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# DETERMINATION OF THIAZOLE-4-CARBOXAMIDE ADENINE DINUCLEOTIDE (TAD) LEVELS IN MONONUCLEAR CELLS OF LEUKEMIC PATIENTS TREATED WITH TIAZOFURIN

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**Abstract**—Tiazofurin is an oncolytic agent which has shown therapeutic activity in end-stage acute nonlymphocytic leukemia (ANLL) and blast crisis of chronic granulocytic leukemia (CGL-BC). Tiazofurin is anabolized to the active metabolite, thiazole-4-carboxamide adenine dinucleotide (TAD), which inhibits IMP dehydrogenase activity, leading to reduction of guanylate pools and cessation of cancer cell proliferation. The concentration of TAD in neoplastic cells of patients treated with tiazofurin should be a good indicator of sensitivity to the drug and also might herald the emergence of drug-resistant cells. Therefore, the precise quantitation of TAD in cancer cells during tiazofurin treatment is essential. In this paper we report a highly sensitive method for the determination of TAD in biological samples. With this technique, in addition to TAD, thirteen other biologically relevant adenine, guanine, cytosine and uridine nucleotides can be separated and quantitated accurately. TAD standard was separated on a Waters Partisil 10-SAX column in a RCM-10 module using an ammonium phosphate buffer system. TAD eluted at 21 min with a limit of detection of 15 pmol and linearity up to 3 nmol. The coefficient of variation was 0.6 ± 0.1% for retention time and 2 ± 0.3% for TAD concentration. Recovery of TAD was 96% with reproducibility of 98%. To examine the applicability of this method to a clinical setting, blood samples were obtained from a patient with CGL-BC and leukocytes were separated on a Ficoll–Hypaq gradient, extracted with trichloroacetic acid, and an aliquot was analyzed on HPLC. The TAD peak was identified by comparing the retention time and spectral analysis of the standard. After the patient was treated with a 2200 mg/m$^2$ (12.7 mM) dose of tiazofurin, the TAD concentrations in the mononuclear cells at 2, 6, and 24 hr were 23.1, 13.6, and 0.8 μM. TAD levels at 2, 6, and 24 hr after a tiazofurin dose of 3300 mg/m$^2$ (21.1 mM) were 42.8, 26.1, and 1.4 μM respectively.

Tiazofurin, a C-nucleoside antimetabolite, has potent oncolytic activity in murine and human tumor cell systems [1–5]. The mechanism of action of the drug is due to its anabolism to thiazole-4-carboxamide adenine dinucleotide (TAD§), an analog of NAD, wherein the nicotinamide moiety is replaced by thiazole-4-carboxamide [6, 7]. TAD is a strong inhibitor of IMP dehydrogenase activity, causing decreases in guanylate concentrations, leading to inhibition of tumor cell proliferation [8–10]. Recently, tiazofurin was shown to exhibit therapeutic activity in leukemic patients [11–14]. We developed a sensitive HPLC technique to measure TAD concentrations *in vitro* and *in vivo*. The clinical applicability of this method is demonstrated by quantitating TAD concentrations in mononuclear cells in a leukemic patient undergoing tiazofurin treatment.

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§ Abbreviations: ANLL, acute nonlymphocytic leukemia; CGL-BC, blast crisis of chronic granulocytic leukemia; tiazofurin, 2-β-D-ribofuranosylthiazole-4-carboxamide, NSC 286193; TAD, thiazole-4-carboxamide adenine dinucleotide; TCA, trichloroacetic acid; and TOA, tri-*n*-octylamine.

## MATERIALS AND METHODS

*Materials.* Nucleotides, snake venom phosphodiesterase (0.22 units/mg solid) and alkaline phosphatase (2200 units/mg protein) were purchased from the Sigma Chemical Co., St. Louis, MO. Ammonium phosphate and methanol (HPLC grade) were from the Fisher Scientific Co., Itasca, IL. Synthetically prepared TAD [15] was provided by Dr. Victor Marquez of the National Cancer Institute, N.I.H., Bethesda, MD. RCM Partisil 10-SAX column and HPLC apparatus consisting of an NEC computer (APC IV series, Power Mate 2 mode), a 990 photodiode array detector, a 600 E multisolvent delivery system, and a refrigerated 712-WISP autoinjector were purchased from Waters Associates, Milford, MA.

*Sample preparation.* Preparation of bone marrow and peripheral blood mononuclear cells and determination of GTP concentrations were carried out as reported [16]. Briefly, mononuclear cells were separated by a Ficoll–Hypaq density gradient. An aliquot of cells was then extracted with ice-cold 10% trichloroacetic acid (TCA), neutralized immediately with 0.5 M tri-*n*-octylamine (TOA) in freon, and then analyzed by HPLC for TAD concentrations.

*Chromatographic analysis.* Aliquots (50–100 μL) of the neutralized samples were analyzed on a Waters Partisil 10-SAX column in an RCM-10 module pre-equilibrated with 5 mM ammonium phosphate

Table 1. Chromatographic method for the separation of TAD and nucleotides

| Column | RCM Partisil-10 SAX | | | |
|---|---|---|---|---|
| Buffers | | | | |
| (A) | 5 mM $NH_4H_2PO_4$ pH 3.0 | | | |
| (B) | 650 mM $NH_4H_2PO_4$ pH 3.7 | | | |
| | Chromatographic condition | | | |
| Time (min) | Flow rate (mL/min) | Buffer (A) (%) | Buffer (B) (%) | Gradient curve |
| Initial | 1.2 | 100 | 0 | — |
| 5.0 | 1.2 | 100 | 0 | 6 |
| 5.5 | 2.0 | 100 | 0 | 6 |
| 15.0 | 2.0 | 85 | 15 | 7 |
| 20.0 | 2.0 | 40 | 60 | 9 |
| 20.1 | 1.0 | 40 | 60 | 6 |
| 22.0 | 1.0 | 0 | 100 | 6 |
| 23.0 | 1.0 | 0 | 100 | 6 |
| 24.0 | 1.2 | 0 | 100 | 6 |
| 30.0 | 1.2 | 0 | 100 | 6 |
| 35.0 | 2.0 | 0 | 100 | 6 |
| 36.0 | 2.0 | 100 | 0 | 6 |
| 45.0 | 2.0 | 100 | 0 | 6 |

Total time of analysis was 45 min.

buffer, pH 3.0. A gradient with 650 mM ammonium phosphate buffer, pH 3.7, was applied and the conditions for analysis are shown in Table 1. TAD and nucleotide concentrations were quantitated using a photodiode array detector which scanned and stored spectral data between 190 and 390 nm.

*Identification of TAD.* Four different methods were used for identification of TAD: (1) *retention time*—TAD and biologically important nucleotides were identified by comparing the retention time of the sample with standards; (2) *spectral analysis*—TAD and nucleotides were recognized in the sample and standards on the basis of their UV spectra between 190 and 390 nm; (3) *additive effect*—graded concentrations of authentic TAD (25–250 pmol) were added to aliquots of neutralized TCA extracts of leukemic cells from patients who did or did not receive tiazofurin treatment and analyzed on HPLC as detailed earlier; and (4) *enzymatic analysis*—aliquots (40 μL) of samples from a patient (2 hr after tiazofurin infusion) or standard mixture containing TAD (0.02 mM) and nucleotides (0.2 mM each of CMP, CDP, CTP, UMP, UDP, UTP, AMP, ADP, ATP, GMP, GDP, GTP, IMP, XMP and NAD) in Eppendorf test tubes were treated with 5 μL of alkaline phosphatase (1.6 units) or snake venom phosphodiesterase ($1.02 \times 10^{-4}$ units) and 5 μL of 0.2 M Tris–HCl buffer, pH 8.4, with 0.1 M $MgCl_2$. The mixture was incubated at 37° for 20 min, and the reaction was terminated by heating for 1 min at 95°. Then the tubes were centrifuged for 3 min at 12,000 *g*, and an aliquot (30 μL) was analyzed by HPLC. Control tubes contained 5 μL of water instead of enzyme.

*Tiazofurin assay.* An HPLC method was used for the analysis of tiazofurin.* Briefly, plasma from patients was extracted with cold TCA (final concentration 10%) and neutralized with 0.5 M TOA in freon. An aliquot of the neutralized extract was loaded onto a Radial Pak 8C18 5 μm column (Waters Associates) equilibrated with 20 mM sodium acetate, pH 4.5, containing 0.75% acetonitrile. Tiazofurin was eluted isocratically with the same buffer at a flow rate of 2 mL/min. Under these conditions tiazofurin eluted at 13.4 min.

*Dose and administration.* Tiazofurin in sterile injection form was obtained from the National Cancer Institute, Bethesda, MD. The drug was administered over a period of 1 hr by an infusion pump (Imed Co., San Diego, CA) under sterile conditions through a permanent central line set up in patients [11, 12].

## RESULTS

*Chromatographic separation of standard TAD and nucleotides.* The HPLC elution profile of standard TAD and biologically relevant nucleotides is illustrated in Fig. 1. Tiazofurin and its active metabolite (TAD) eluted at 3.5 and 21.2 min well-separated from nucleotides, CMP, AMP, NAD, UMP, IMP, GMP, XMP, CDP, UDP, ADP, GDP, CTP, UTP, ATP and GTP.

*Validation of the method.* The TAD peak in the biological samples was identified on the basis of the following properties: (a) the elution time was identical to that of the authentic compound; (b) the spectral profile of TAD peak in the sample was identical to that of the standard; (c) when authentic TAD was added to aliquots of leukemic cells from patients who were or were not treated with tiazofurin and immediately processed as described in Materials and Methods, the TAD concentration in the samples showed a proportional increase (Fig. 2); and (d) the TAD peak in the sample disappeared when the sample was incubated with snake venom

* Jayaram HN, Lapis E, Tricot GJ, Kneebone P, Paulik E, Zhen W, Hoffman R and Weber G, Clinical pharmacokinetic study of tiazofurin administered as a 1-hr infusion. Submitted for publication.

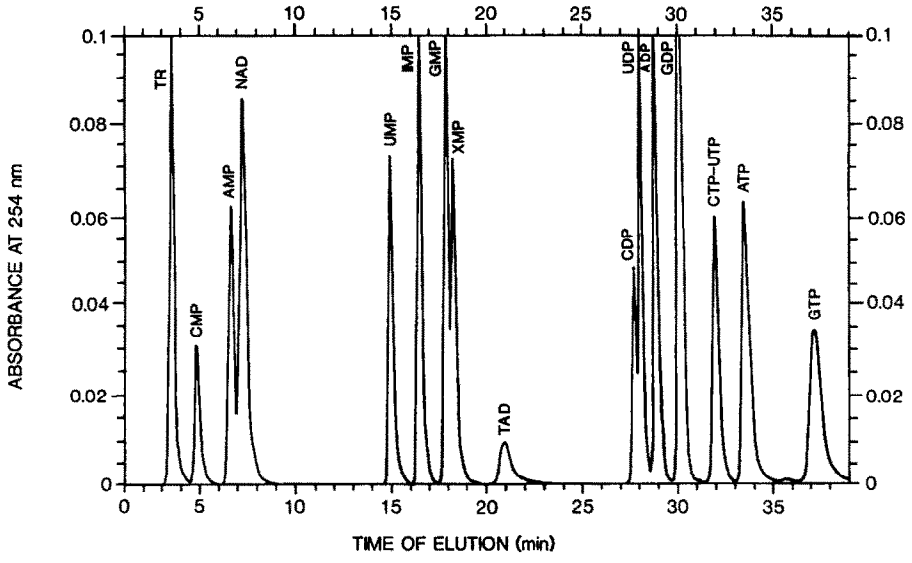


Fig. 1. Chromatographic elution profile of standard TAD and fifteen nucleotides.

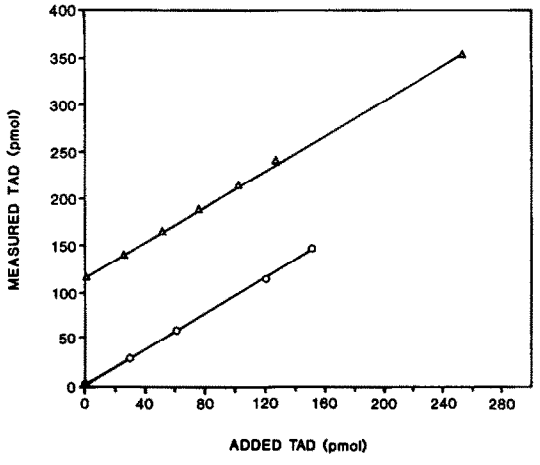


Fig. 2. Additive effect of TAD on its recovery. Graded concentrations of TAD were added to neutralized TCA extracts of leukemic cells obtained from a patient 2 hr after treatment with tiazofurin (3300 mg/m$^2$) (△—△). Graded amounts of TAD were also added to neutralized TCA extracts of leukemic cells from a patient who had no chemotherapy (○—○).

phosphodiesterase; treatment of samples with alkaline phosphatase did not influence the peak area of TAD. These results indicate that the peak identified in the samples was TAD.

*Sensitivity and linearity of the TAD assay.* The limit of detection of TAD under our experimental conditions was 15 pmol (Fig. 3). Quantitation of TAD concentration was based on the chromatographic area and was determined for ten different concentrations of authentic TAD. TAD measurements were linear from 15 pmol to 3 nmol (Fig. 3). The concentration of TAD in the mononuclear cells of the leukemic patient after therapeutic doses of tiazofurin (2200 and 3300 mg/m$^2$) ranged between 0.8 and 42.8 $\mu$M and was within the sensitivity and linearity of the present method.

*Reproducibility of TAD measurement.* The reproducibility of the elution profile of one analysis to another was examined both in standards and in samples from the leukemic patient. When 250 or 500 pmol each of standard TAD were injected five times on the HPLC column, the mean variation and standard error of retention time was 0.6 ± 0.1%, and the average variation of measured TAD concentration was 2 ± 0.3%. When a sample of leukemic cell extract obtained from a patient treated with tiazofurin (containing 128 pmol TAD) was injected five times on the HPLC column, a similar retention time was observed with an average variation in the concentration of TAD of 1.1%.

*Recovery of TAD.* Leukemic cells from patients who did not receive any chemotherapy were obtained and transferred into groups of four Eppendorf tubes ($9.9 \times 10^6$ cells/tube). TAD was added (250 or 500 pmol) to each tube, immediately extracted with 300 $\mu$L of cold 10% TCA, and an aliquot of neutralized extract was analyzed for TAD concentrations on HPLC. Recovery of TAD averaged 96% with a standard error of 2.3%.

*TAD concentrations in the mononuclear cells of a leukemic patient treated with tiazofurin.* The applicability of this method was tested in mononuclear cells of a patient with CGL-BC who received tiazofurin in a 1-hr infusion by pump. Eligibility of patients, the schedule of administration, toxicity, and the clinical outcome of tiazofurin treatment have been reported [11–14]. A chromatographic profile of TAD and nucleotides after a tiazofurin dose of 3300 mg/m$^2$ is provided in Fig. 4. Samples were obtained prior to the start of infusion and at 2, 6 and 24 hr post-infusion. TAD concentration was high at 2 hr and decreased by 6 hr; low concentrations persisted at 24 hr. TAD levels in the mononuclear

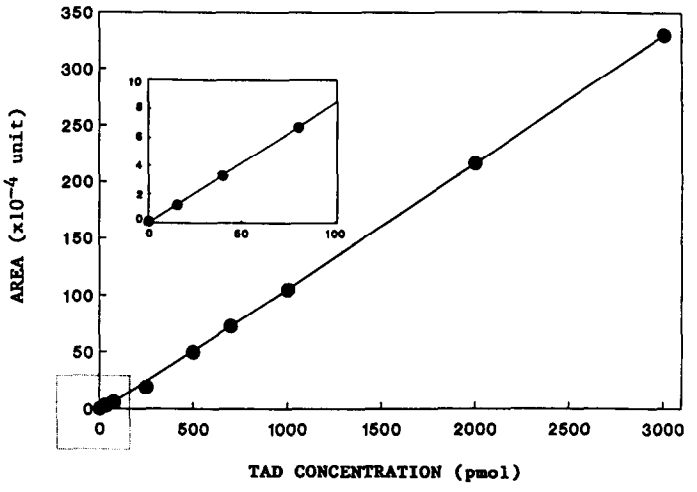


Fig. 3. Sensitivity and linearity of TAD assay. In these measurements, the area was used for quantitation. The detector response at the lowest level of detection (15 pmol) was 0.126 mV. The optimal wavelength for obtaining this degree of sensitivity was at 254 nm.

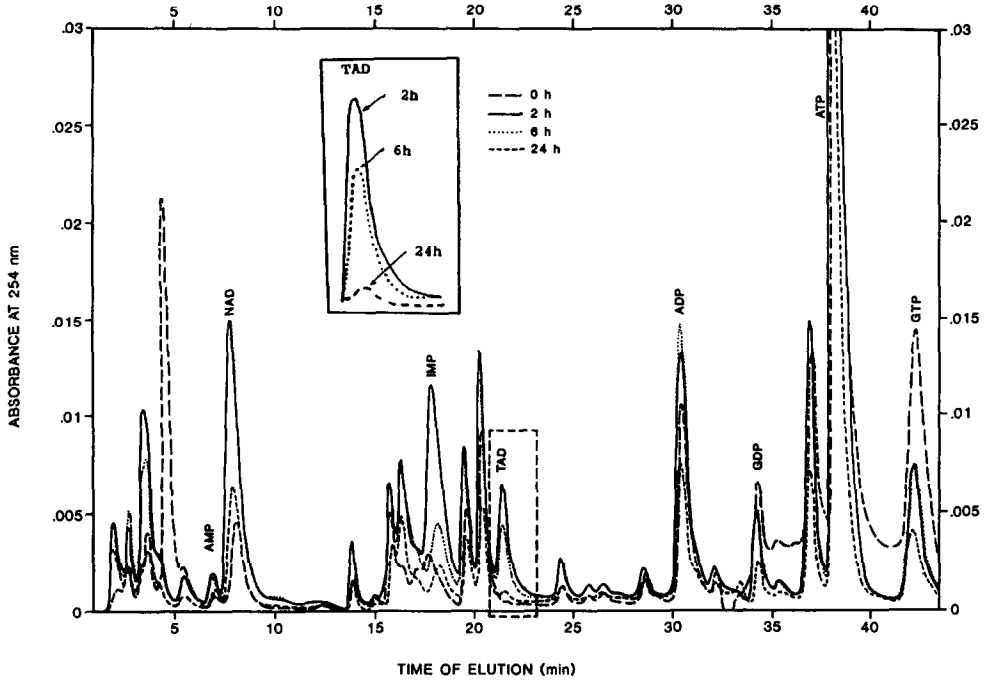


Fig. 4. Chromatographic elution pattern of TAD and nucleotides before and after treatment with tiazofurin (3300 mg/m$^2$).

cells of a patient receiving a series of tiazofurin treatments are shown in Fig. 5. After three doses of 2200 mg/m$^2$, if GTP concentrations were not decreased to <80% of pretreatment value, the dose was then escalated to 3300 mg/m$^2$ according to the clinical treatment protocol [12]. Tiazofurin concentration in plasma and TAD level in mononuclear cells were proportional to the dose of the drug (Fig. 5). Mean tiazofurin concentrations of 225 and 383 $\mu$M were measured in the plasma of the patient 2 hr after receiving 2200 and 3300 mg/m$^2$ of tiazofurin respectively. Mean TAD levels of 23.1 and 42.8 $\mu$M were measured in the mononuclear cells of the leukemic patient at 2 hr after the administration of 2200 and 3300 mg/m$^2$ tiazofurin respectively. About 5–13% of the plasma tiazofurin

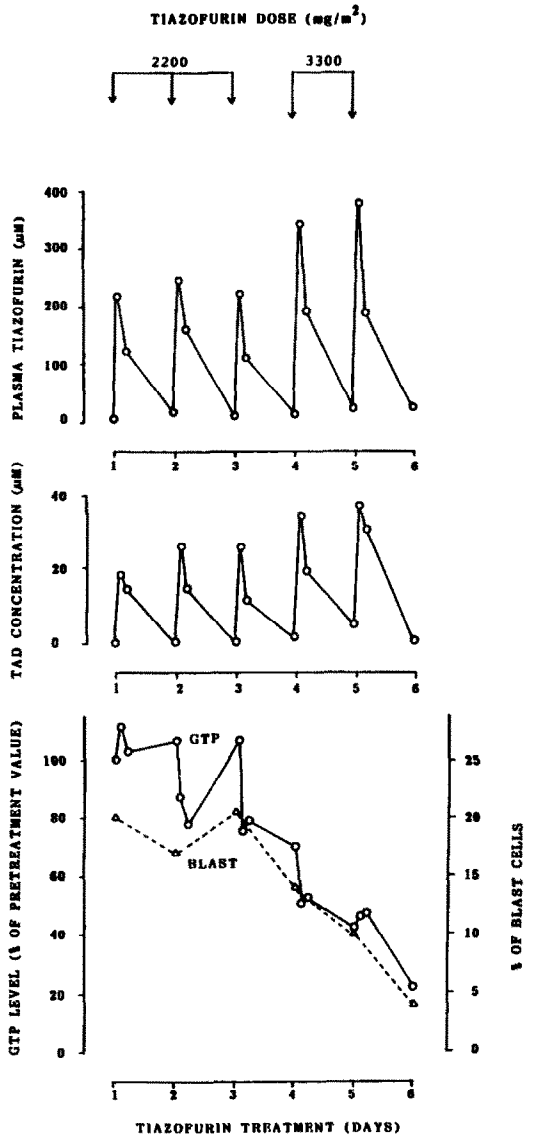


Fig. 5. Concentrations of tiazofurin, TAD, GTP and leukemic cells in peripheral blood samples following tiazofurin treatment. Values for tiazofurin in plasma were expressed as micromolar. TAD in mononuclear cells in the peripheral blood sample was calculated as nmol/mL sample ($\mu$M). Leukemic cell counts (blasts) were given as percent present in the peripheral blood. The pretreatment value of white blood cells was $41.7 \times 10^9$/L; leukemic cells, $8.3 \times 10^9$/L; and GTP concentration, 315.4 nmol/$10^9$ leukemic cells.

levels were accountable as TAD in mononuclear cells.

## DISCUSSION

We encountered the following problems in measuring TAD concentrations in the leukemic cells of patients treated with tiazofurin with the existing method for the separation of TAD [16]. In the earlier method, mononuclear cells from leukemic patients were incubated *in vitro* with radiolabeled tiazofurin which was converted to radioactive TAD and was then quantitated by scintillation spectrometry. In the Phase I/II studies conducted at Indiana University, non-radioactive tiazofurin was administered to leukemic patients [11–14]. Many of these patients were leukopenic with very low cell numbers and only small concentrations (pmol) of TAD could be detected, whereas the concentrations of endogenous nucleotides were magnitudes higher than that of TAD. Thus, we needed to develop an ultrasensitive method which separated TAD from other nucleotides.

The applicability of this method was demonstrated in the mononuclear cells of a leukemic patient who was treated with tiazofurin. TAD concentrations following tiazofurin doses of 2200 and 3300 mg/m$^2$ at 2 hr were 23.1 and 42.8 $\mu$M; at 6 hr were 13.6 and 26.1 $\mu$M; and at 24 hr were 0.8 and 1.4 $\mu$M respectively. TAD level reached its nadir at 24 hr and was still 8- and 14-fold higher than the $K_i$ for TAD of the IMP dehydrogenase of leukemic cells (0.1 $\mu$M) [17]. Thus, the TAD concentrations reached in leukemic cells should be sufficient to exert potent inhibition of IMP dehydrogenase activity which decreased guanylate pools resulting in a decline in blast cell number.

These studies indicate that our method is capable of determining exact TAD concentrations in target cells during chemotherapy with tiazofurin. Precise information on the concentration of TAD in neoplastic cells of patients should help in making effective and efficient protocol changes tailored to individual pharmacokinetics and IMP dehydrogenase activity. Since TAD is the active metabolite of tiazofurin, a decrease in the TAD concentration during treatment may be a good predictor for the possible emergence of a tiazofurin-resistant cell population.

Tiazofurin is a prodrug and only those neoplastic cells which form sufficient concentration of the active metabolite, TAD, should be sensitive to tiazofurin therapy. Tiazofurin is a promising agent in the treatment of leukemia [11–14]. There is potential for reopening tiazofurin trials in solid tumors based on their capacity to form high levels of TAD. The technique described here for the measurement of TAD should aid in the ongoing and future tiazofurin trials for predicting sensitivity, monitoring response, and recognizing the development of drug resistance.

*Novel aspects of this study.* The novel aspects include the following: (1) an ultrasensitive HPLC method was developed for the measurement of TAD in biological samples; (2) this technique was reproducible (98%) with a recovery of 96%; and (3) for the first time TAD concentrations were measured in samples from patients undergoing tiazofurin therapy.

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