Eur J Cancer Clin Oncol, Vol. 20, No. 7, pp. 933–938, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

A Sensitive Assay for the Pharmacokinetic Investigation of a Rapidly Hydrolyzing Alkylating Agent Lycurim® (Ritrosulfan; R-74; NSC-122 402)

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Abstract—*Lycurim® [1,4-di(2'-methanesulfonyloxyethylamino)-1,4-dideoxymesoerythritol dimethane sulfonate] is rapidly hydrolyzed in aqueous media to inactive products according to a second-order reaction. The respective rate constants are:* $k_1 = 9.82 \times 10^{-2}$ *and* $k_2 = 1.76 \times 10^{-2}$ *μmol/ml/min. The concentrations of the parent compound and alkylating intermediate(s) were measured by chemical trapping with N,N-diethyldithiocarbamic acid (DDTC). The rate of this reaction is substantially higher:* $k_1 = 2.61 \times 10^{-1}$ *and* $K_2 = 4.76 \times 10^{-2}$ *μmol/ml/min. By using* ^{35}*S-labeled DDTC, alkylating compounds in concentrations as low as 0.04 μg/ml could be detected in spiked plasma samples. After intracavitary application of 60 mg Lycurim no alkylating activity could be demonstrated in the plasma of patients at any time point.*

INTRODUCTION

LYCURIM [1,4-di-(2'-methanesulfonyloxyethylamino)-1,4-dideoxymesoerythritol dimethane sulfonate] (Fig. 1.I.) is a member of the alkylating sugar alcohol derivatives developed and synthesized by Horváth and Vargha in Hungary [1]. The biological properties were studied by Csányi and his co-workers [2,3] and by Sandberg and Goldin [4]. In clinical studies it was found to be effective especially against chronic lymphoid leukemias, lymphomas and for intracavitary treatment of tumorous exudates [5–7]. In the present study we describe the reaction kinetics of Lycurim with ^{35}S-*N,N*-diethyldithiocarbamic acid sodium salt (DDTC) [8–10] for the trapping of the parent compound and its alkylating intermediates in biological fluids.

MATERIALS AND METHODS

Lycurim was kindly supplied by the Chemical Works of Gedeon Richter Ltd, Budapest, Hungary. [^{35}S]-DDTC was purchased from Amersham, U.K., with a specific activity of 262.6 MBq/mmol.

Accepted 3 January 1984.

*Methanesulfonyloxy = mesyloxy.

Chromatography

The separation of the solvolytic products of Lycurim as well as their DDTC derivatives were studied by one-dimensional TLC on precoated plates (Kiselgel F254, 0.25 mm thickness, Merck, Darmstadt, F.R.G.) in two different solvent systems (Table 1). The separated compounds were detected on the plates using the following methods: under the u.v. lamp at 254/366 nm; colour reaction with nitrobenzylpyridine (NBP) reagent for alkylating derivatives; [11] potassium permanganate and benzidine for hydrolytic products containing a primary hydroxyl group [12]; phosphomolybdic acid for DDTC derivatives [13]; and generally with 5% sulfuric acid in methanol solution by heating at 125°C for 15 min.

The radiochromatograms were evaluated by a Berthold Radiochromatogram Scanner and the radioactivity of the separated spots was measured by an LKB Wallac Liquid Scintillation Counter (Type 81000) in Aquasol® scintillation cocktail (New England Nuclear).

The kinetic study of the reaction between Lycurim and DDTC

Solutions: 3 mg/ml Lycurim (5.4×10^{-3} mmol/ml) in water; 2.97 mg/ml [^{35}S]-DDTC (1.3×10^{-2} mmol/ml), specific activity 262.6 MBq/mmol. Two milliliters from both solutions were

Table 1. Chromatographic data of Lycurim and its derivatives in solvent systems S_1 and S_2

Lycurim derivatives	R_F values							
	S_1†	S_2†	*	†	‡	§	‖	¶
1,4-di-(2'-mesyloxy-ethylamino)-1,4-dideoxy-*m*-erythritol dimethanesulfonate (I.) Lycurim	0.47	0.51	+	+	-	+	-	-
1-(2'-mesyloxy-ethylamino)-4-(2"-hydroxy-ethylamino)--1,4-dideoxy-*m*-erythritol (II.)	0.40	0.43	+	+	-	+	+	-
1,4-di-(2'-hydroxy-ethylamino)-1,4-dideoxy-*m*-erythritol (III.)	0.30	0.36	+	+	-	-	+	-
1,4-di-(2'-)N,N-diethyl-dithiocarbamoyl(-ethylamino)-1,4-dideoxy-*m*-erythritol (V)	0.78	0.85	+	+	+	-	-	+
1-(2'-)N,N-diethyl-dithiocarbamoyl(-ethylamino-4-)2'-mesyloxy-ethylamino(-1,4-dideoxy-*m*-erythritol) (IV.)	0.69	0.73	+	+	+	+	-	+
1-(2'-)N,N-diethyl-dithiocarbamoyl(-ethylamino-4-)2'-hydroxy-ethylamino)-1,4-dideoxy-*m*-erythritol (VI.) (derived from II)	0.55	0.65	+	+	+	-	+	+

*5% sulfuric acid in methanol, and heating at 125°C for 15 min.
†u.v. light, 254 nm.
‡u.v. light, 366 nm.
§Colour reaction with NBP.
‖Potassium permanganate and benzidine.
¶Phosphomolybdic acid.
S_1 = (isopropyl alcohol–ammonium hydroxide 35%–sodium hydrogen phosphate solution, pH = 4.75) (80:5:15); S_2 = butyl acetate–chloroform–acetone–benzene–pyridine (6:5:4:3:1).

mixed at room temperature and after 0, 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 min standing 100-μl samples were applied for TLC separation in solvent system S_1. The interaction proceeded according to the second-order consecutive reactions:

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C; \qquad C + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} D,$$

where A = original Lycurim (I) concentration, B = original [^{35}S]-DDTC (VII) concentration, C = concentration of the intermediate product (IV) and D = final product (V). The reaction rate constants were calculated according to the methods published by Laidler [14] and Benson [15].

The kinetic study of the hydrolysis of Lycurim

Immediately after the dissolution of Lycurim and 5, 10, 20, 30, 40, 50, 60, 90 and 120 min later 100-μl samples were removed from the solution. The samples were added to 1 ml 2.97 mg/ml (1.32×10^{-2} mmol/ml) of [^{35}S]-DDTC solution, mixed and allowed to stand for 1 hr at room temperature.

After that a 200-μl sample was used for the determination of the unchanged drug and its alkylating metabolite as mentioned earlier. Lycurim (I) was recovered as compound V and the intermediate (II) as compound VI (see Figs 1, 2 and Table 1). The concentration of Lycurim was 3 mg/ml (5.4×10^{-3} mmol/ml).

Determination of Lycurim and its alkylating metabolites in form of [^{35}S]-DDTC-derivatives from human plasma

Five hundred microliters of [^{35}S]-DDTC solution were added to the mixture of 2 ml human plasma and 200, 100 and 10 μl of Lycurim, mixed and allowed to stand for 1 hr at room temperature (Table 2). For the extraction of DDTC derivatives of the parent compound and its intermediate products the pH was increased to over 8.4 by 0.1 N sodium hydroxide. Afterwards it was extracted five times with 2 ml of chloroform. The chloroformic suspension was separated by centrifugation at 0°C, 5000 rev/min for 15 min.

The combined chloroform layer was dried on sodium sulfate. After the filtration the chloroform solution was evaporated to dryness. The residue was dissolved in 500 μl chloroform and 100 μl solution was separated by TLC in solvent system S_1. The Lycurim concentration was calculated on the basis of the specific activity of [^{35}S]-DDTC.

Clinical pharmacologic studies

Two patients with pleural effusion undergoing intracavitary Lycurim therapy were evaluated for blood levels of alkylating compounds. Neither of the patients had received chemotherapy for several months before the study. In both cases 60 mg Lycurim, dissolved in 60 ml of saline immediately before application, was injected after tapping the exudate. Five milliliters of blood samples were taken immediately after Lycurim administration and 30, 60, 90, 120, 150, 180 and 360 minutes later. The blood was collected in

Table 2. Recovery of Lycurim added to plasma samples

Concentration of Lycurim solution:							
0.5 mg/ml	200 μl	100 μl	20 μl				
0.05 mg/ml				100 μl	20 μl		
0.005 mg/ml						100 μl	20 μl
Human plasma	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
2 mg/ml [^{35}S]-DDTC solution	500 μl	500 μl	500 μl	500 μl	500 μl	500 μl	500 μl
Original Lycurim content of sample	100 μg	50 μg	10 μg	5 μg	1 μg	500 ng	100 ng
Calculated radioactivity (DMP)	5.6614×10^6	2.8307×10^6	5.6614×10^5	2.8307×10^5	5.6614×10^4	2.8307×10^4	5.6614×10^3
Found radioactivity at $R_F = 0.74$ (DPM)	5.4349×10^6	2.6609×10^6	5.0386×10^5	2.4344×10^5	4.4159×10^4	2.0947×10^4	3.1704×10^3
Found Lycurim content/ according to above radioactivity	96 μg	47 μg	8.9 μg	4.3 μg	780 ng	380 ng	56 ng
Ratio of found/calculated	0.96	0.94	0.89	0.86	0.79	0.74	0.56

tubes containing 10 μl of heparin and 500 μl of [^{35}S]-DDTC, 2 mg/ml concentration, to trap immediately the alkylating compounds. After centrifugation the plasma was allowed to stand at room temperature for 1 hr. The DDTC derivatives were determined as described above.

RESULTS

The dissociation and hydrolysis of Lycurim (I) is supposed to proceed at slightly alkaline pH according to the scheme shown in Fig. 1. During the reaction four molecules of methane sulfonic acid per mol of Lycurim are formed. If these are neutralized by the addition of a base, the reaction can proceed until the formation of the final, biologically inactive hydrolytic products (III). During this process an intermediate, monofunctional alkylating agent containing only one methanesulfonyloxy group is formed (II). The rate constants of the two consecutive reactions of hydrolysis were found to differ almost with one order of magnitude: $k_1 = 9.82 \times 10^{-2}$ and $k_2 = 1.76 \times 10^{-2}$ μmol/ml/min.

DDTC applied to great excess is able to trap both the mono- and bifunctional alkylating compounds due to its high nucleophilic activity (Fig. 2). According to our determination, the rate constant of the first step of the reaction is 2–3 times higher than the corresponding rate constant of hydrolysis. Rate constants for the DDTC reactions with Lycurim are: $k_1 = 2.61 \times 10^{-1}$ and $k_2 = 4.76 \times 10^{-2}$ μmol/ml/min.

The R_F values of Lycurim derivatives in different solvent systems are summarized in Table 1.

The limit of detection of Lycurim in human plasma was 0.04 μg/ml under ten-fold excess of a stoichiometric [^{35}S]-DDTC quantity relative to the calculated maximum concentration of the drug. At or above 0.4 μg/ml of Lycurim the recovery is 79–96% (Table 2).

No Lycurim or other alkylating compounds could be detected in the blood at any time points after intracavitary drug application. In the sample of residual pleural exudate, which was obtained immediately after Lycurim injection, 85% of the calculated drug concentration was detected. The 15% decrease might have been due to dilution by the residual fluid and chemical interactions.

DISCUSSION

The pharmacokinetic basis of the intracavitary application of Lycurim has been investigated with radioactive drug uniformly labeled with ^{14}C at the ethylamino side chains [16, 17]. After intracavitary administration of 30 mg Lycurim, the peak plasma concentration of radioactivity was lower than 0.5 μg/ml and occurred around 5 hr. The absorption half-life of radioactivity was calculated to be around 1 hr. After intracavitary administration in the plasma Lycurim or alkylating metabolites could not be detected by the 4-(4′-nitrobenzyl)-pyridine reaction. It was assumed that a part of Lycurim would react with nucleophiles other than hydroxyl ions present in the exudate. Other part of Lycurim would be converted mostly or entirely to inactive hydrolytic products locally, due to the long absorption time. The values of rate constants of hydrolysis calculated in our present study are large enough to permit alone the almost complete conversion of Lycurim to the final hydrolytic product before absorption. The reaction with SH-containing nucleophiles in tissues or body fluids is probably even quicker.

The rate constants of the interaction of DDTC with Lycurim were found to be one order of magnitude higher than those of hydrolysis.

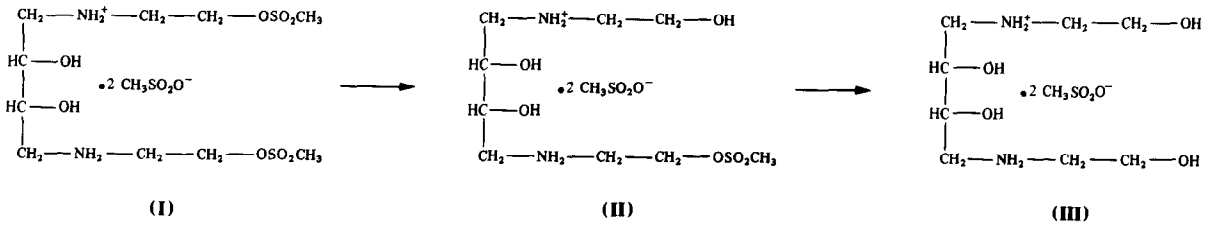

Fig. 1. Scheme of hydrolysis of Lycurim.

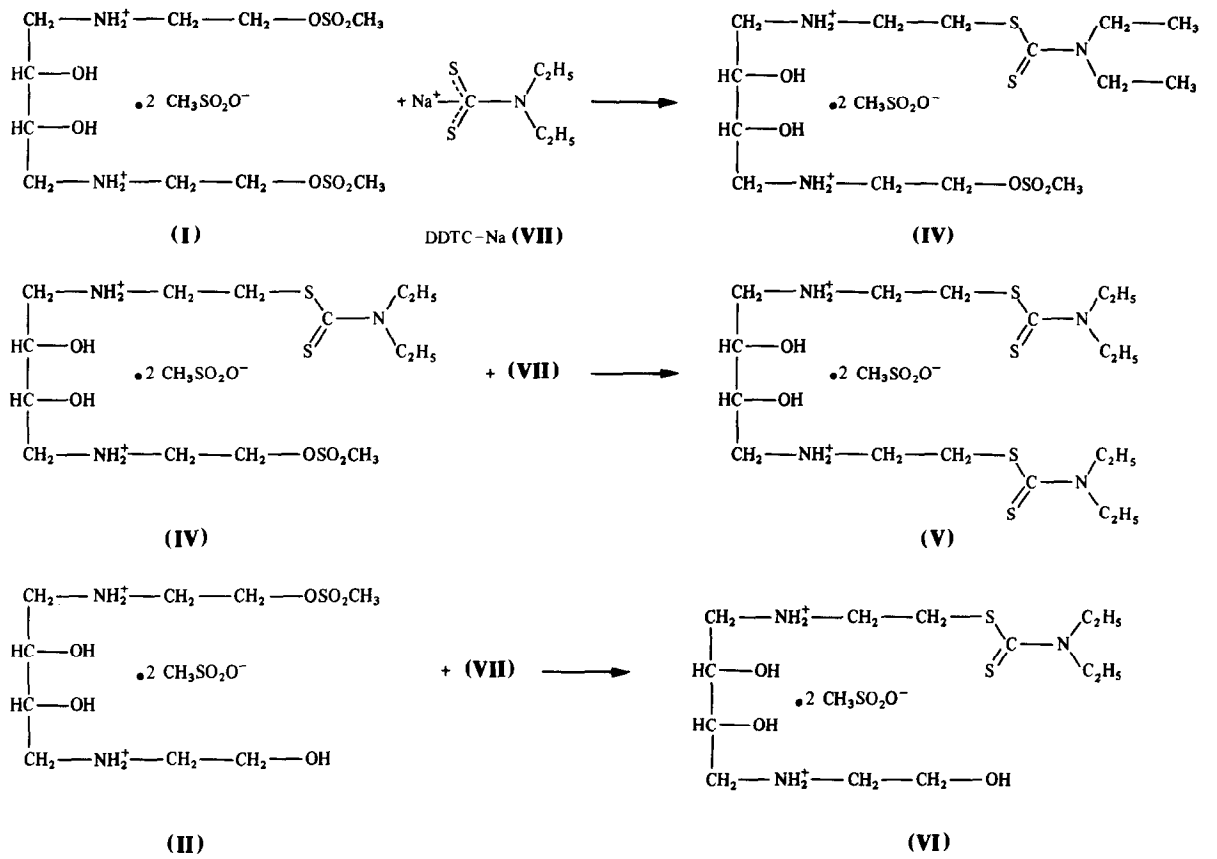

Fig. 2. Interaction of Lycurim with DDTC.

Accordingly, this reaction is suitable for trapping the alkylating compounds in biological fluids before hydrolysis. Nevertheless, no radioactive spots corresponding to Lycurim–DDTC derivatives could be demonstrated in the plasma for 6 hr after the intracavitary application of 60 mg Lycurim, which is the clinically tolerable and effective dose if applied in a small volume. Consequently, the concentration of alkylating agents in the blood remained lower than 0.4 μg/ml, which is below the 1 μg/ml threshold inhibitory concentration of the drug determined in tissue culture [2]. The observation of Ban *et al.* [18] showing that Lycurim remained active in phosphate-buffered saline solution at a pH >7.4 even if stored at 37°C for 72 hr does not contradict our conclusions since under their conditions the alkalinity would primarily favor the cyclization of the ethylamino side chains into much more stable aziridine rings [19].

On the basis of the pharmacokinetic results it might be concluded that this rapidly hydrolyzing alkylating agent is particularly suitable for intracavitary treatment because only inactive products reach the systemic circulation. Indeed, according to out ongoing phase I study, even 180 mg of Lycurim dissolved in 2 l of fluid can be safely administered in the form of a 'belly bath' therapy described by Jones *et al.* [20].

Acknowledgement—The authors are indebted to Chemical Works Gedeon Richter, Ltd, for kindly providing Lycurim and ^{35}S-labeled DDTC for the study.

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APPENDIX: CHEMICAL SYNTHESES

Synthesis and identification of the final product of the hydrolysis of 1,4-*di*-(2'-*hydroxyethylamino*)-*1,4-dideoxy*-m-*erythritol* (III)

Fifty-six milligrams (0.1 mmol) of Lycurim were dissolved in 5 ml water and 2 ml 0.1 N sodium hydroxide solution was added dropwise so that the pH could be kept between 7 and 7.4. During the addition the solution was stirred, then allowed to stand for 2 hr. After reaching the steady state, the pH of the reaction mixture was adjusted to 8.5–9 by the addition of 0.1 N sodium hydroxide solution. The product was extracted three times with 5 ml chloroform. The combined chloroformic layer was dried on sodium sulfate and evaporated to dryness. The oily product was dissolved in 0.5 ml isopropyl alcohol, added with a few drops of concentrated hydrochloric acid and rubbed until crystallization. The white crystalline product was recrystallized from boiling isopropyl alcohol and dried at room temperature.

The resulting product (26.4 mg; 94%; Mp: 135–137°C) was controlled by TLC, u.v. and IR spectroscopy.

Elemental analysis for $C_8H_{22}N_2O_4Cl_2$. Mol. wt: 281.28; calculated: C, 34.17%; H, 7.89%; N, 9.96%; Cl, 25.22%; found: C, 33.6%; H, 8.1%; N, 9.5%; Cl, 24.9%.

Synthesis of the Lycurim-DDTC derivative, 1,4-*di*-(2'-N,N-*diethyl-dithiocarbamoyl*)-*ethylamino*-1,4-*dideoxy*-m-*erithrytol* (V)

Two hundred milligrams (0.8 mmol) of DDTC were dissolved in 10 ml water. One hundred and twenty milligrams of Lycurim were added while stirred and then allowed to stand for 2 hr. The pH of the reaction mixture was then adjusted to 8.5–9 by the addition of 0.1 N sodium hydroxide solution. The

reaction product precipitated in the form of a free base. It was dissolved in 10 ml chloroform and the supernatant was extracted two times with 10 ml chloroform. The product was prepared as described in the synthesis of the hydrolytic product (93.4 mg, 86%), Mp: 122–124°C.

Elemental analysis for $C_{18}H_{40}N_4O_2S_4Cl_2$. Mol. wt: 543.71; calculated: C, 39.76%; H, 7.42%; N, 10.30%; S, 23.59%; Cl, 13.04%; found: C, 39.6%; H, 7.7%; N, 10.2%; S, 23.6%; Cl, 13%.

The product was crystallized easily in the form of formate salt from isopropyl alcohol by the addition of a few drops of formic acid (Mp: 114–116°C).

Elemental analysis for $C_{20}H_{42}N_4O_6S_4$. Mol. wt: 562.85; calculated: C, 42.68%; H, 7.52%; N, 9.95%; S, 22.78%; found: C, 42.5%; H, 7.7%; N, 9.8%; S, 22.6%.