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Simultaneous LC determination of tiazofurin, its acetyl and benzoyl esters and their active metabolite thiazole-4-carboxamide adenine dinucleotide in biological samples

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Abstract

A rapid and sensitive HPLC-RP method for simultaneous determination of tiazofurin, its 5′-*O* acetyl and benzoyl esters and their active metabolite thiazole-4-carboxamide adenine dinucleotide was developed and validated. The method allowed determination and quantification of nanomolar quantities of these substances in cell extracts of treated cells, and was also used in kinetic studies of cellular uptake of tiazofurin and its esters from the cultivation medium. Separation of the analyzed substances from unidentified peaks from both biological materials was achieved by gradient elution, thus reducing the possibility of interference. The mobile phase consisted of a 0.1 M sodium–hydrogen phosphate, pH 5.1 and methanol. Run time was 22 min, with 5 min equilibration time.

Keywords: HPLC; Tiazofurin; Acetyl and benzoyl esters; TAD

1. Introduction

Synthetic C-nucleoside tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) is a potent antitumor agent that inhibits inosine-monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme of the guanylate synthesis pathway which catalyses the conversion of IMP to XMP [1]. In sensitive cells tiazofurin is transformed to its active metabolite thiazole-4-carboxamide adenine dinucleotide (TAD), an analogue of NAD, which binds to the enzyme, thus inhibiting its activity [2]. Acetyl-tiazofurin [2-(5-*O*-acetyl-β-D-ribofuranosyl)thiazole-4-carboxamide] and benzoyl-tiazofurin [2-(5-*O*-benzoyl-β-D-ribofuranosyl)thiazole-4-carboxamide] are 5′-*O* esters of tiazofurin, synthesized in order to improve its biological activity, but their biochemical effect is still under investigation. Previous experiments showed that these tiazofurin derivatives had different influence on cell proliferation and induction of apoptosis in C6 rat glioma cells in vitro [3].

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C6 rat glioma cell line is a well-known experimental model in neurooncology, whose sensitivity to treatment by tiazofurin was established in earlier experiments [4]. Since this cell line has very high activity of IMPDH [5], it represents a good model for studies of its inhibition.

One of the first steps towards the elucidation of the mechanism and kinetics of action of acetyl- and benzoyl-tiazofurin was the attempt to determine the concentrations of tiazofurin and/or its 5′-*O* esters and their active metabolite TAD in extracts of in vitro treated cells. Tiazofurin and TAD in normal human leukocytes [6] and mononuclear cells of leukemic patients treated with tiazofurin [7] were previously determined by reverse-phase and anion-exchange HPLC with UV detection, but none of the proposed methods could be applied for quantification of acetyl- and benzoyl-tiazofurin. For that purpose we developed and validated a sensitive HPLC method for simultaneous determination of the three nucleoside analogues and their active metabolite TAD.

2. Materials and methods

2.1. Chemicals and reagents

Acetyl-tiazofurin and benzoyl-tiazofurin were synthesized in Laboratory for synthesis of ICN Galenika (unpublished data), as well as tiazofurin and TAD. The structures of tiazofurin and its acetyl and benzoyl esters was confirmed by ^{1}H and ^{13}C NMR, mass spectroscopy and elemental analysis, and their purity, determined by HPLC, was more than 99%. TAD structure was confirmed by ^{1}H and ^{13}C NMR and its purity was 96.7%, determined by HPLC.

All cell cultivation reagents were purchased from ICN Pharmaceuticals, Costa Mesa, CA. Inorganic chemicals (potassium chloride, sodium phosphate monohydrate) were of high purity grade, obtained from Merck. Methanol (HPLC grade) was also purchased from Merck.

2.2. Cell culture

C6 rat glioma cells were cultivated and maintained as previously described [4]. Cells were incubated in a medium containing 60 μM of tiazofurin, acetyl-tiazofurin or benzoyl-tiazofurin. After previously defined incubation time, cells were harvested by trypsinization and centrifugation. The supernatant was discarded and the cells were frozen until further analysis.

2.3. Sample preparation

Samples of treated cells were prepared according to the modified literature method for HPLC nucleoside determination in biological materials [9]. Cells were homogenized in 0.15 M KCl, pH 7.4 (0.5 ml of KCl was added to 100 mg of cells). Cell homogenate was centrifuged at 40 000 rpm at 40 °C for 35 min. After centrifugation, the tubes with the supernatant were placed in boiling water for 1 min and centrifuged at 15 000 rpm for 10 min. The supernatant was filtered through Sartorius filter 0.2 μ and the clear filtrate was used for injection. Cell cultivation media were prepared according to the same procedure, without prior homogenization and first centrifugation step.

2.4. Instrumentation

Analyses were performed on HPLC system Hewlett-Packard 1100 equipped with the quaternary pump and a diode-array detector. Temperature was maintained at 25 °C in thermostated column compartment.

2.5. Chromatographic conditions

1) Column: Supelcosil LC-18, 25 cm × 4.6 mm, 5 μm
2) Guard-column: Pelliguard LC-18, 2 cm × 4.6 mm, 5μm
3) Temperature: 25 °C
4) Flow: 1 ml/min
5) Injection volume: 100 μl
6) Detection: 254 nm
7) Mobile phase:

Table 1
Gradient elution programme for HPLC determination of tiazofurin, its acetyl and benzoyl esters and their active metabolite TAD

Time (min)	Solvent A (%)	Solvent B (%)
Initial	95	5
10.0	85	15
15.0	40	60
22.0	40	60

Solvent A: 0.1 M sodium-phosphate, pH 5.1; Solvent B: methanol. Equilibration of the column was performed with the initial mobile phase composition for 5 min.

Solution A: 0.1M Na_2HPO_4, pH 5.1
Solution B: methanol

8) Gradient elution programme is given in Table 1
9) Equilibration time: 5 min with initial mobile phase composition

3. Results

3.1. Method validation

(a) Peak identification was performed by comparison of retention times and UV spectra of the eluting peaks with known standards. Retention times of the analyzed substances were: 8.6 min for tiazofurin, 9.4 min for TAD, 16.7 min for acetyl-tiazofurin and 19.8 min for benzoyl-tiazofurin.

(b) Comparison of chromatographic profiles of the extracts of treated and untreated cells, as well as of cell cultivation media with and without nucleoside analogues, showed no interference of the peaks of interest with the unidentified peaks from biological matrices. The peaks of the four analyzed substances were well separated from the unidentified peaks deriving from cellular matrix and cell cultivation media. Elution profiles of the representative tested samples and standard mixture are shown in Fig. 1.

(c) The linearity of the method was determined by six-point calibration, in two different concentration ranges; the lower concentration range (1.25–12.5 μM) was used for quantification of tiazofurin, TAD and tiazofurin derivatives in cell extracts, whereas the higher concentration range calibration included predicted concentrations for the analyses of tiazofurin and its esters in cell cultivation media (10–100 μM). Standard mixture was prepared by dissolving standards of the four analyzed substances in water. Standard concentrations for lower concentration range were 1.25, 2.5, 5.0, 6.25, 8.75 and 12.5 μM. Higher concentration range calibration included standards of 10, 25, 35, 50, 75 and 100 μM. Linearity curves, correlation coefficients and residual standard deviations (RSD) values for calibrations in both concentration ranges are given in Table 2.

(d) The precision of measurements was examined by six replicate injections of the same standard solution (concentration of the analyzed substances in the standard solution was 5.0 μM) added to the homogenate of untreated cells prepared as described in Section 2, thus fully mimick-

Table 2
Linearity curves, correlation coefficients and RSD for lower and higher concentration range calibration for HPLC determination of tiazofurin, its acetyl and benzoyl esters and their active metabolite TAD

Concentration range (μM)	Compound	Linearity curve	Correlation coefficient	RSD
1.25–12.5	Tiazofurin	$y = 24.70x - 0.936$	0.99993	0.25
	TAD	$y = 1.38x + 0.197$	0.99991	0.27
	Acetyl-tiazofurin	$y = 21.35x + 0.042$	0.99923	1.14
	Benzoyl-tiazofurin	$y = 26.37x - 2.880$	0.99899	1.06
10–100	Tiazofurin	$y = 18.97x - 0.643$	1.00000	1.54
	Acetyl-tiazofurin	$y = 15.81x - 0.012$	1.00000	0.84
	Benzoyl-tiazofurin	$y = 19.27x - 0.397$	0.99998	1.64

Linearity curves are given according to the equation: $y = ax + b$. Linearity for both concentration ranges was determined by six-point calibration.

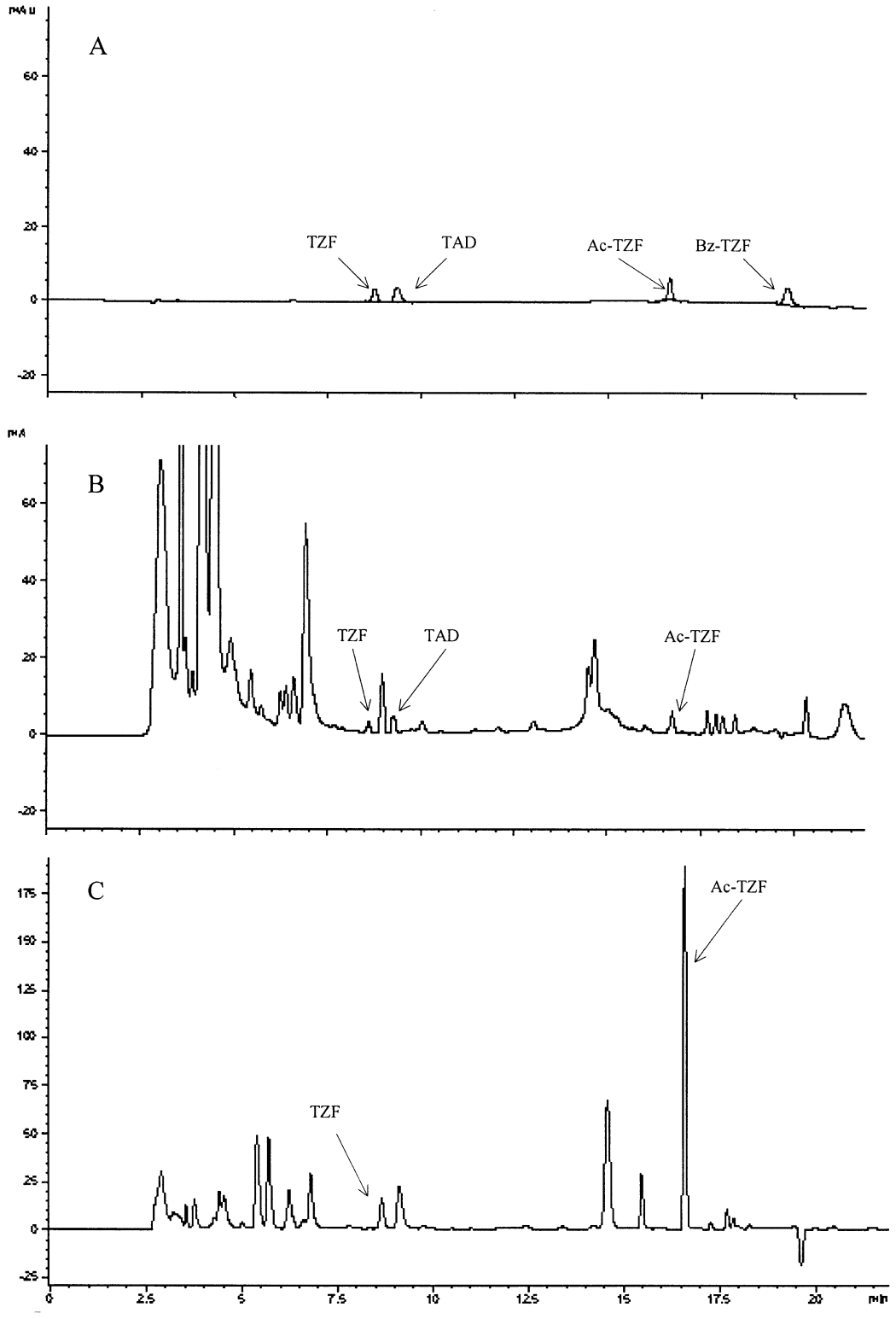

Fig. 1

Table 3
Accuracy of the method for simultaneous determination of tiazofurin, its acetyl and benzoyl esters and their active metabolite TAD

Recovery (%)				
Added (μmol)	Tiazofurin	TAD	Acetyl-tiazofurin	Benzoyl-tiazofurin
2.258	96.97	103.22	107.17	101.77
2.941	100.58	102.52	100.75	92.76
3.846	103.64	102.29	102.70	99.58
4.146	104.68	100.92	100.10	96.72
4.545	98.71	93.53	107.94	96.04
RSD (%)	3.21	3.96	3.50	3.55

The accuracy of the method was determined by addition of known quantities of the four tested substances to the homogenate of untreated cells, which was further prepared and analyzed according to the procedure described in Section 2. The table shows recoveries and relative standard deviation for determination of tiazofurin, TAD and tiazofurin esters in five analyzed samples.

Table 4
LD and LQ for tiazofurin, its derivatives and TAD (in nanomoles applied to the column)

	Tiazofurin	TAD	Acetyl-tiazofurin	Benzoyl-tiazofurin
LD	2.60	10.3	2.38	8.54
LQ	8.68	34.3	7.96	28.5

The LD and LQ were determined mathematically as three and ten times the standard deviation of noise for the time period of the eluting peak.

ing the experimental conditions of HPLC analysis. Coefficients of variation of retention times for the four substances under investigation were 0.046, 0.075, 0.015 and 0.014% for tiazofurin, TAD, acetyl-tiazofurin and benzoyl-tiazofurin, respectively. Coefficients of variation of peak areas for the same substances were 0.146, 0.232, 0.111 and 0.085%, respectively.

(e) The accuracy of the method was determined by addition of known quantities of the four analyzed substances to homogenate of untreated cells. The samples were further prepared and analyzed as described. Recoveries and relative standard deviations for all substances under investigation, for five tested samples, are given in Table 3.

(f) The limits of detection (LD) and quantification (LQ) for tiazofurin, TAD, acetyl- and benzoyl tiazofurin are shown in Table 4. LD and LQ were determined mathematically, as three and ten times the standard deviation of noise over the time range of the eluting peak, respectively.

(g) The stability of the analytes was tested by leaving the prepared cell extracts and cell cultivation media overnight, on room temperature. None of the four tested substances degraded under these conditions.

(h) The method ruggedness was tested by changing the pH of the solution A below and above the proposed pH value. These small changes did not affect the retention times and shapes of the peaks of interest.

3.2. Concentrations of tiazofurin, tiazofurin esters and TAD in treated C6 cells

The new HPLC method was applied for quantification of tiazofurin, tiazofurin esters and TAD in cell extracts of treated C6 rat glioma cells. The

Fig. 1. Elution profiles of the standard mixture and representative samples. (A) standard mixture (standards were dissolved in initial mobile phase; concentration of tiazofurin and its esters was 1.25 μM, and TAD concentration was 2 μM; (B) a sample obtained from C6 cells treated with acetyl-tiazofurin; (C) a sample of cell cultivation medium with acetyl-tiazofurin. *Abbreviations:* TZF, tiazofurin; Ac-TZF, acetyl-tiazofurin; Bz-TZF, benzoyl-tiazofurin.

Table 5
Determined concentrations of tiazofurin, its esters and their active metabolite TAD in treated C6 cells after 2 h of incubation

	Concentration (nmol/mg cells)			
Treatment	Tiazofurin	TAD	Acetyl-tiazofurin	Benzoyl-tiazofurin
Tiazofurin	14.5±0.3	21.5±3.9		
Acetyl-tiazofurin	14.2±3.1	21.7±0.7	29.6±3.9	
Benzoyl-tiazofurin	17.6±2.7	11.2±0.6		2.4±0.3

Mean values±standard deviations of three independent samples are presented.

cells were treated with tiazofurin, acetyl- or benzoyl-tiazofurin (60 μM) for 2 h and the samples were prepared as described in Section 2. The results are given in Table 5.

The obtained results indicate that both tiazofurin esters undergo intracellular hydrolization to tiazofurin and subsequently metabolize to the active metabolite TAD, to a different degree.

4. Discussion

Esterification of tiazofurin was aimed to enhance the lipophylic profile, and subsequently the cellular uptake of the pro-drug tiazofurin, thus increasing its antineoplastic potential. It is expected that tiazofurin 5′-*O* esters share the same metabolic pattern with tiazofurin after penetration through the cell membrane. Metabolic pathway of both esters most probably involves hydrolyzation to tiazofurin, which is further phosphorylated and metabolized to active metabolite TAD. Hence, simultaneous determination of tiazofurin acetyl and benzoyl esters, tiazofurin and their active metabolite became a necessity in experiments designed to investigate biochemical activity of these new tiazofurin analogues. For the purpose of their quantification in biological materials, a rapid RP-HPLC method was developed and validated.

The applicability of the method was tested by simultaneous quantification of tiazofurin, acetyl or benzoyl ester of tiazofurin and their active metabolite TAD in treated C6 rat glioma cells and in cell cultivation media. Experiments were designed in such a manner as to enable method testing in two different concentration ranges, as well as on different biological materials.

Sample preparation was modified with regard to the chemical stability of the examined substances. Previously proposed procedures for quantification of nucleosides in different tissue or cell extracts included protein precipitation in highly acidic environment [7–9]. Since this treatment caused significant acid hydrolysis of both tiazofurin 5′-*O* esters, especially acetyl-tiazofurin, sample proteins were precipitated by short-term denaturation in boiling water. Literature data show that very brief exposure to this temperature should not affect TAD stability in prepared extracts [10], which justified the use of this sample preparation procedure.

Gradient elution is a common feature in RP-HPLC methods for determination of nucleotides, nucleosides and their analogues in biological materials [9,11,12]. A rather sharp increase of organic solvent content in the mobile phase used in our method enabled faster elution of hydrophobic benzoyl derivative, together with certain matrix components, thus preventing deterioration of the column due to uneluting peaks. The choice of the chromatographic column and the proposed mobile phase composition represent a standard in liquid chromatography, thus making this method relatively inexpensive and available. None of the four tested substances degraded overnight in prepared cell extracts or in cell cultivation media samples, which allowed sample injection from the autosampling device. Small changes of pH value of the mobile phase did not significantly affect the retention times and shapes of the peaks of interest in samples of C6 cell extracts and cell cultivation medium. However, content of other biological

matrices could influence or interfere with the eluting peaks, and this parameter should be carefully considered with each new application.

In conclusion, the obtained results show that this HPLC method could be used for simultaneous determination and quantification of tiazofurin, its acetyl and benzoyl esters and their active metabolite TAD in different biological materials. The method is also stability-indicating, since both tiazofurin esters could degrade to tiazofurin under certain conditions. The method is linear within a wide range of concentrations and quantification of the analyzed substances is accurate and reliable. Peaks of interest are well separated from unidentified peaks from the both studied biological materials. LD and LQ are in nanomolar range, which makes the method applicable for determination of very small concentrations of the analyzed substances.

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