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Determination of nicotinamide-adenine dinucleotide and thiazole-4-carboxamide-adenine dinucleotide in human leukocytes by reversed-phase high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic assay for cellular nicotinamide-adenine dinucleotide and thiazole-4-carboxamide-adenine dinucleotide is presented that is appropriate for analysis of these dinucleotides in extracts of Ficoll-purified human leukemic cells. The separation, which is effected by reversed-phase chromatogrpahy, is highly reproducible and the limit of quantitation is as low as 10–15 pmol. The stability of these compounds in neutralized perchloric acid extracts is addressed and the applicability of the procedure to clinical specimens is demonstrated.

INTRODUCTION

The antitumor activity of tiazofurin reflects cellular metabolism of the agent to form thiazole-4-carboxamide-adenine dinucleotide (TAD), an analog of nicotinamide-adenine dinucleotide (NAD) [1,2]. The formation of TAD, a highly effective inhibitor of IMP dehydrogenase (EC 1.1.1.205) [3,4], has been observed in most mammalian cells with the exception of erythrocytes. Its presence results in depletion of cellular guanylate pools [1–5] with accompanying growth inhibition and has been suggested as being useful in evaluating the sensitivity of leukemic cells to tiazofurin [6]. Tiazofurin is reported to be a useful agent in the treatment of chronic myeloid leukemia (CML) in blast crisis [7,8], a disease with a very poor prognosis [9]. It is, therefore, important to develop a simple and reliable procedure for determination of the active form of tiazofurin, TAD, in human leukocytes.

Measurement of TAD concentrations in cultured cells is easily accomplished by ion-exchange high-performance liquid chromatographic (HPLC) techniques [1,2]. Its analysis in human leukemic leukocytes, however, has been somewhat compromised by interference with UV-absorbing substances not generally observed in extracts from cultured cells. Using anion exchange with a Waters Partisil 10-SAX column and a highly complex gradient scheme, Zhen *et al.* [10] have recently described the HPLC quantitation

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of TAD in mononuclear cells of leukemia patients. Their method, however, employs a highly complex gradient scheme producing a marginal separation of TAD from other components that could be easily disturbed by minor fluctuations in pump efficiency.

This communication describes a relatively simple reversed-phase HPLC procedure that effectively allows analysis of both NAD and TAD levels in extracts of purified human leukocytes.

EXPERIMENTAL

Chemicals

Tiazofurin was obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, MD, USA). Authentic TAD was generously provided by Roland K. Robins (Costa Mesa, CA, USA). Triammonium phosphate was purchased from Crescent Chemical (Hauppauge, NY, USA).

Patients

This study was carried out in conjunction with a clinical and pharmacology study of tiazofurin in the treatment of CML in blast crisis at M.D. Anderson Cancer Center. Drug was administered as a 1-h infusion at a dose level of 2200 mg/m^2. A pre-treatment blood sample was drawn prior to start of the infusion and a post-treatment sample obtained 2 h after the end of the infusion.

Purification and extraction of cells

Cells were purified from heparinized peripheral blood samples by Ficoll-Hypaque gradient centrifugation as described by Plunkett *et al.* [11]. The cells were washed twice with phosphate-buffered saline (pelleted each time by centrifugation for 5 min at 500 *g*), and $5 \cdot 10^7$ cells were resuspended in 0.5 ml of cold water. Perchloric acid, 0.5 ml (0.8 *M*), was added and, after 10 min at 0°C, the precipitate was removed by centrifugation as above and washed with 1 ml of cold 0.4 *M* perchloric acid. The pH of the combined supernatant solutions was adjusted to 6.5–7.0 with 10 *M* potassium hydroxide solution. After centrifugation again the supernatant solution was employed for direct analysis.

Chromatography

Cell extracts were analyzed using a Waters system (Waters Assoc. Milford, MA, USA) equipped with two Model 510 pumps, a Model 720 system controller, a data module and a 300 mm × 3.9 mm I.D. Resolve C_{18} (5 μm) reversed-phase column at room temperature. Samples, 100–200 μl, were injected with a Waters U6K injector and elution was carried out with a linear gradient from 100% buffer A to 30% buffer B in 30 min followed by an additional 5 min at 100% buffer A. The flow-rate was 1 ml per min. Buffer A was 0.1 *M* triammonium phosphate at pH 5.1; buffer B was 25% methanol. Prior to beginning determinations each day the column was equilibrated with the final conditions (30% buffer B) for 15 min and then with starting buffer (100% buffer A). This routine produced a stable baseline. All buffers were filtered through a 0.22-μm Millipore filter prior to use. Compounds of interest were detected by their absorbance at 254 nm. This method is a modification of one described previously by Hunting *et al.* [12] for measurement of NAD in extracts of cultured cells.

Identification of TAD

The peak appearing in extracts of cells from tiazofurin-treated patients at a retention time of 31.5 min was identified as TAD by comparison with an authentic standard and further by quantitation of the products formed after degradation by nucleotide pyrophosphatase (EC 3.6.1.9). To accomplish the latter the putative TAD peak was collected from five fractionations of $5 \cdot 10^6$ cell equivalents and removed from the buffer by adsorption onto activated charcoal (25 mg added to approximately 15 ml of combined peak fractions). The charcoal was removed by filtration and the TAD eluted from it by washing with ammoniacal ethanol [1.5% ammonium hydroxide in 50% ethanol (v/v)]. The resulting solution was evaporated under nitrogen and the residue dissolved in 1 ml of water. This solution contained 716 pmol of putative TAD (assuming a molar extinction coefficient of 18 500 at a wavelength of 252 nm [13]). Aliquots of this (0.25 ml) were treated with 0.5 U of nucleotide pyrophosphatase

in the presence of 0.25 *M* Tris–HCl, pH 7.5 and 0.025 *M* magnesium chloride for 30 min at 37°C. The reaction was terminated by immersion in a boiling water bath for 1 min followed by HPLC analysis of the products by the method described above.

RESULTS

The effectiveness of this procedure for separation of the major cellular metabolites of importance in tiazofurin therapy is demonstrated by the profile of a group of standards shown in Fig. 1. TAD is cleanly separated from all other compounds, suggesting that the method may be particularly suitable for quantitative determinations of cellular TAD concentrations during therapy. The most likely interference with NAD measurements by this procedure would come from inosine, however, it is probable that this compound would be quickly removed by the action of purine nucleoside phosphorylase and thus would not accumulate in the cells and present a problem. The retention times of these and other compounds relevant to the metabolism of tiazofurin and NAD are summarized in Table I. Quantitation of a relatively broad range of TAD concentrations can be carried out in extracts of human leukocytes using this procedure. This was demonstrated by the addition of precisely measured concentrations of authentic TAD to an extract of Ficoll-purified cells obtained from a patient prior to therapy with tiazofurin (Table II). HPLC analysis of the TAD-containing extracts yielded a linear response with close to 100% recovery for all but the lowest concentration, 6.2 pmol. Measurement of NAD in the same extracts was not com-

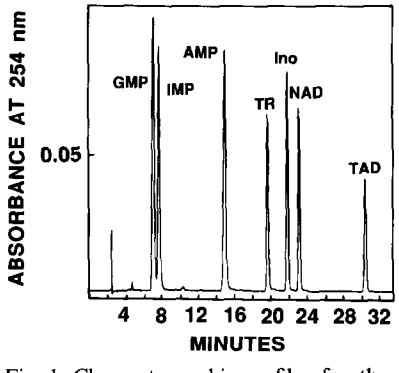

Fig. 1. Chromatographic profile of authentic NAD, TAD and other relevant nucleotides and derivatives. The procedure is described in the text.

TABLE I

RETENTION TIMES OF RELEVANT NUCLEOTIDES AND RELATED COMPOUNDS

Compound[a]	Retention time (min)
EDTA	2.30
NMN	5.50
Nicotinic acid	6.29
IMP	7.04
Tiazofurin 5'-monophosphate	7.10
Nicotinamide ribonucleoside	7.31
ADP-ribose	9.60
AMP	14.83
NADP	17.37
NaAD	17.54
Tiazofurin	19.41
NAD	23.39
Nicotinamide	25.31
TAD	31.48

[a]The chromatographic properties of these compounds were determined as described in Experimental using the same column and buffer solutions for all.

TABLE II

TAD ANALYSIS: PRECISION AND RECOVERY IN A PERCHLORIC ACID EXTRACT OF FICOLL-PURIFIED HUMAN LEUKEMIC LEUKOCYTES

Authentic TAD was added to aliquots of an extract of Ficoll-purified cells from a CML patient in blast crisis. The blood sample was drawn prior to treatment and the cells were purified as described in Experimental. Extract equivalent to $2.5 \cdot 10^6$ cells was analyzed in triplicate for each determination.

Sample	TAD added (pmol)	TAD determined (pmol)	S.D. (%)	Recovery (%)
1	6.2	8.1	1.1	131
2	15.5	15.9	7.5	102
3	93.0	92.5	2.9	99.5
4	155.0	153.3	2.0	98.9
5	310.0	300.6	2.4	97.0

promised by the varying concentrations of TAD.

This procedure was successfully applied to determination of cellular TAD and NAD in the leukocytes of a patient undergoing therapy with tiazofurin (Fig. 2). The middle graph of Fig. 2 shows the HPLC profile of a perchloric acid extract of pre-treatment cells; a sharp peak of NAD was evident, with essentially no peaks in the region of TAD elution. An extract of cells from the same patient 2 h after treatment with tiazofurin (top graph) contained substantial peaks of both NAD and another substance, presumably TAD,

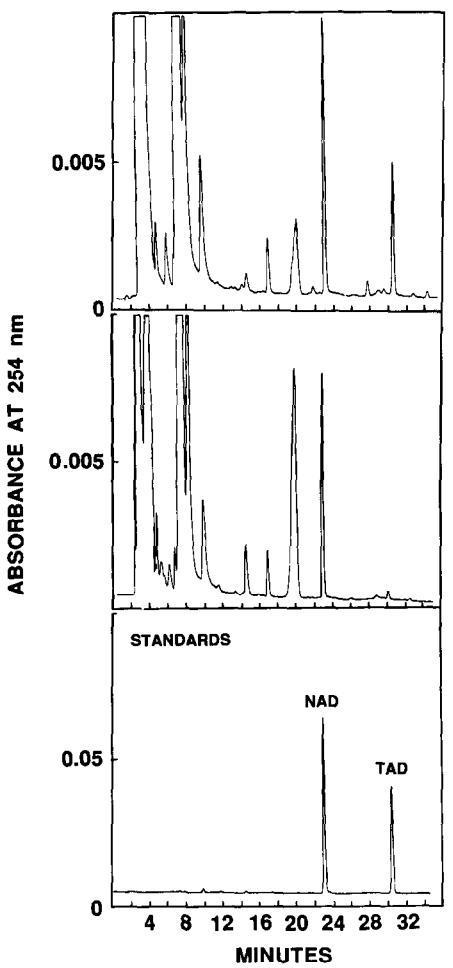

Fig. 2. Determination of TAD accumulation in the cells of a patient during the first course of treatment with tiazofurin. Bottom, HPLC analysis of standards; middle, pre-treatment HPLC profile of lymphoblast extract; top, analysis of cells from the same patient 2 h after infusion with tiazofurin, which were found to contain 92 pmol TAD per $2.5 \cdot 10^6$ cells.

clearly resolved from other cellular components. To establish the identity of the second peak the fractions containing it were pooled, and the metabolite was removed from the solvent and concentrated by a conventional procedure involving adsorption onto activated charcoal (described in Experimental). Treatment of this material with nucleotide pyrophosphatase yielded two new peaks, one eluting at a retention time consistent with tiazofurin 5'-monophosphate (125 pmol) and the other with that of AMP (142 pmol). In the control reaction without enzyme the TAD (179 pmol) remained intact. Allowing for some breakdown of products during the 30-min incubation, the formation of AMP and tiazofurin 5'-monophosphate in similar molar amounts to the initial TAD verifies the identity of this peak with that of authentic TAD.

In carrying out pharmacological studies it is frequently essential, because of the number of samples, to store them frozen for future analysis or to leave them overnight in an autosampling device. The stabilities of NAD and TAD in neutralized perchloric acid cell extracts were assessed at two storage temperatures, 4°C and −10°C (Table III). Both NAD and TAD were stable for three days at 4°C in neutralized extracts (pH 6.8) which is sufficient for analysis with a temperature-controlled autosampling device. Extracts can safely be held at −10°C for at least a week

TABLE III

STABILITY OF NAD AND TAD IN NEUTRALIZED PERCHLORIC ACID EXTRACTS

Authentic TAD was added to a perchloric acid extract of cells as described for Table II. It was divided into several aliquots that were stored as indicated. At the times shown, aliquots were removed and analyzed for NAD and TAD as described. Values given are the mean ± S.D. for duplicate samples.

Storage conditions	Percentage remaining	
	NAD	TAD
One day, 24°C	47.7 ± 0.7	54.5 ± 1.8
Three days, 4°C	96.6 ± 1.5	101.5 ± 1.6
Six days, 4°C	45.9 ± 0.6	48.1 ± 0.7
Six days, −10°C	96.8 ± 2.0	101.0 ± 1.0

without degradation of either compound. At room temperature, however, substantial degradation occurred in one day.

DISCUSSION

Pharmacological studies of drug metabolism and action *in vivo* are dependent upon the availability of assay procedures that are appropriate for multiple samples and have a minimum time requirement. Recent interest in the activity of tiazofurin toward CML in blast crisis has stimulated the design of a pharmacologically directed therapy [7]. Although cellular TAD determinations have not generally been done in these studies, there is increasing interest in doing so. TAD is the only known active metabolite of tiazofurin and thus its concentration in the cell is probably the best pharmacological indicator of antimetabolic activity *in vivo*. The ability of bone marrow or peripheral leukocytes to form TAD from radioactive tiazofurin *in vitro* has been shown by Jayaram *et al.* [6] to be useful in the selection of patients that will respond to the drug. It would be possible to accomplish this with unlabeled drug as well, employing an assay such as that described here.

In comparing the two HPLC methods of TAD determination, anion-exchange and reversed-phase, it is clear that each has its attributes. The anion-exchange procedure is useful in that GTP concentration can be determined at the same time, which is not possible with the reversed-phase method. However, the gradient procedure is very complicated and could be very frustrating in the case of minor changes in pump efficiency, etc. An additional problem that we have encountered in using the Partisil 10-SAX column for TAD determinations is that residual EDTA, used in routine wash procedures and as a stabilizer in Ficoll–Hypaque solutions, appears to interfere in the TAD region of the chromatographic profile and is a particular problem with extracts having low TAD concentration. For these reasons we have chosen to use the reversed-phase system and have found it to be reasonably simple and dependable. The Resolve C_{18} column is quite stable, allowing processing of numerous samples before any loss of peak integrity begins.

In summary, the estimation of TAD concentrations in human leukocytes can be carried out with relative ease using reversed-phase HPLC. Although this method lacks the advantage of allowing GTP estimation it is sensitive and reproducible, and allows processing of a relatively large number of sample without interruption for column cleaning.

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