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Note

Reversed-phase high-performance liquid chromatographic determination of anaxirone in biological specimens

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Anaxirone (α,β-1,2,4-triglycidylurazol; TGU; Henkel-SIS 43410; NSC 332 488) is a novel triepoxide derived from teroxirone (α-triglycidyltriazinetrione; TGT; Henkel's compound; NSC 296 934) with antineoplastic activity [1,2] (Fig. 1). This group of agents is currently undergoing clinical testing in Phase II studies and has shown thrombophlebitis at the injection site and myelosuppression as toxic side-effects [3–7]. Formerly reported high-performance liquid chromatographic (HPLC) assays allowed detection down to

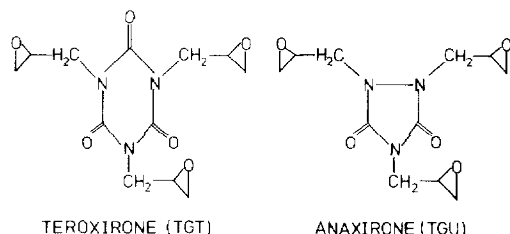

Fig. 1. Chemical structures of teroxirone [1,3,5-triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-tris-(oxiranylmethyl)-, (α)-; TGT] and anaxirone (α,β-1,2,4-triglycidyl-1,2,4-triazolidin-3,5-dione; TGU).

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15 μg/ml, and the reported terminal half-life in plasma was 8.63 min in man and 5.0 min in mice [8,9]. Since clinical studies revealed protracted bone marrow toxicity [4, 9, 10], a sensitive assay was needed to evaluate the pharmacokinetic behaviour in patients over several half-lives as well as their preclinical distribution pattern in animals.

EXPERIMENTAL

Reagents

TGU was obtained from Degussa Pharma Gruppe, Asta Werke (Frankfurt, F.R.G.; Lot No. 84 567). HPLC-grade methanol, water, chloroform and acetonitrile were supplied by Merck (Darmstadt, F.R.G.).

Chromatographic equipment

The chromatographic system consisted of a Waters Model 45 solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) at a flow-rate of 1 ml/min, a Rheodyne injector (Rheodyne, Berkeley, CA, U.S.A.) with a 100-μl loop, a precolumn Waters C_{18} Guardpak, a Waters μBondapak C_{18} column (300 $\times$ 3.9 mm I.D., particle size 10 μm), a Waters Model 481 detector set at 216 nm, and a Servogor 120 strip-chart recorder (BBC Metrawatt, Vienna, Austria).

Solutions

TGU stock solutions containing 2.0 mg/ml were prepared every second day in deionized water and stored at 2°C. Stock solutions were serially diluted into the working range of 10 ng/ml to 2.0 mg/ml. These solutions were injected directly into the column.

Plasma standards contained 1.0 ml of normal plasma (University Hospital Blood Bank, Essen, F.R.G.) and 1.0 ml of TGU aqueous standard over a concentration range of 10 ng/ml to 200 μg/ml. This mixture was vortex-mixed for 10 s (Scientific Industries, Springfield, MA, U.S.A.). Standards of TGU were used to establish a calibration curve for the assay of TGU concentration in the patients' plasma.

Mobile phase

A 150-ml volume of acetonitrile was added to 850 ml of water. Prior to use, the mobile phase was degassed by stirring under vacuum for 15 s.

Extraction procedure

Blood samples were chilled in ice immediately after collection, centrifuged at 1270 *g* for 10 min (Hettich, Tuttlingen, F.R.G.; Model Rotanta S) and the plasma was stored at −18°C until analysis. Plasma (1 ml) was added to 9 ml of chloroform in a test tube, vortex-mixed for 10 s, shaken for 10 min (Köttermann, Hänigsen, F.R.G.), then centrifuged at 1270 *g* for 10 min and the supernatant removed. The chloroform layer was evaporated in a water bath at 40°C under a stream of nitrogen, and the residue was redissolved in 100 μl of methanol. A 20-μl aliquot was injected onto the column. A similar procedure was used for ascitic fluid.

Patients received 600 or 800 mg/m^2 TGU as a bolus intravenous injection.

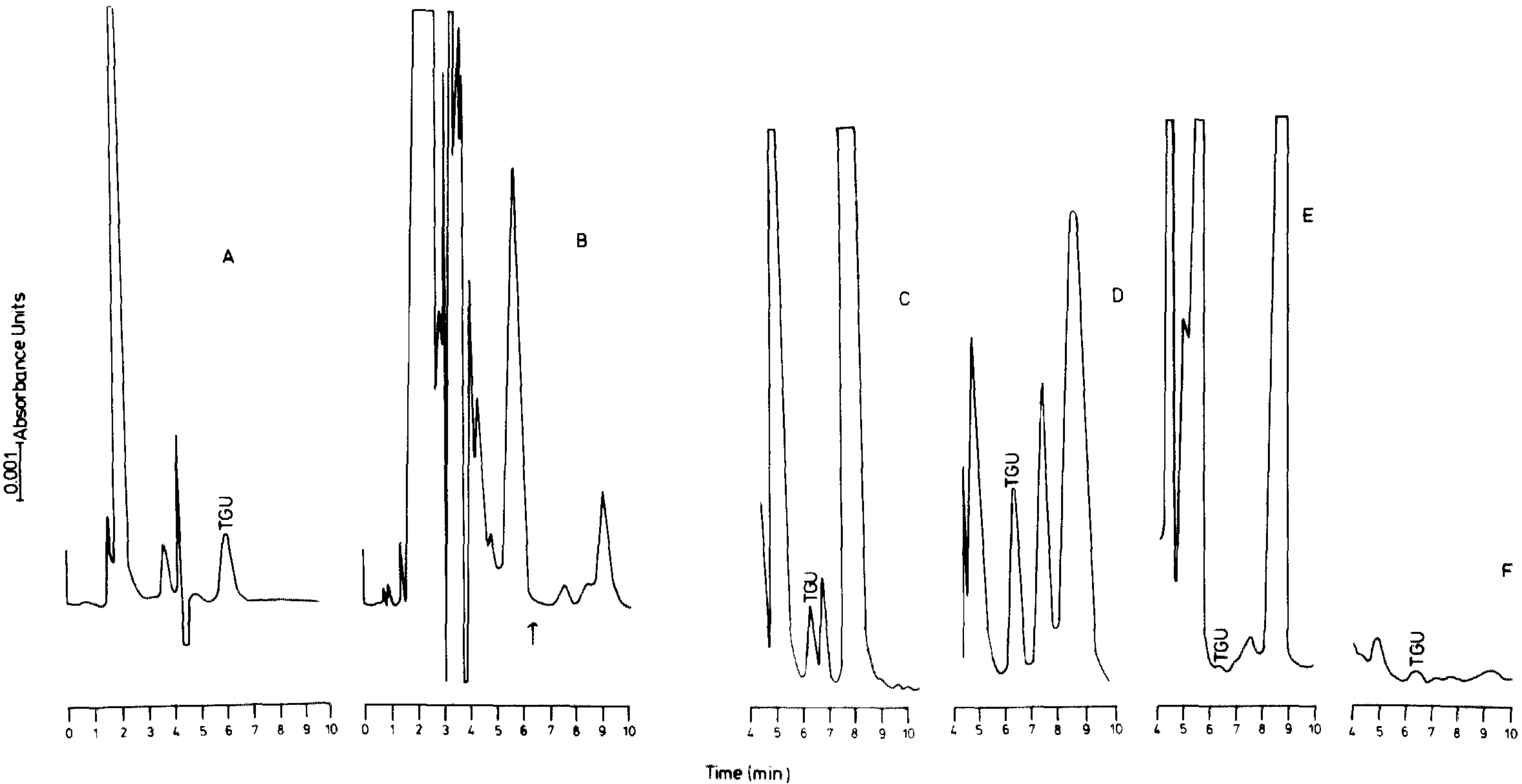

Fig. 2. Chromatograms obtained from (A) aqueous standards prepared with 1 μg/ml TGU; (B) control blank plasma; (C) control plasma containing 1 μg/ml TGU, processed 1 h after preparation; (D) patient's plasma 1 min (concentration 2800 ng/ml) and (E) ascitic fluid 20 min after treatment with a single dose of 600 mg of TGU per m^2 body surface area (concentration 120 ng/ml); and (F) cardiac tissue in mice removed seven days after second dose of 72.24 mg of TGU per kg body weight intraperitoneally. The retention time for TGU was 6.2 min.

Blood samples were collected via an intravenous catheter into EDTA-containing test tubes before therapy and at selected times thereafter.

Four female F_1 mice were obtained from the University of Hannover (Hannover, F.R.G.). All animals were anaesthetized with 50 mg of pentobarbital per kg body weight, given intraperitoneally. Tissue samples from two animals served as blanks. One mouse was given 72.24 mg/kg TGU intraperitoneally weekly for three weeks and another animal received 144 mg/kg by a rapid injection into the tail vein, and the animals were sacrificed after 1 h by cervical dislocation. Selected tissue specimens were removed and mechanically disintegrated (Sonifier B-12, Branson Sonic Power, Danbury, CT, U.S.A.), and an aliquot was taken, centrifuged, extracted and analysed as for plasma samples (Table II).

Calculations

The concentration of TGU in plasma or tissue was calculated by dividing the peak height by the slope of the appropriate plasma standard curve. The slope of each standard curve was determined as the mean ±S.D. of response factors. Extraction efficiency (recovery) was determined by comparing peak heights of extracted plasma standards to peak heights of directly injected aqueous standards of corresponding concentrations.

RESULTS

Stability of aqueous standards

At 2°C, TGU standards in aqueous solutions were stable for up to three days, but long-term studies revealed a 60% decomposition after two months. Aqueous standards (2 μg/ml) were unaltered by elevated temperature (35 and 40°C) but were decomposed by heat (60°C) over 4 h. Light-protected samples were stable for one week at room temperature and for two weeks when stored at 2°C. Fresh aqueous standards were routinely prepared every two days and stored at 2°C.

Stability of plasma standards

In heparinized plasma, TGU (1.0 μg/ml) was degraded with a half-life of 90 min. The disappearance of TGU was accompanied by the appearance of two additional peaks with retention times of 3.8 and 4.2 min.

Chromatograms

Sample chromatograms of aqueous standard (A), control blood bank plasma (B), spiked blood bank plasma (C), and plasma and ascitic fluid from patients (D and E) are shown in Fig. 2. No interfering peaks were seen in the control plasma, the plasma obtained before administration of TGU, or in any of the blank tissue samples obtained from mice. The retention time of TGU was 6.2 min.

Standard curves

Representative standard curve values for TGU are shown in Table I. The correlation coefficient of 0.999 indicates a high degree of linearity in the concen-

TABLE I

LINEARITY AND PRECISION OF THE HPLC ASSAY OF TGU IN PLASMA

TGU concentration (ng/ml)		Coefficient of variation (%)	Recovery*** (%)
Theoretical*	Found** (mean ± S.D.)		
10	9.9 ± 0.5	1.0	104
30	28.7 ± 0.9	4.3	102
100	84.6 ± 1.2	15.4	92
200	211.5 ± 10.3	5.8	114
2000	1813.4 ± 97.3	9.3	102
10000	8510.9 ± 521.1	14.9	86
200000	197139.2 ± 2605.4	1.4	108
Mean ± S.D.		7.4 ± 5.5	101 ± 9

*Theoretical concentrations were based on the amount of TGU in water added to plasma.
**The concentration found was calculated from the calibration curve for each of two plasma samples per concentration. The slope calculated was 2.840 ± 0.039 mm/(ng/ml) (mean ± S.D.).
***The percentage recovery was calculated from peak heights of plasma standards divided by peak heights of standard solutions in water containing the corresponding concentrations.

TABLE II

TGU CONCENTRATIONS IN VARIOUS BODY TISSUES OF TWO MICE 1 h AFTER RECEIVING 72.24 mg TGU PER kg INTRAPERITONEALLY (THIRD COURSE) AND 144 mg TGU PER kg INTRAVENOUSLY AS A BOLUS INJECTION

Material	TGU concentration (ng/mg)	
	Intraperitoneally	Intravenously
Heart	408.1	156.6
Lung	163.9	195.8
Kidney	132.6	139.4
Liver	149.1	134.3
Muscle	124.7	68.6
Bone	N.D.*	–
Bone marrow	N.D.	–

*N.D. = Not detected.

tration range from 10 to 200 000 ng/ml. The mean coefficient of variation of 7.4% showed good precision over this wide concentration range. The recovery of TGU from plasma was 101 ± 9% and the limit of detection was 10 ng/ml in plasma.

Table II describes the tissue distribution of TGU in mice and demonstrates the high concentrations in heart and lung tissues.

DISCUSSION

A variety of mobile phases revealed short retention times below 5 min and were insufficient to separate the drug from interferences.

A previously described HPLC method for TGT employed diethyldithiocarbamate for derivatization, and another assay using methanol–water as mobile phase revealed insufficient separation [1,11]. Polar solvents, e.g. methanol and toluene, allowed recovery of only 30% of added TGU from plasma. An octadecyl silica column (J.T. Baker, Phillipsburg, NJ, U.S.A.) conditioned with methanol and water and eluted with methanol showed a drug recovery of only 35%. The most reproducible, sensitive results were obtained using a liquid–liquid extraction step.

The method described here was not subject to interferences from a number of concomitantly administered drugs such as digitalis, barbiturates or penicillin. In a further application of this assay, we have measured concentrations of TGU as low as 100 ng/ml used in diffusion chamber assays. Our HPLC method is practical for clinical plasma and tissue TGU level measurement, as well as for animal and in vitro studies.

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