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HPLC methods for the determinatiuon of acetyl- and benzoyl-tiazofurin in rat plasma

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Abstract: Reverse-phase HPLC methods for determination of 5'-*O*-acetyl-tiazofurin (AT) and 5'-*O*-benzoyl-tiazofurin (BT) in rat plasma were developed and validated in terms of specificity, linearity, precision, accuracy and sensitivity. Linear calibration curves were constructed in the concentration range of 2.50 – 100.00 μmol/L for both compounds. The separations were achieved on a Supelcosyl LC-18-DB analytical column (250 × 4.6 mm; 5 μm particle size) by isocratic elution, with a mixture of 0.1 M disodium hydrogen phosphate – methanol. UV detection was performed at a wavelength of 254 nm. The proposed methods enable the detection and quantification of nanomolar concentrations of AT and BT in rat plasma.

Keywords: HPLC, acetyl-tiazofurin, benzoyl-tiazofurin, rat plasma.

INTRODUCTION

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) is a synthetic C-nucleoside which has demonstrated clinical efficacy as an antitumor agent.[1] Tiazofurin exhibits antitumor activity against several human and murine tumors.[2–6] It was found that tiazofurin in sensitive cells inhibits inosine-monophosphate dehydrogenase (IMPDH) activity and decreases the cellular concentration of GTP, resulting in the inhibition of cell proliferation and the induction of differentiation and apoptosis.[7–9] The drug induces IMPDH inhibition after intracellular conversion to the active metabolite thiazole-4-carboxamide adenine dinucleotide (TAD), which binds tightly at the NAD^+ site and inhibits IMPDH activity.

Two novel 5'-*O* esters of tiazofurin, 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], were synthesized in order to improve the biological ac-

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tivity of the patent compound.[10] Previous experiments showed that these tiazofurin derivatives had different effects on cell proliferation and induction of apoptosis *in vitro*.[11]

The purpose of this study was to develop simple and sensitive HPLC methods for the determination of AT and BT in rat plasma, which could be used to study the pharmacokinetics of these tiazofurin derivatives. The previously published methods for tiazofurin analysis in plasma[12–14] usually included reverse-phase and anion-exchange HPLC with UV detection, but none of the proposed methods could be applied for quantification of AT and BT. A recently developed method for the simultaneous determination of tiazofurin, its acetyl and benzoyl esters and their active metabolite TAD in rat glioma cells and in cell cultivation media[15] was not applicable for the quantification of AT and BT in rat plasma, due to overlapping by peaks of substances from this biological material.

EXPERIMENTAL

Chemicals and reagents

AT and BT were synthesized in the Laboratory for Synthesis of Galenika a.d., their structures were confirmed by ^{1}H and ^{13}C-NMR mass spectroscopy and elemental analysis, and their purity, determined by HPLC, was more than 99 %. Disodium hydrogen phosphate monohydrate was of high purity, purchased from Merck. HPLC-grade methanol was also purchased from Merck. Distilled, deionised water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA).

Experimental animals

Male Wistar albino rats (2 – 2.5 months old, 180 – 220 g) were used in the experiments. The rats were maintained in accordance with the principles declared in the Guide for Care and Use of Laboratory Animals, NIH publication No 85-23. The rats were intraperitoneally (i.p.) administered with AT or BT (125 mg/kg).

Sample preparation

Blood samples were taken five minutes after drug administration using heparinized glass vials and immediately centrifuged at 4000 rpm at 4 ºC for 5 min. The plasma samples (500 μL) were transfered to 1.5-mL polypropylene Eppendorf tubes and placed in boiling water for 2 min to achieve protein precipitation. After boiling, the plasma was vortexed with 500 μL of water and centrifuged at 10000 rpm at 4 ºC for 10 min. The supernatant was filtered through a Sartorius filter 0.2 μm and the filtrate was used for analysis.

HPLC Instrumentation

Determination of AT and BT was performed on a Hewlett-Packard 1100 HPLC system equipped with a binary pump and diode-array detector. The analytical column was maintained at 25 ºC using a thermostated column compartment.

Chromatographic conditions

The mobile phase for determination of AT consisted of a mixture of 0.1 M disodium hydrogen phosphate (pH 5.1) – methanol (75 : 25, v/v) at a flow-rate of 1 mL/min. Determination of BT required an increase of the organic solvent content in the mobile phase. The mobile phase in this case consisted of a mixture of 0.1 M disodium hydrogen phosphate (pH 5.1) – methanol (35 : 75, v/v) at a flow-rate of 0.7 mL/min. The other chromatography conditions were the same for both compounds: Column: Supelcosil LC-18-BD, 250 × 4.6 mm, 5μm. Guard-column: Pelliguard LC-18, 20 × 4.6 mm, 5 μm. Temperature: 25 ºC. Injection volume: 20 μL. Detection: 254 nm.

RESULTS

Validation of the methods

1) Peak identification was performed by comparision of retention times and UV spectra of the peaks of interest with known standards. The retention times of the ana-

lysed substances were: 7.2 min for AT and 5.7 min for BT.

2) The specificity of the assays was tested by visual inspection and by comparison of the chromatograms of prepared plasma samples of treated and untreated animals. The peaks of unidentified plasma components did not elute in the same region as AT or BT. The HPLC chromatograms obtained for the AT standard, a plasma sample with AT and a control sample (untreated animal) are shown in Fig. 1. The HPLC chromatograms for the BT standard, a plasma sample with BT and a control sample (untreated animal) are shown in Fig. 2.

3) The linearity of the methods was assessed by standard ranging from 2.5 to 100 μM for both compounds. The standard solutions were prepared by dissolving standards in distilled water. The linearity curves, correlation coefficients and residual standard deviation values for both analysed substances are given in Table I.

TABLE I. Linearity curves, correlation coefficients and residual standard deviation values (*RSD*) for the HPLC determination of acetyl-tiazofurin and benzoyl-tiazofurin

Compound	Conc. range/μM	Linearity curve	Correl. coeff.	*RSD*
Acetyl-tiazofurin	2.50 – 100.00	$y = 3.81\,x + 0.184$	0.99999	1.01
Benzoyl-tiazofurin	2.50 – 100.00	$y = 18.76\,x - 0.325$	1.00000	1.42

The linearity curves are given according to the equation: $y = ax + b$ (a – slope, b – intercept with the y-axis). The linearity for both analysed substances was determined by a six-point calibration

4) The within-assay precision was evaluated by six repeated, separate measurements of the 10 μM standard for both analysed substances. The standard substances were added to the plasma of untreated animals and the samples were prepared as described in Experimental. The coefficients of variation of the retention times for AT and BT were 0.065 and 0.039 %, respectively. The coefficients of variation of the peak areas for the same substances were 0.042 and 0.015 %, respectively.

5) The accuracy of the methods was expressed as percentage recovery of known added amounts of AT or BT to the plasma of untreated animals. The samples were further prepared and analysed as described. The recoveries and relative standard deviations for both substances, for nine tested samples are given in Table II.

6) The limits of detection (LD) for AT and BT were 0.46 and 0.33 μmol/L, respectively. The limits of quantification (LQ) for the same substances were 1.52 and 1.11 μmol/L, respectively. The LD and LQ were determined mathematically, as three and ten times the standard deviation of noise over the time range of the eluting peak.

7) A robustness test was performed in order to examine the influence of the operational parameters on the chromatographic separation. The temperature (25 ± 2 ºC) and pH value of the disodium hydrogen phosphate (5.1 ± 0.05) were varied in order to confirm the robustness of both methods. These parameter changes did not affect the retention times and shape of the peaks of interest.

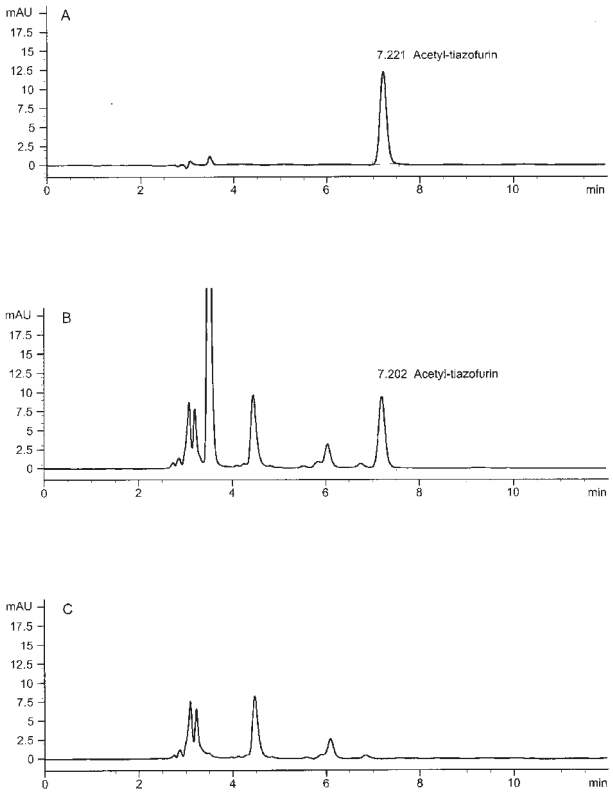

Fig. 1. HPLC Chromatograms for (A) the acetyl-tiazofurin standard, (B) a plasma sample with acetyl-tiazofurin five minutes after drug administration and (C) a control sample (untreated animal).

TABLE II. Accuracy of the HPLC methods for the determination of acetyl- and benzoyl-tiazofurin in rat plasma

Acetyl-tiazofurin		Benzoyl-tiazofurin	
Added/μmol L^{-1}	Recovery/%	Added/μmol L^{-1}	Recovery/%
15	102.05	5	96.40
15	99.31	5	104.41
15	98.00	5	95.62
30	105.62	10	96.61
30	100.61	10	98.48
30	103.59	10	101.51
60	104.02	25	98.49
60	105.09	25	101.24
60	97.64	25	92.76
RSD/%	2.81		3.38

The accuracy of the methods was expressed as percentage recovery by the assay of known added amounts of acetyl-tiazofurin or benzoyl-tiazofurin to the plasma of untreated animals. The Table shows the recovery and relative standard deviation values (*RSD*) for the determination of both substances, for nine tested samples of three different concentrations

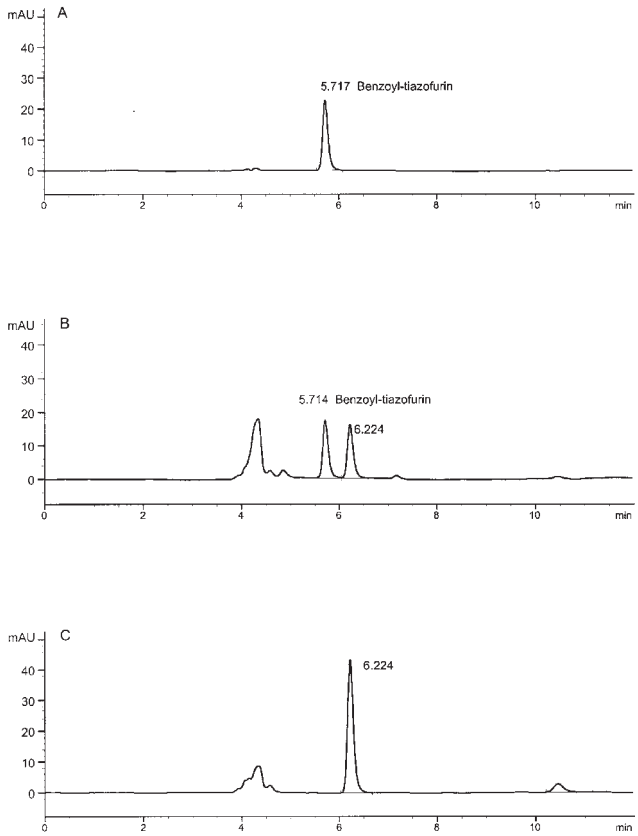

Fig. 2. HPLC Chromatograms for (A) the benzoyl-tiazofurin standard, (B) a plasma sample with benzoyl-tiazofurin five minutes after drug administration and (C) a control sample (untreated animal).

Concentrations of acetyl- and benzoyl-tiazofurin in plasma of treated rats

The described HPLC methods were applied to determine the concentration of AT and BT in rat plasma. The plasma levels of both analysed substances were determined five minutes after administration (125 mg/kg, i.p.) to male Wistar albino rats. The samples were prepared as described in Experimental, and the results are shown in Table III.

TABLE III. Concentrations of acetyl- and benzoyl-tiazofurin in rat plasma obtained five minutes after their i.p. administration (125 mg/kg)

Substance	Concetration/nmol mL^{-1}
Acetyl-tiazofurin	10.4 ± 1.7
Benzoyl-tiazofurin	5.8 ± 2.6

Data represent mean ± *SD* (*n* = 3)

The obtained results indicate that both esters of tiazofurin could be detected and quantified in rat plasma 5 min after administration. However, both derivatives undergo

hydrolysis to tiazofurin in plasma, as was shown by determination of tiazofurin in the same samples using a previously described HPLC method[15] (data not shown).

DISCUSSION

This paper describes two novel reverse-phase HPLC methods for the determination of 5'-*O*-acetyl-tiazofurin and 5'-*O*-benzoyl-tiazofurin in rat plasma, which were developed and validated in terms of specificity, linearity, precision, accuracy and sensitivity. The applicability of the methods was tested by the detection and quantification of AT and BT in rat plasma 5 min after their i.p. administration.

The previously proposed method for the quantification of acetyl- and benzoyl-tiazofurin in extracts of C6 rat glioma cells and in cell cultivation media[15] could not be applied to rat plasma samples as the peaks of interest could not be separated from the plasma matrix peaks.

A simple sample preparation procedure, which included the precipitation of plasma proteins by short-term denaturation in boiling water,[15] was used. Previously proposed procedures involving protein precipitation in an acid environment[13,14] caused adic hydrolysis of both tiazofurin esters.

The choice of extraction procedure, mobile phase and column for both methods was based on our previous experience using other extraction procedures in combination with several mobile phases, including methanol as organic modifier, and different reverse-phase C_{18} columns. Initially attempts were made to use gradient elution for the determination of AT and BT in the same run. However, the highly non-polar BT could not be eluted without significant increase in the content of organic solvent in the mobile phase. This required much longer analysis run times in order to prevent column deterioration. These considerations showed the necessity of the application of two separate methods for the quantification of AT and BT.

The standard curves of both analysed substances were linear ($r > 0.99$) within a wide range of concentrations. The intercepts with the *y*-axis did not significantly deviate from zero. The relative standard deviation of AT and BT recoveries (2.81 and 5.36 %, respectively) confirmed the accuracy of the methods. The selectivity of the methods can be seen from the sharp resolution of the peaks, while no interfering peaks from endogenous compounds were detected. The LD and LQ values of both analysed substances were in nanomolar range, indicating the possibility of determination and quantification of very small concentrations of AT and BT in rat plasma.

The proposed analytical procedures for the determination of AT and BT in rat plasma are sensitive and reproducible. A large number of samples can be extracted and prepared for analysis in a single day. An automatic injector allows the samples to be run overnight. The run time of 12 min for both methods enables the fast analysis of a large number of samples.

ИЗВОД

HPLC МЕТОДЕ ЗА ОДРЕЂИВАЊЕ АЦЕТИЛ- И БЕНЗОИЛ-ТИАЗОФУРИНА У ПЛАЗМИ ПАЦОВА

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За потребе одређивања 5'-*O*-ацетил-тиазофурина (АТ) и 5'-*O*-бензоил-тиазофурина (ВТ) у плазми пацова, развијене су и валидиране нове RP-HPLC методе. Валидација метода укључила је испитивање селективности, линеарности, прецизности, тачности и осетљивости. Линеарност метода је потврђена у опсегу концентрација од 2,50 до 100,00 μmol/L, за обе супстанце. У обе методе је хроматографско раздвајање постигнуто изократским елуирањем, на колони Supelcosyl LC-18-DB (250 × 4,6 mm; 5 μm) и мобилном фазом коју сачињава мешавина 0,1 M Na_2HPO_4 и метанола у одговарајућем односу. UV детекција је вршена на таласној дужини од 254 nm. Описане методе омогућавају детекцију и квантификацију наномоларних концентрација АТ и ВТ у плазми пацова.

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