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QUANTIFICATION OF TIAZOFURIN IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RAYMOND W. KLECKER, Jr.* and JERRY M. COLLINS

Pharmacokinetics Section, Clinical Pharmacology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20205 (U.S.A.)

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SUMMARY

A simple and sensitive method was developed for the separation and quantification of tiazofurin (TCAR) in plasma using 2-β-D-ribofuranosylthiazole-5-carboxamide (iso-TCAR) or [5-^{3}H]TCAR as an internal standard. The procedure uses C_{18} extraction columns to clean-up the plasma samples for measurement by high-performance liquid chromatography. A sensitivity of 0.33 μ*M* (0.08 μg/ml) was easily achieved for 0.5-ml plasma samples. When human whole blood was incubated in vitro with TCAR for 2 h, the plasma concentrations were decreased by 10% (4°C) and 25% (23°C). TCAR could still be measured 22 h after injection of 220 mg/kg into mice. For a two-compartment pharmacokinetic model, the half-lives of TCAR were 18.8 and 412 min.

INTRODUCTION

Tiazofurin (TCAR, 2-β-D-ribofuranosylthiazole-4-carboxamide, NSC 286193, Fig. 1), a thiazole-C-nucleoside, has shown promise as a new anticancer drug. It has demonstrated antitumor activity against several murine leukemias, as well as Lewis lung carcinoma [1,2]. TCAR has recently started Phase I trials in humans at several institutions. The pharmacokinetic studies associated with these Phase I trials require a simple and sensitive method for analysis of TCAR in plasma. Previously published methods for TCAR analysis are either inadequately sensitive for complete pharmacokinetic studies [3,4] or require multiple and/or lengthy procedures [4,5].

This paper describes a simple procedure for the analysis of TCAR in human plasma. The procedure utilizes C_{18} extraction columns for plasma clean-up and measurement by high-performance liquid chromatography (HPLC). Surface-

*Correspondence address: NIH, Building 10, Room 6N119, Bethesda, MD 20205, U.S.A.

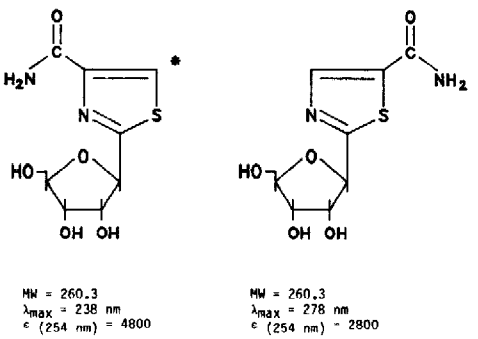

Fig. 1. Structures of TCAR (left) and iso-TCAR (right). The asterisk denotes the position of the tritium label on TCAR, when present.

modified materials (such as the C_{18} bonded phase) are commonly used for biological sample clean-up [6,7], and in some cases are more efficient than classical liquid—liquid extractions [8]. Iso-TCAR (NSC 363223, Fig. 1) is one of several structurally similar C-nucleosides which were synthesized concurrently with TCAR [9,10]. Either tritiated TCAR or iso-TCAR can be used as an internal standard for the procedure.

EXPERIMENTAL

Materials

TCAR, iso-TCAR and [5-^{3}H]TCAR were obtained from the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD, U.S.A.). The organic solvents, methanol and acetonitrile, were HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S A.). The glacial acetic acid was ACS grade (Fisher, Fair Lawn, NJ, U.S.A.). Double-distilled water was used for all solution preparations. The C_{18} extraction columns used were Sep-Pak (Waters Assoc. Milford, MA, U.S.A.) and Baker-10 SPE (J.T. Baker, Philipsburg, NJ, U S.A.).

HPLC conditions

The HPLC mobile phase contained 40 m*M* acetic acid with 1.5% (v/v) acetonitrile. The mobile phase was passed through a 0.45-μm filter to remove any particulate material and to degas the solvent. The solvent was pumped via a Waters Model 6000A pump, isocratically at 2 ml/min, through a Waters Radial-Pak C_{18} column, 5-μm particle size. The column was washed with approximately 450 μl acetonitrile after each injection of extracted plasma by increasing the acetonitrile concentration to 45% for 30 sec via a second pump. There was a 10-min delay after the acetonitrile wash before the next sample injection. A waters 440 fixed-wavelength detector was used to monitor the absorbance at 254 nm.

Sample preparation

On each day of use, a stock TCAR solution was diluted in water to the required concentrations. The standard curves in plasma were prepared by adding aliquots of the diluted stock solutions to pooled plasma obtained from normal

volunteers. The plasma standard solutions ranged from 0.1 μM to 1000 μM TCAR. The volume of plasma standards used for the extraction was 200 μl on the Baker columns and 500 μl on the Waters columns. Each point of the standard curve was processed in triplicate.

All plasma samples and standards received 50 μl of 10^{-4} M iso-TCAR. Alternatively, plasma standard curves were prepared with 50 μl [5-^{3}H]TCAR (100,000 dpm per 50 μl) as internal standard.

Samples of the mouse study with expected plasma TCAR concentrations of 100 μM or greater were diluted 10- or 100-fold in water to avoid overloading of the extraction or analytical columns.

Extraction procedure

A Baker-10 extraction system attached to an unregulated vacuum supply (house vacuum) was used for the extraction. All the washes and sample additions were eluted through the columns by vacuum.

Waters extraction columns

The columns (330 mg C_{18} resin) were first conditioned by passing 4 ml of methanol through the columns, followed by 4 ml of water. The samples were added to the columns and the columns were washed with 4 ml of water. TCAR and iso-TCAR were eluted from the C_{18} columns into collection tubes with 2 ml of methanol. The methanol collections were brought to dryness under a steady stream of nitrogen and redissolved in 150 μl of the HPLC mobile phase.

Baker extraction columns

The columns (107 mg C_{18} resin) were conditioned with 1 ml of methanol, followed by 1 ml water. The samples were added to the columns and 0.5 ml of water was used to wash the columns. Then 0.5 ml of a methanol—water (50:50) solution was eluted through the C_{18} extraction columns into the collection tubes. The methanol collection was dried under a stream of nitrogen and redissolved as before.

Recovery of TCAR and iso-TCAR

The extraction recovery of TCAR and iso-TCAR was determined by extracting a mixture of 100 μM TCAR and 100 μM iso-TCAR in water and normal plasma. For the aqueous samples TCAR and iso-TCAR were measured in each of the extraction column washes. The extraction efficiencies of TCAR and iso-TCAR in the methanol wash were measured for both the aqueous and the normal plasma samples.

To determine whether the extraction efficiency was concentration-dependent, normal plasma was spiked with TCAR and [5-^{3}H]TCAR to give final concentrations of 0.1 μM and 100 μM TCAR. The redissolved extraction residue was mixed with 15 ml liquid scintillation cocktail and counted on a Searle Analytic Mark III (Chicago, IL, U.S.A.) liquid scintillation counter.

TCAR stability in plasma and whole blood

TCAR was added to fresh human plasma to give plasma concentrations of

500 μM and 5 μM. The plasma was gently mixed and 0.5-ml aliquots were transferred into 4-ml soda-lime glass vials. Three of the vials at each TCAR concentration were frozen at −15°C and served as the controls at zero-time. Three samples, from each plasma concentration of TCAR, were gently shaken at 4°C and room temperature (23°C) for 24 h.

Fresh human blood was collected in heparinized tubes and used to prepare 500 μM and 5 μM TCAR solutions. The blood solutions were gently mixed and 1-ml aliquots from each were added to heparinized collection tubes. The tubes were gently shaken at either 4°C or room temperature. Three sample tubes at each TCAR concentration and each temperature were centrifuged at zero-time, 2 h and 24 h. The plasma was separated by centrifugation, transferred into glass vials, and stored frozen at −15°C until analyzed.

Mouse pharmacokinetic study

Plasma levels of TCAR were measured after a single bolus injection into the tail vein of CDF_1 mice, 24—27 g. TCAR powder was dissolved in 5% dextrose in water (Abbott Labs., North Chicago, IL, U.S.A.) and passed through a 0.45-μm filter. The dose of TCAR was 220 mg/kg, which is 1/13 of the single dose LD10 for CDF_1 mice [11]. Each mouse was sacrificed by decapitation and the blood was allowed to flow into a heparinized tube through a polypropylene funnel. Each tube was centrifuged immediately after blood acquisition. The plasma was separated and transferred to a 4-ml soda-lime glass vial. The samples were stored frozen at −15°C until they were analyzed. The volume of standards and samples used for the analysis was 150 μl.

Calculations

Both the TCAR standard curves and the plasma disappearance data from the mice were analyzed on MLAB, a non-linear-fitting program [12].

The standard curve data were fit to the straight-line equation of $y = mx + b$, where m is the slope of the line and b is the y-intercept. The y-value is the peak height ratio (PHR) of TCAR/iso-TCAR and the x-value is the concentration of TCAR. All the points of the standard curve were weighted using $1/(\mathrm{PHR})^2$ as the weighting factor.

The plasma concentration—time data were fitted to biexponential and triexponentional equations:

$$C(t) = A_1 e^{-\lambda_1 t} + A_2 e^{-\lambda_2 t}$$

$$C(t) = A_1 e^{-\lambda_1 t} + A_2 e^{-\lambda_2 t} + A_3 e^{-\lambda_3 t}$$

where $C(t)$ is the plasma TCAR concentration at time t. The half-lives of each of the phases can be calculated by dividing the natural logarithm of 2 by each of the apparent first-order elimination rate constants (λ_1, λ_2, λ_3). The weighting factor used to fit the exponential equations was $1/(\text{concentration})^2$. The area under the concentration—time curve(AUC) and total body clearance (Cl_{TB}) were calculated by:

$$\mathrm{AUC} = \Sigma\ (A_i/\lambda_i) \text{ and}$$

$$Cl_{TB} = \text{dose/AUC}.$$

RESULTS

Plasma extracts

Fig. 2 shows the chromatograms of normal human plasma extracts. In the

blank plasma (Fig. 2a), the peaks were unidentified plasma components. They did not elute, however, in the same region as TCAR or iso-TCAR (Fig. 2b). The retention times of iso-TCAR and TCAR were 7.8 and 8.6 min, respectively and had a peak-to-peak separation of 45 sec. The peaks of TCAR and iso-TCAR overlapped near their base but were separated enough such that the peak height measurement of either peak was not hindered by the presence of the other (Fig. 2b).

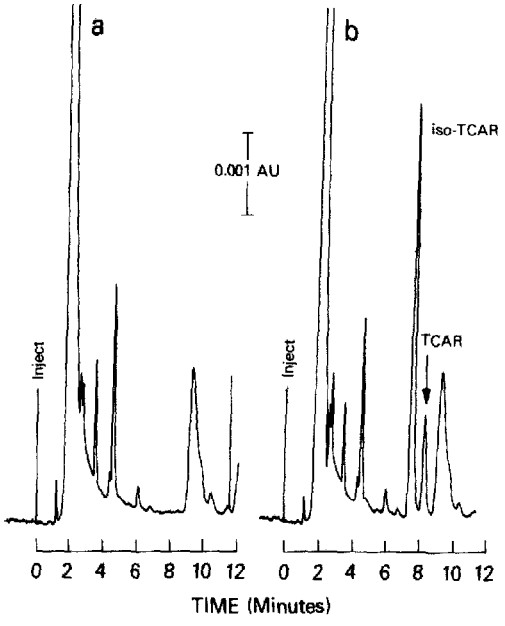

Fig. 2. Chromatograms for 0.5-ml samples of (a) normal human plasma and (b) normal human plasma with TCAR (1 μM) and iso-TCAR (10 μM). The absorbance was measured at 254 nm at an attenuation of 0.01 absorbance units (AU) full-scale. The retention times of TCAR and iso-TCAR were 7.8 min and 8.6 min, respectively. The extracted residue was redissolved in 150 μl HPLC mobile phase, and 50 μl were injected.

Linearity and sensitivity of extraction

The standard curve on the Waters columns with iso-TCAR as internal standard was linear between 0.1 μM and 1000 μM TCAR. The limit of detection was 0.33 μM (0.08 μg/ml). The standard curve obtained from the same standard solutions extracted on the Bakers columns was also linear over the same range. However, the limit of detection was 0.67 μM (0.17 μg/ml). The limit of detection for the two brands of extraction columns was proportional to the initial sample volume extracted on each brand. The standard curves that used [5-^{3}H]TCAR as an internal standard were also linear over the same range.

The size of the TCAR peak at the limit of detection for each of the procedures was 3.3—4% of the full-scale absorbance of 0.01 AU and was approximately ten times greater than the baseline noise.

The coefficient of variation for the standard curves was usually less than 5%. The maximum variation was 11% and occurred at concentrations less than 1 μM.

The y-intercept of the fitted line for each standard curve was less than 1/6

of the mean peak height ratio of the lowest TCAR standards. The deviation from the fitted line for 65 of 69 standard solutions was less than 6%. The maximum deviation from the fitted line was 15% and also occurred at concentrations less than 1 μM.

Extraction recovery

The aqueous 100 μM TCAR—iso-TCAR mixture was used for a total accounting of TCAR and iso-TCAR throughout the extraction procedure (Table I). The cumulative recovery of TCAR and iso-TCAR was close to 100% for both extraction procedures.

The mean recovery of TCAR and iso-TCAR in the methanol washes of the extracted normal plasma using both procedures was within 5% of the value for the methanol washes of the aqueous sample extraction. Also, the recovery of TCAR for each extraction procedure was not concentration-dependent. There was less than 5% difference in the recovery of TCAR from the 100 μM and 0.1 μM plasma solutions.

TABLE I

PERCENTAGE RECOVERY OF TCAR AND ISO-TCAR FROM EXTRACTION COLUMNS

	Waters column				Bakers column			
	Sample addition 0.5 ml	Water wash 4 ml	100% methanol wash 2 ml	Cumulative percent recovery	Sample addition 0.2 ml	Water wash 0.5 ml	50% methanol wash 0.5 ml	Cumulative percent recovery
TCAR	0	28	76	104	2	12	79	93
iso-TCAR	0	33	72	105	3	16	79	98

Dilution of samples

Six of the samples used in the in vitro blood study were measured, diluted 100-fold, extracted and remeasured. The undiluted samples were 500—700 μM. After remeasuring, and correcting for the dilution, there was a deviation of 6 ± 3% between the diluted and non-diluted measurements.

TCAR stability in normal human plasma

In normal plasma TCAR was stable for at least 24 h, regardless of the incubation temperature or the TCAR concentration. TCAR concentrations measured at the end of 24-h incubations were 100 ± 5% of the zero-time samples. In previous studies, TCAR was found to be stable for at least one week at room temperature in solution with mannitol and sodium chloride or 5% dextrose in water [13].

Apparent red blood cell uptake

Table II shows the concentrations of TCAR measured in the plasma of the blood samples. Even at zero-time, TCAR had entered red blood cells. If the red blood cells totally excluded TCAR, the expected plasma concentrations at

TABLE II

PLASMA TCAR CONCENTRATION (μM) OVER TIME FOR GENTLY MIXED HEPARINIZED HUMAN WHOLE BLOOD

Temperature (°C)	Incubation time		
	0	2 h	24 h
4	617 ± 8	556 ± 7	408 ± 4
23		463 ± 0	281 ± 2
4	4.82 ± 0.07	4.39 ± 0.01	3.14 ± 0.04
23		4.33 ± 0.04	2.56 ± 0.06

zero-time would have been 880 μM and 8.8 μM (for the measured hematocrit of 43%). At the end of the 24-h incubation, the mean concentration of TCAR in the separated plasma of the 4°C samples was 66% of the zero-time samples in the 500 μM case and 65% in the 5 μM case. At room temperature the 24-h 500 μM sample was 46% of the initial zero-time sample while the 24-h 5 μM sample was 53%.

Mouse pharmacokinetic study

Plasma samples from the mouse study had measurable TCAR concentrations for the entire 22-h sampling period (Fig. 3). A three-compartment fit to the data resulted in half-lives of 11.5, 38.4 and 497 min. At this dose, a two-compartment model also fit the data. The initial and terminal half-lives for the two-compartment model were 18.8 and 412 min. Both fits gave identical AUC and Cl_{TB} values (Table III) and similar values for the initial and terminal half-lives.

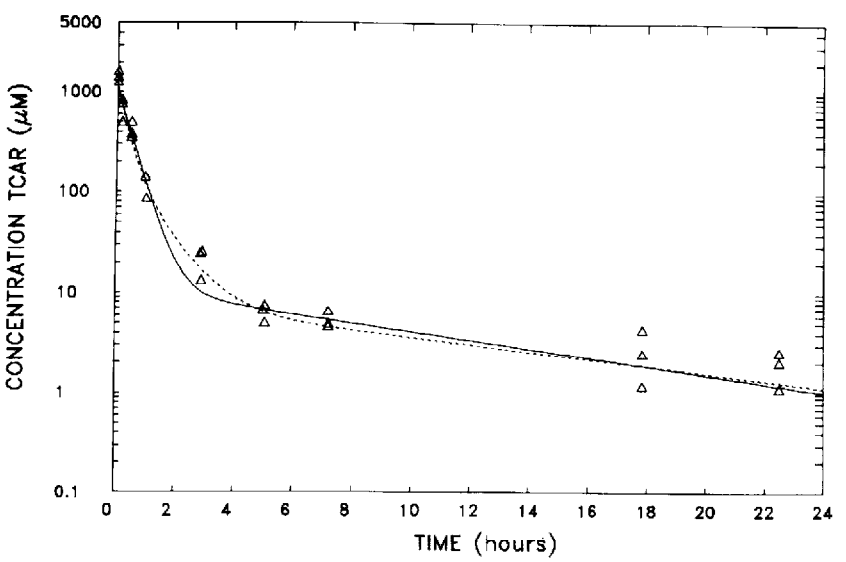

Fig. 3. The disappearance of TCAR in CDF_1 mouse after intravenous bolus injection (220 mg/kg). The triangles represent the measurement of plasma TCAR in a single mouse. The curves are the non-linear fits of the data for two-compartment (solid) and three-compartment (dashed) models.

TABLE III

SUMMARY OF THE PHARMACOKINETIC PARAMETERS OF TCAR IN CDF, MICE FOR BOTH TWO-COMPARTMENT AND THREE-COMPARTMENT MODELS

Model	AUC (m*M*—min)	Cl_{TB} (ml/min/m^2)	λ_1 (min^{-1})	λ_2 (min^{-1})	λ_3 (min^{-1})
Two-compartment	35.9	70.6	0.037	0.0017	—
Three-compartment	36.0	70.4	0.060	0.018	0.0014

DISCUSSION

The new analytical procedure for the measurement of TCAR in plasma is simple, sensitive, and reproducible. A large number of samples can be extracted and prepared for injection in a single day. An automatic injector allows the samples to be run on the high-performance liquid chromatograph overnight and the results can be calculated the following day. Either iso-TCAR or [5-^{3}H]-TCAR can serve as an internal standard, but iso-TCAR is more practical, especially for automated analyses.

The limit of detection for the extraction of 0.5 ml plasma is 0.33 μM (0.08 μg/ml). This is obtained by redissolving the extraction residue in 150 μl of HPLC mobile phase, injecting 50 μl into the HPLC system and measuring the absorbance at 254 nm. The limit of detection could be further lowered by injecting a larger fraction of the extract and/or by measuring the absorbance at the TCAR λ_{max} value of 238 nm.

In many clinical settings, blood samples are drawn and stored for several hours before the plasma is separated by centrifugation. If this occurs with blood samples of TCAR the results obtained from the analysis may be misleading. The fate of plasma TCAR in whole blood was shown in the in vitro blood study. After 2 h of incubation at 4°C the concentration of TCAR in the separated plasma was 10% less than the measured TCAR concentration in the zero-time sample, at the higher TCAR concentration. The room temperature sample showed a 25% decrease in plasma TCAR concentration.

The plasma disappearance of TCAR in animals has usually been described by a three-compartment model [14—16]. In the present study, there was little difference between the fits of the two-compartment and the three-compartment models. The major difference occurs at the 3-h time point, where the three-compartment model fit the data more accurately. However, the AUC and Cl_{TB} values were the same for either of the models.

Other HPLC procedures for the measurement of TCAR have been reported. One method proposes multiple liquid—liquid extractions for clean-up of plasma and uses and automated HPLC column-switching technique [5]. Although the column-switching technique can be a powerful tool, it is not readily available to the average researcher. The other methods are apparently less sensitive than the present method. Mouse plasma TCAR was detectable for less than 3 h [3] and 6 h [4] following doses of 200 mg/kg and 250 mg/kg, respectively. The present method measured TCAR in mouse plasma up to 22 h after injection of 220 mg/kg. The plasma TCAR was 1—10 μM in the samples between 3 and

22 h. The importance of this increased sensitivity is underscored by cell culture studies which have shown that TCAR concentrations as low as 5 μM produce 80% inhibition of its target enzyme, inosine monophosphate dehydrogenase [10].

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