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Short communication

HPLC determination of tiazofurin in the rat brain

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Abstract

A sensitive, selective and reproducible HPLC method for determination of tiazofurin in rat brain was developed and validated. The method allowed determination and quantification of nanomolar concentrations of tiazofurin in brain and its regions (hippocampus, cortex and striatum) of treated animals. Separation of tiazofurin from other peaks from brain tissue was achieved by isocratic elution on reverse phase chromatographic column. The mobile phase consisted of 0.05 M sodium acetate pH 4.6. Run time was 15 min.

Keywords: HPLC; Tiazofurin; Rat brain

1. Introduction

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) is effective growth inhibitor of a variety of animal and human tumors [1–3]. Tiazofurin is also active against both rat and human glioma cells in vitro, causing inhibition of cell proliferation and induction of differentiation and apoptosis [4–6]. In tiazofurin-sensitive tumors, the drug is metabolized to thiazole-4-carboxamide adenine dinucleotide (TAD), an analogue of NAD, which strongly inhibits inosine-monophosphate dehydrogenase (IMPDH), the rate limiting enzyme of guanylate synthesis pathway [7,8]. Elevated IMPDH activity recorded in some brain tumors [9] makes them a good biochemical target for tiazofurin action. Transport studies showed that tiazofurin penetrates the blood–brain barrier [10] and suggested its possible role in brain tumor treatment [11]. These results initiated investigation of tiazofurin effects on animal model of brain tumor developed in our laboratory [12], which requested tiazofurin quantification in brain tissue. Furthermore, our experiments showed that tiazofurin modifies central regulation of the motor activity in the rat, possibly acting on adenosine A1 receptors [13]. More detailed investigation of these effects also required tiazofurin quantification in the brain.

Previously proposed methods for determination of tiazofurin content in tissue extracts usually included radioactive labeling of the drug [14]. The available literature describes several methods

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with UV detection of tiazofurin in plasma, erythrocytes and urine [15–17], which in our hands were not applicable for its determination in brain tissue. Our recently published method for simultaneous determination of tiazofurin, its 5′-O esters and TAD in biological samples [18] also failed to detect tiazofurin content in brain, due to the overlapping peaks from this complex biological material. These results indicated the necessity for development of a new method for quantification of tiazofurin in brain tissue.

2. Materials and methods

2.1. Chemical and reagents

Tiazofurin was synthesized in Laboratory for synthesis of Galenika a.d., and its structure was confirmed by ^{1}H and ^{13}C NMR, mass spectroscopy and elemental analysis. The purity of tiazofurin was more than 99% determined by HPLC. Sodium acetate and potassium chloride were of high purity grade, obtained from Merck.

2.2. Experimental model

Male Wistar albino rats (2–2.5 months old, 180–220 g) were injected with tiazofurin dissolved in distilled water (125 mg/kg i.p.). Animals were anaesthetized with thiopentone sodium (30 mg/kg i.p.) and sacrificed by decapitation.

Rats were maintained in accordance with principles enunciated in the Guide for Care and Use of Laboratory Animals, NIH publication No 85-23.

2.3. Sample preparation

Brain samples were removed and homogenized in 0.15 M KCl (pH 7.4 adjusted with 0.1 M KOH; 1 ml of KCl was added to 1 g of tissue). Brain homogenate was centrifuged at 40000 rpm at 4 °C for 30 min. After centrifugation, the tube with the supernatant was placed in boiling water for 3 min to achieve protein precipitation, and subsequently centrifuged at 40000 rpm for 20 min. Supernatant was filtered trough Sartorius filter 0.2 μm and the clear supernatant was used for analysis. Tiazofurin concentrations were also determined in brain regions: hippocampus, cortex and striatum. For that purpose, brain regions of five animals were carefully separated, pooled and prepared using the same procedure.

2.4. Instrumentation

Analyses were performed on HPLC system Hewlett–Packard 1100 with the quaternary pump and diode-array detector. Temperature was maintained at 35 °C in thermostated column compartment.

2.5. Chromatographic conditions

Column: Zorbax SB-C18, 4.6 × 250 mm, 5 μm. Guard-column: Pelliguard LC-18, 2 cm × 4.6 mm, 5 μm. Temperature: 35 °C. Flow: 2 ml/min. Injection volume: 100 μl. Detection: 254 nm. Mobile phase: sodium acetate 0.05 M, pH 4.6 adjusted with glacial acetic acid.

3. Results

3.1. Method validation

Peak identification was performed by comparison of retention times and UV spectra of the eluting peaks with the known standard. Retention time for tiazofurin was 9.0 min.

Comparison of chromatographic profiles of the prepared brain samples of tiazofurin treated and untreated animals showed no interference of the peak of interest with the unidentified peaks from biological matrix. Elution profiles of the representative sample (treated animals), the control sample (untreated animals) and tiazofurin standard are shown in Fig. 1.

The linearity of the method was determined by six-point calibration. Concentrations of standards were 0.5, 2.5, 7.5, 10.0, 12.0 and 15 μg/ml of tiazofurin. Linearity curve, correlation coefficients and residual standard deviation values are given in Table 1.

The precision of measurements was examined by six replicate injections of the same standard

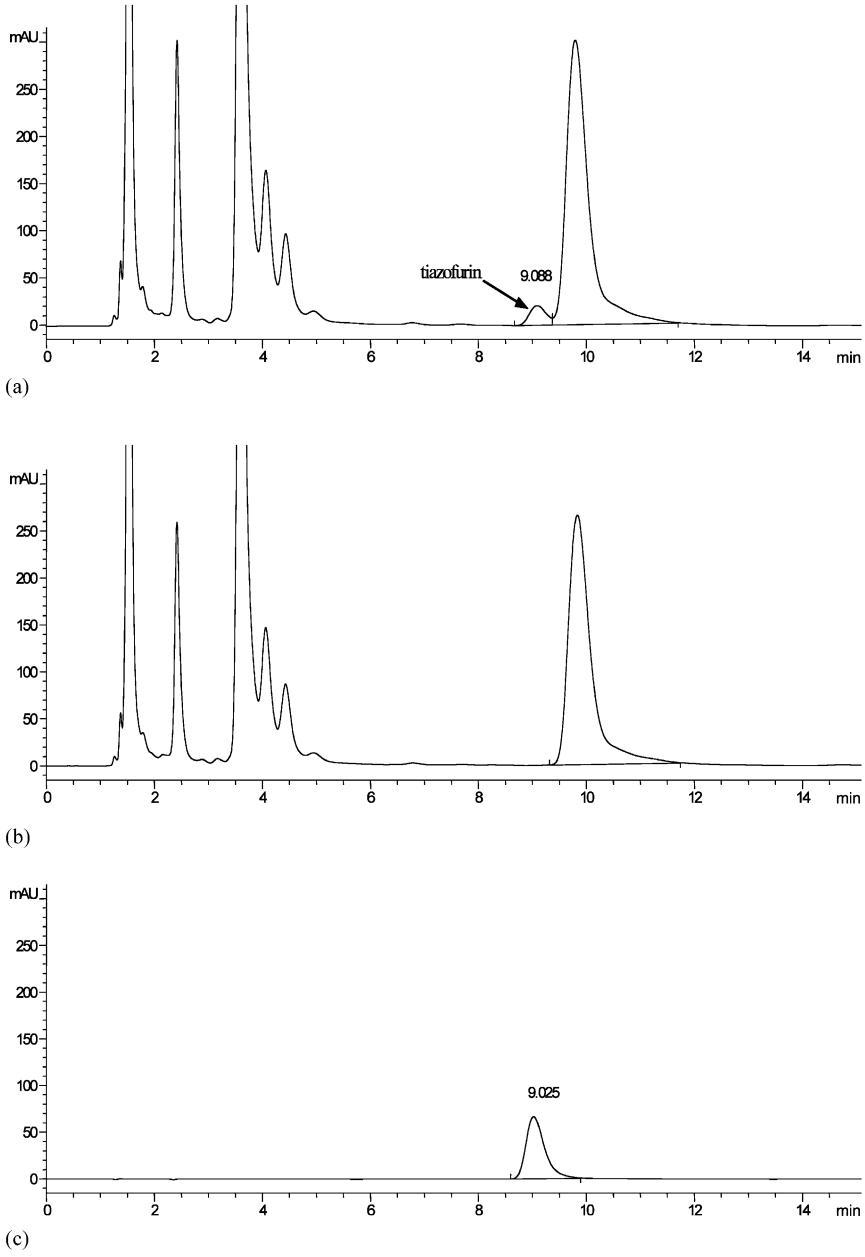

Fig. 1. Elution profiles of the representative samples of: (a) treated animal, (b) control untreated animal and (c) tiazofurin standard.

solution (concentration of tiazofurin in the standard solution was 10 μg/ml). Coefficient of variation of retention times for tiazofurin peak was 0.098%, and coefficient of variation of peak area was 0.669%.

The accuracy of the method was determined by addition of known quantities of tiazofurin standard to the prepared brain extract of untreated animals. The samples were further prepared and analyzed as described. Recoveries and relative

Table 1
Linearity curve, correlation coefficient and residual standard deviation (RSD) for HPLC determination of tiazofurin in the rat brain

Concentration range (μg/ml)	Linearity curve	Correlation coefficient	RSD
0.5–15.0	$y = 31.42x - 1.16$	0.99991	2.84

Linearity curve is given according to the equation: $y = ax + b$. Linearity was determined by six-point calibration.

standard deviations for tiazofurin, for nine tested samples, are given in Table 2.

The limits of detection and quantification for tiazofurin were 0.12 and 0.33 μg/ml, respectively. Limits of detection and quantification were determined mathematically, as three and ten times the standard deviation of noise over the time range of the eluting peak.

3.2. Concentration of tiazofurin in the brain of treated rats

This method allowed determination of tiazofurin concentration in the whole brain as well as in the brain regions: hippocampus, cortex and striatum. The results are given in Table 3.

The results obtained in this study confirmed tiazofurin penetration through the blood–brain barrier. Tiazofurin was detectable in rat brain after 15 min, reached the highest concentration 2 h after drug administration and was still present in tested samples 6 h after drug injection (data not shown). Since tiazofurin concentration reached its maximum 2 h after administration, the same period was chosen for determination of this drug in brain regions. Obtained results show the highest concentration of tiazofurin in cortex (Table 3).

Table 2
Accuracy of the HPLC method for determination of tiazofurin in rat brain

Added (μg/ml)	Found (μg/ml)	Recovery (%)
15.00	14.86	99.06
15.00	14.38	95.86
15.00	15.26	101.73
7.50	7.66	102.13
7.50	7.28	97.07
7.50	6.74	89.86
1.00	1.09	109.00
1.00	1.11	111.00
1.00	1.01	101.00
RSD (%)		6.42

The accuracy of the method was determined after addition of known quantities of tiazofurin to the brain homogenate of untreated rats, which was further prepared and analyzed according to the procedure described in Section 2. Table 2 shows recoveries and relative standard deviation (RSD) of tiazofurin determination for nine analyzed samples, in three concentrations.

Table 3
Determined concentrations of tiazofurin in the whole brain and brain regions 2 h after drug administration

Brain region	Concentration (nmol/g of tissue)
Whole brain	10.43 ± 0.82
Cortex	1.59 ± 0.13
Hippocampus	1.14 ± 0.09
Striatum	0.67 ± 0.07

Concentration of tiazofurin is presented as mean values ± standard deviations of three independent samples.

4. Discussion

This paper describes a simple and rapid procedure for HPLC determination of tiazofurin in the rat brain.

None of the previously proposed HPLC methods for tiazofurin quantification by UV detection [15–17] was applicable for brain tissue samples, due to the inability to achieve acceptable separation of tiazofurin peak from the brain matrix peaks.

The proposed method uses previously described sample preparation procedure [18] which avoids the use of organic solvents and aggressive acids. In comparison to the previously published [18], the presented method differs in mode of elution, solvent composition, column temperature and flow rate. The sensitive and selective HPLC

separation is based on isocratic elution, while the mobile phase without organic solvents protects the operator, as well as the environment. Faster separation was achieved with increased column temperature (run time 15 min) and higher flow rate, which enables analysis of a large number of samples.

Even though the method proved to be linear in very low concentrations (0.5–15 μg/ml), it was necessary to pool the brain regions of five animals to achieve the detectable concentration of tiazofurin under these conditions. On the other hand, the brain extract of a single experimental animal was used for quantification of tiazofurin in the whole brain.

Relative standard deviation of tiazofurin recoveries (6.42%) confirmed the adequacy of sample preparation procedure and chromatographic separation. Limit of detection and limit of quantification values indicate the possibility of determination and quantification of very low tiazofurin concentrations in a complex biological material.

The results obtained using the described method confirm that tiazofurin penetrates the blood–brain barrier and enters the rat brain to a certain extent. Tiazofurin was detected in all of tested brain regions: hippocampus, cortex and striatum. These results are a clear evidence of presence of tiazofurin in brain, as well as of its uneven distribution in brain regions of treated animals. Determined concentrations of tiazofurin were in nanomolar range, which is in good correlation with previous findings [14]. Furthermore, our method enabled tiazofurin quantification in liver, spleen and kidney of the treated rats (data not shown), which confirmed its applicability in different tissues.

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