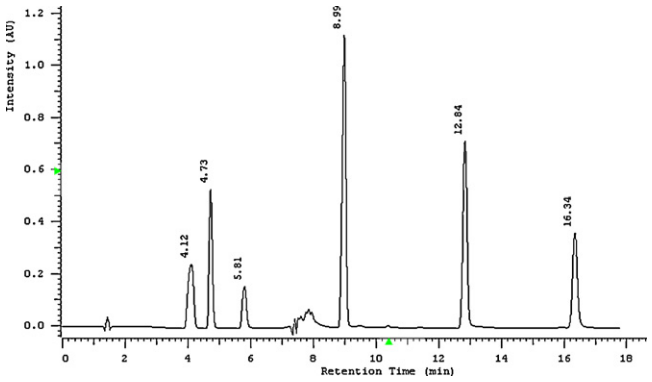

Fig. 2. Separation of tested compounds. Conditions as in Table S1.

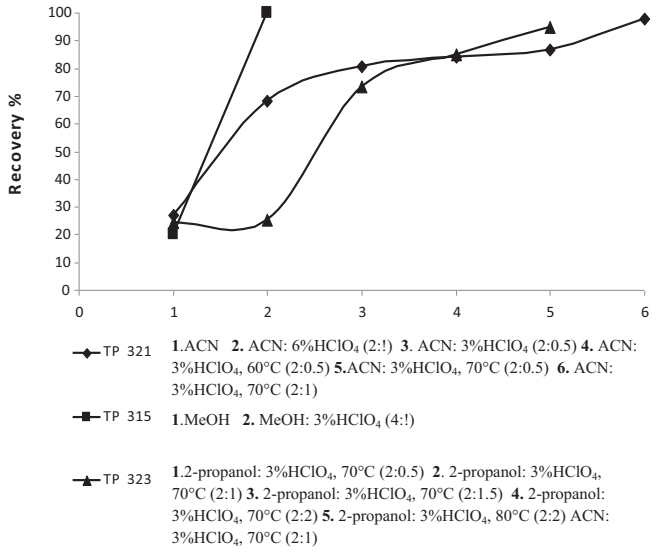

Fig. 3. Influence of the protein precipitation conditions on the extraction yield. Recoveries of the compounds were examined by using procedure described in Section 2.5. At each case, solution containing 15 μg of appropriate compound was added to blank brain tissue homogenate (200 μL).

2-propanol was reduced preventing them from splitting. The obtained results are presented on chromatogram in Fig. 2. As it can be seen, the applied eluent system permitted complete resolution of the whole analyzed group in an analysis time lower than 20 min. All peaks parameters are collected in Table S1 (the supplementary materials). The proposed method can be used for multi-component sample analysis in the study of a synergistic effect. Our preliminary studies have demonstrated such a synergistic action of newly synthesized compounds with conventional anticonvulsant drugs [10]. Thus, there exists a real possibility of utilizing newly-synthesized compounds in complex formulations containing reduced therapeutic doses while maintaining efficacy.

The second strategy is devoted to analysis of a sample containing only one component. In such case, we can use isocratic elution mode with organic–aqueous mobile phase to accelerate the analyses. The concentration and type of organic modifier depend on the lipophilicity of the examined compound. So, compound TP-315 required mobile phase containing 80% methanol, TP-321 85% acetonitrile whereas TP-323 according to the highest lipophilicity needed 80% 2-propanol to achieve an acceptable retention time. The peak parameters are collected in Table S2 (the supplementary materials) for the investigated compounds in their optimal chromatographic systems. The developed appropriate conditions were applied in further quantitative studies.

3.2. Method validation

3.2.1. Method linearity, LOD and LOQ values

Calibration curves were set up on investigated biological matrix, in our case – brain tissue homogenizate by addition of analyte standard solutions at six different concentrations. The obtained mixtures were further analyzed by the SPE procedure. Good linearity values with $r^2 > 0.995$ were found for all examined compounds. Complete results of linearity, LOD, and LOQ are presented in Table 2.

3.2.2. Optimization of sample preparation strategy (absolute recovery, precision)

Extraction yield experiments were carried out on blank brain tissue homogenate spiked with analyte standard solution with the concentration for each analyte equal to 10 μg mL^{-1}. Different kinds of sample preparation methods were tested for improving their absolute recovery. Taking into account solubility of the analyzed compounds each of them requires different organic solvent to be best soluble. Thus, to precipitate proteins methanol, acetonitrile and 2-propanol with addition of different amount of 6% $HClO_4$ (v/v) in water and different temperatures (20, 60, 70, and 80 °C) were tested. Obtained mixture was vortexed, centrifuged and supernatant was subjected to the SPE procedure according to method described in Section 2.6. On the basis of the obtained results presented in Fig. 3, the key matter to increase the extraction efficiency is the right proportion of organic solvent to perchloric acid and appropriate temperature of these processes. On the basis of these experiments, the optimal conditions for further studies were chosen for each analyte.

Analytical recovery was examined at three different concentration levels representing low, middle and high analyte contents. Recovery was determined via comparison of the theoretical content to the measured one as percentage. The mean recoveries determined from three different concentrations were above 85%. Obtained precision results were satisfactory. RSD% for repeatability

Table 2
Linearity, LOQ, and LOD parameters for the investigated compounds.

Compound	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Conc. range (μg mL^{-1})	Linearity equation: $y = ax + b$			
				$a \pm$ SD	$b \pm$ SD	R^2	F
TP-315	0.01039	0.03467	0.2–20	239,818 ± 4068	−70,353 ± 6305	0.9988	3473
TP-321	0.01305	0.04351	0.2–20	28,902 ± 583	−2986 ± 6303	0.9980	2451
TP-323	0.1020	0.3400	0.2–20	41,412 ± 164	−1119 ± 132	0.9999	63,923

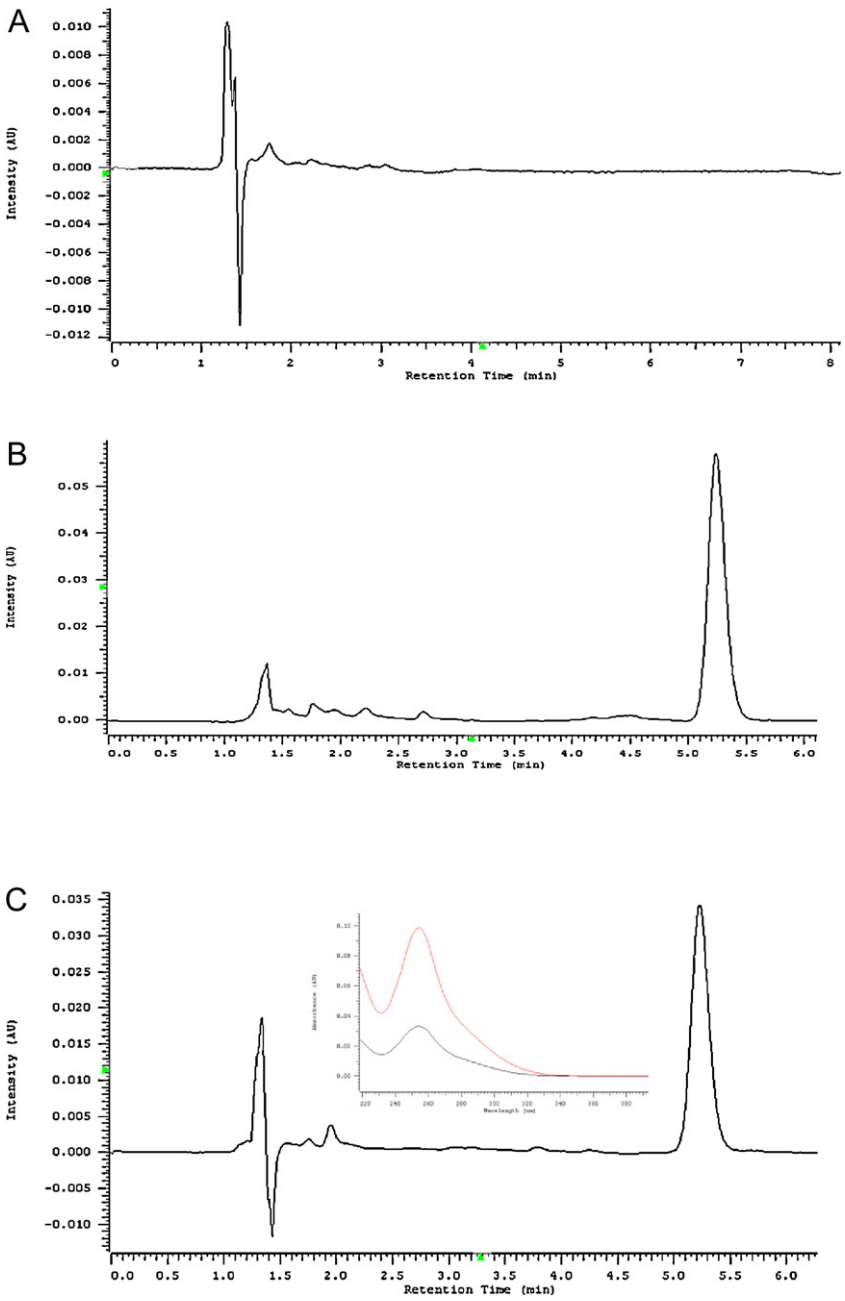

Fig. 4. Chromatograms of (A) a blank brain homogenate sample after the SPE procedure; (B) a blank brain homogenate sample spiked with TP-315 (50 μg mL^{-1}), subjected to the SPE procedure; (C) brain homogenate sample from mouse treated with TP-315 300 mg kg^{-1}. Small picture: UV-absorption spectra of TP-315 standard (upper line) and compound extracted from mouse brain treated with TP-315 from 220 to 400 nm. Conditions: stationary phase: Zorbax Extend-C18 (150 mm × 4.6 mm I.D; 5-μm), mobile phase 80% MeOH/water; flow rate 1 mL min^{-1}, detection 254 nm.

Table 3
Precision and accuracy assay results for investigated compounds on spiked brain tissue.

Analyte	Added μg	Found μg	Recovery (%)	Repeatability RSD%	Intermediate precision RSD%
TP-315	5.0	4.91	98.28	±2.03	±3.21
	7.5	7.49	99.98	±2.6	±4.2
	15.0	14.36	95.7	±2.1	±3.4
TP-321	0.5	0.44	87.0	±2.3	±4.2
	5.0	4.82	96.4	±2.6	±3.2
	15.0	14.7	98.0	±2.1	±2.8
TP-323	0.5	0.48	95.2	±2.6	±3.3
	5.0	4.86	97.2	±2.6	±2.6
	10.0	9.68	96.8	±1.2	±2.6

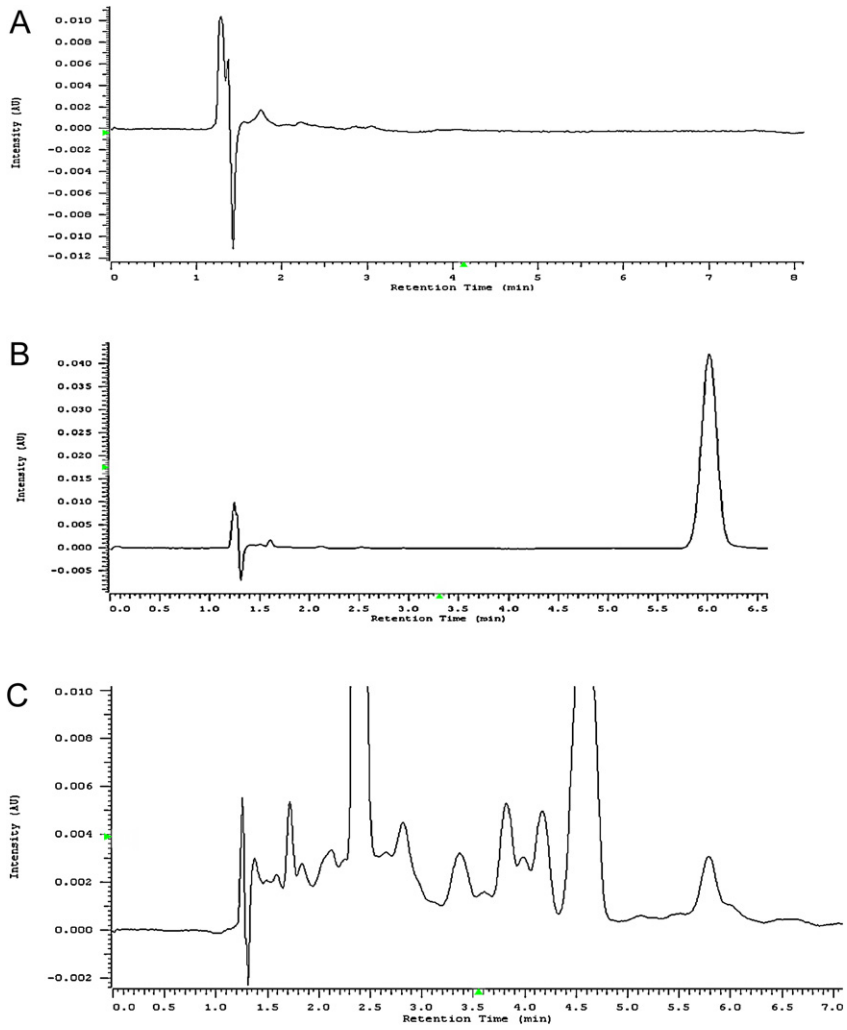

Fig. 5. Chromatograms of (A) a blank brain homogenate sample after the SPE procedure; (B) a blank brain homogenate sample spiked with TP-321 (8 μg mL^{-1}), subjected to the SPE procedure; (C) brain homogenate sample from mouse treated with TP-321 300 mg kg^{-1}. Conditions: stationary phase: Zorbax Extend-C18 (150 mm × 4.6 mm I.D; 5-μm), mobile phase 85% ACN/water; flow rate 1 mL min^{-1}, detection 254 nm. Identification of compound by overlapping the spectrum of isolated compound and standard TP-321.

was always lower than 3% and for intermediate precision lower than 5%. All results are summarized in Table 3.

3.2.3. Degradation study

For the acidic hydrolysis, 1 mL of 6% $HClO_4$ per 1 mL of test solution with concentration 50 μg mL^{-1} was added and kept in ambient temperature for 2 h. For the thermal degradation, 1 mL of water was added to 1 mL of 50 μg mL^{-1} test solutions. Obtained mixture was kept for 1 h in water batch at appropriate temperature, cooled and injected into the HPLC system. Additionally, the stability of standard solutions after storage for 1 month in refrigerator at 4 °C was determined. In each case, a fresh solution was analyzed for comparison. According to presented degradation studies, there is no evidence that tested stress conditions lead to major degradation of the investigated compounds (Table S3, the supplementary materials). The total recovery of each substance after stress testing was not lower than 85%. Only a long storage time (30 days) can damage the substances identified as TP-321 and TP-323. Additional peaks appeared after this time.

3.3. Analysis of brain sample

The described validated method was applied to the analysis of brain tissue of mice treated with investigated compounds with 300 mg kg^{-1} dose as well as brain tissue of control mice which received only saline. Results are presented in Table 4. Chromatograms of a blank sample, standard solution and the brain sample obtained by the use of appropriate procedure for each investigated compound are presented in Figs. 4–6. To identify the compounds overlaided spectra of isolated peaks and appropriate standards were performed.

Table 4
Quantified analyte concentrations in real biological samples.

Investigated compound	μg of compound found	Average mass of analyte (μg) per 1 mg of the brain tissue homogenate belonging to the mouse treated by the dose of 300 mg kg^{-1} (mean ± SD)	*n*
TP-315	1.8074–13.3031	7.2017 ± 0.10	6
TP-321	0.6939–0.9704	0.7029 ± 0.09	6
TP-323	Not found	Not found	6

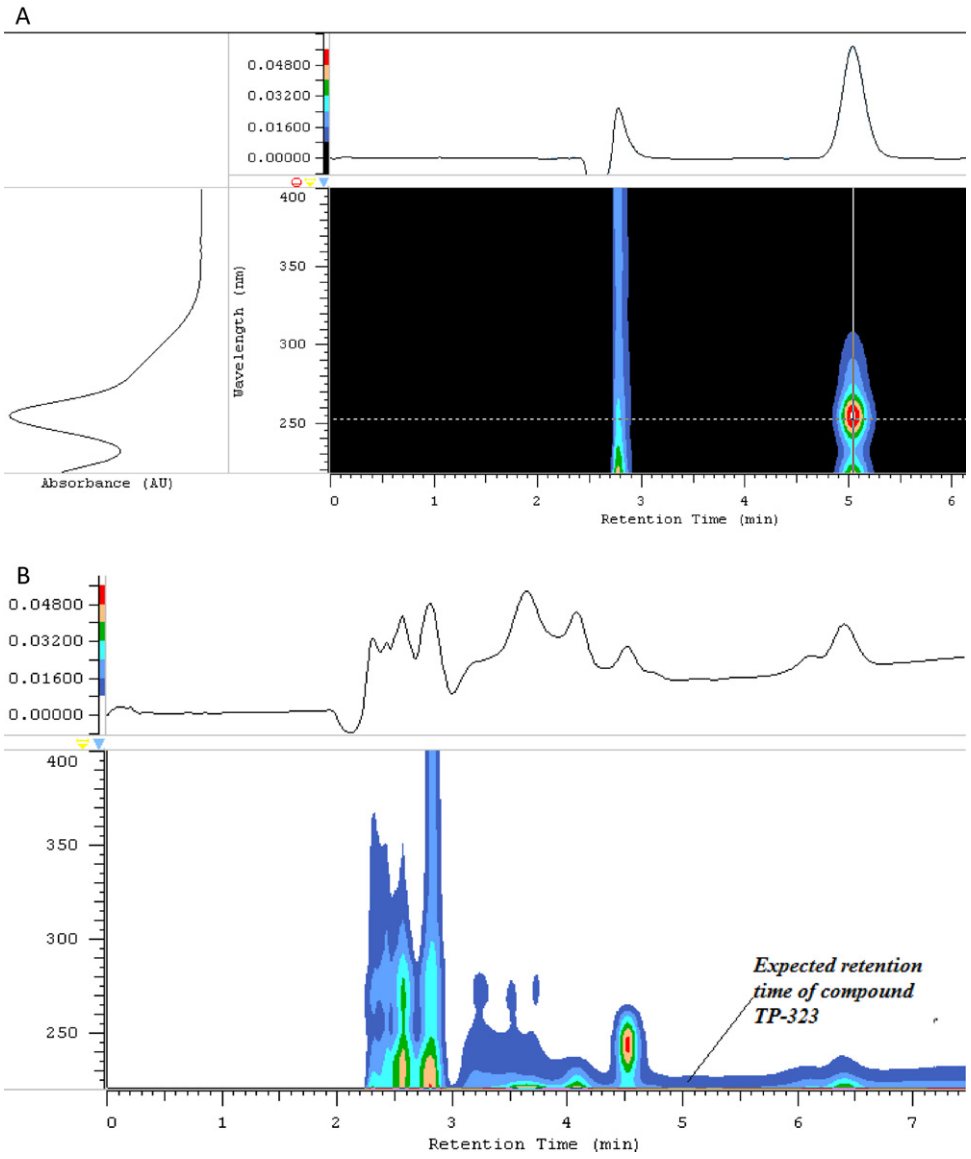

Fig. 6. Chromatograms of (A) a blank brain homogenate sample spiked with TP-323 (50 μg mL^{-1}), subjected to the SPE procedure; (B) brain homogenate sample from mouse treated with TP-323 300 mg kg^{-1}. Conditions: stationary phase: Zorbax Extend-C18 (150 mm × 4.6 mm I.D; 5-μm), mobile phase 85% 2-propanol/water; flow rate 0.5 mL min^{-1}, detection 260 nm.

The agreement of compared spectra was always higher than 0.995.

In case of homogenate of the mice brain tissue treated with compounds assigned as TP-323 and TP-321 for which minute activity was previously found, unknown metabolites appeared. We can anticipate that these metabolites are also inactive.

4. Conclusion

A sensitive HPLC method with DAD detection has been developed for the quantification of new synthesized compounds with different anti-epileptic activity in brain tissue of mice treated with a single dose. A rapid SPE procedure was developed. Owing to appropriate sample preparation, proposed strategy provides to suitable sample purification and gives extraction yields higher than 95% with precision lower than 5%.

On the basis of the obtained results we can conclude that the most promising is the compound assigned as TP-315 not only because of its therapeutic potential. Performed quantification of this compound in homogenate of mice brain tissue testify to the best permeability across the blood–brain barrier (BBB). The greatest amount of TP-315 is present in homogenate of the brain of mice treated with this compound in comparison to remaining tested compounds.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.10.011.

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