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RADIOCHEMICAL ASSAY OF STABILITY OF ^{14}C-CYTOSTASAN SOLUTIONS DURING PREPARATION AND STORAGE

V. ŠČASNÁR,* Š. BEZEK,* T. TRNOVEC,* R. GRUPE,**
I. LISSÉ**

**Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, Bratislava (Czechoslovakia)*
***Institute for Pharmacological Research in the Pharmaceutical Industry GERMED, Berlin*

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Cytostasan, 5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazolyl-2-butyric acid, is an antineoplastic agent which degrades spontaneously in water solutions yielding two hydrolysis products, monohydroxy- and dihydroxycytostasan. We developed a stability-indicating radiochemical assay based on ion-pair extraction to investigate the stability of solutions of ^{14}C-cytostasan under conditions that might be expected when the drug is being prepared and stored for pharmacokinetic studies in animals. The possibility of using the distribution coefficient of ^{14}C-cytostasan as an indicator of stability was investigated in the extraction system benzene-dicarbolide of cobalt-0.5N $HClO_4$. The mechanism of extraction is believed to be that of ion-pair forming process between the hydrophobic anion and the protonized cytostasan. Since no extraction of hydroxy derivates was observed the value of the distribution coefficient of the parent drug appears to be a suitable indicator of the stability of ^{14}C-cytostasan solutions.

Introduction

Over the past two years we have been dealing with pharmacokinetics of cytostasan an antineoplastic drug of the nitrogen mustard type, which is clinically used in the treatment of chronic lymphadenosis and multiple myeloma.[1] Chemically, cytostasan is 5-[bis(2-chloroethyl)amino]-1-methylbenzimidazolyl-2-butyric acid and belongs to the group of alkylating agents (melphanal, chlorambucil). The analytical chemistry of cytostasan was described by HESS.[2] He found that the drug degrades spontaneously in water solutions yielding two hydrolysis products, monohydroxy-cytostasan and dihydroxycytostasan. Neither of these degradation products has cytotoxic activity. Kinetic data for the individual hydrolytic steps of cytostasan were obtained by the ^{1}H-NMR method.[3] Upon hydrolysis two chlorine atoms are replaced by an OH group. Because of the high instability of cytostasan in water we focused our attention to its stability under conditions that might be expected when the drug is being prepared and stored prior to administration to animals. We used the drug double la-

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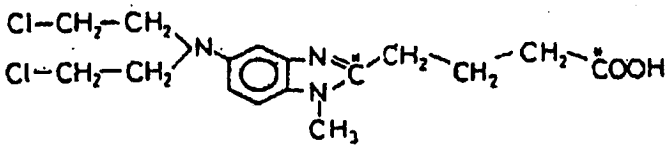

Fig. 1. Chemical structure of ^{14}C-cytostasan (* position of label)

belled with ^{14}C. The structural formula of ^{14}C-cytostasan is shown in Fig. 1. As known, radiochemical purity, the fraction of radioactivity present in the specified chemical form, is a major factor determining the reproducibility in pharmacokinetic studies. Impurities may arise during preparation and storage of radioactively labelled drugs and will modify organ distribution and specificity of the assay, possibly leading to incorrect data. The aim of this work was to propose a simple and specific radiochemical assay for the indication of the stability of water solutions of ^{14}C-labelled cytostasan to be performed before they are used in pharmacokinetic experiments.

Experimental

^{14}C-cytostasan with a specific radioactivity of 290 MBq/mmol was prepared in the Zentralinstitut für Kernforschung, Rosendorf, GDR. The product was supplied in the form of a powder without any traces of humidity in a sealed vial. Its radiochemical purity was 98%. Nonradioactive cytostasan and dihydroxycytostasan were gifts from the Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, GDR. Stock solutions of ^{14}C-cytostasan were prepared by dissolving the proper amount of label in distilled water or saline and aliquots of 10 μl were taken for thin layer chromatography analysis or extraction experiments at various storage time intervals. The polyhedral complex $H^+[(\pi\text{-}(3)\text{-}1,2\text{-}B_9C_2H_{11})_2Co^-]$, further referred to as dicarbolide of cobalt (DC-H^+), was synthesized in the Institute of Inorganic Chemistry, Czechoslovak Academy of Sciences, Prague, and supplied in the form of an orange powder. For extraction the agent was dissolved in benzene. All reagents and organic solvents used were of analytical grade.

Ascending thin layer chromatography was conducted on Silufol UV 254 nm chromatoplates (Kavalier, CSSR) coated with silica gel. The solvent system butanol–acetic acid–water (4:1:1) was used. A small amount of nonradioactive cytostasan and dihydroxy-cytostasan were spotted together with the sample being analyzed in order to visualize the spots under a UV lamp. Monohydroxycytostasan was prepared by storing nonradioactive cytostasan in distilled water for several days. The chromatoplates were analyzed with a scanner equipped with a gas flow proportional detector (Tesla Vráble, CSSR). For quantitative determination of radioche-

mical purity, the chromatoplates were cut into several sections and individual strips were counted for radioactivity in 10 ml of Bray's scintillation cocktail using the Tricarb model 300 CD (Downers Grove, IL, USA) liquid scintillation counter.

The extraction experiments were carried out in glass tubes at ambient temperature by shaking for 5 minutes at the phase ratio org/aq = 1/1. After extraction both phases were separated by centrifugation. The distribution coefficient D was calculated as the radioactivity ratio of aliquots of the organic and aqueous phase.

In studying the effect of temperature on the value of the distribution coefficient of ^{14}C-cytostasan the solutions were kept at 37 °C on a water bath, and at 5 °C and −15 °C using a commercial refrigerator and freezer. Thawing of samples was achieved at ambient temperature, and immediately afterwards the samples were analyzed and then refrozen at −15 °C for at least 30 minutes.

Results and discussion

A representative radiochromatogram of freshly dissolved ^{14}C-cytostasan in distilled water is shown in Fig. 2 (5 min). Its radiochemical purity was found to be 96% and the R_f value 0.66. The same sample analyzed 20 days later provided one radioactive peak with an r_f value of 0.36, as is seen in Fig. 3a. In both cases the radioactive peaks corresponded to the spots of freshly dissolved nonradioactive drug and to the drug stored for 3 months in distilled water and to the spot of synthesized dihydroxycytostasan, the final degradation product of cytostasan.

Figure 2 depicts the radiochromatogram of carrier-free ^{14}C-cytostasan held in distilled water at ambient temperature for 5, 15, 30 and 70 minutes. As is seen, a rapid degradation of the label occurred. The radiochemical purity test revealed the following amounts of the parent drug at the given time intervals: 96, 80, 70 and 65%, respectively. The corresponding spots under UV lamp are shown below. Peak 1 corresponds to ^{14}C-cytostasan, peak 2 is ^{14}C-monohydroxycytostasan and peak 3 is ^{14}C-dihydroxycytostasan.

The degradation rate of ^{14}C-cytostasan was reduced by addition of 100 μg/ml of nonradioactive cytostasan to the ^{14}C-cytostasan stock solution. Even after 2 hour storage, about 90% of the parent drug was present in the solution. The inhibition of degradation to a minimum rate was observed in water solutions saturated with nonradioactive cytostasan. A typical radiochromatogram of slowly hydrolyzed ^{14}C-cytostasan kept for 14 days in saturated water solutions is given in Fig. 3b. It is obvious that the radioactive peaks 1,2 and 3 correspond to the parent drug, monohydroxycytostasan and dihydroxycytostasan, respectively.

From the results presented it is clear that TLC provides an excellent proof on

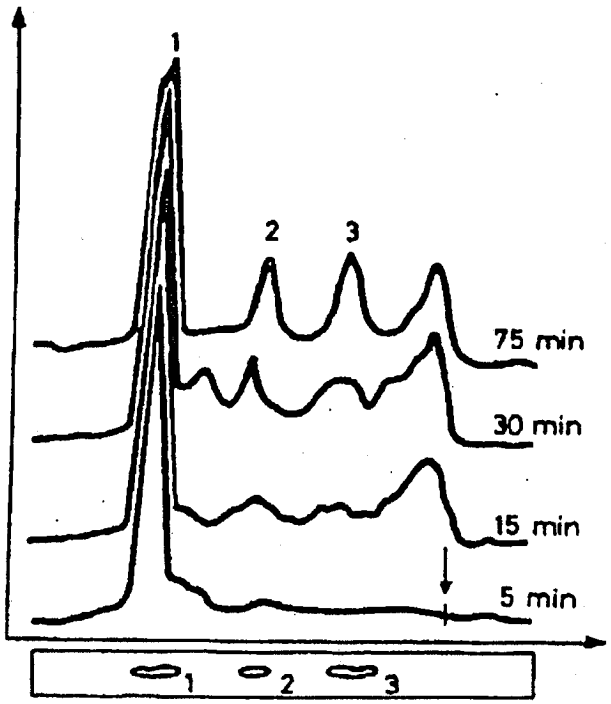

Fig. 2. TLC radiochromatograms showing the effect of storing time on the stability of ^{14}C-cytostasan kept in distilled water. The corresponding nonradioactive spots are shown below. The application point is indicated by an arrow; (1) ^{14}C-cytostasan, (2) ^{14}C-monohydroxycytostasan, (3) ^{14}C-dihydroxycytostasan

the high instability of ^{14}C-cytostasan in water solutions, but on the other hand the whole procedure including spotting, developing and measuring is rather long-lasting and fails to meet the criteria for rapidly degrading cytostasan. Thus, we searched for a much faster method which could give us equivalent information within 10 minutes.

Figure 4 depicts the pH dependence of distribution coefficient of ^{14}C-cytostasan upon extraction from the phosphate buffer into benzene. As is seen the pH does not influence the D value which remains low, approximately 2. We have tested a number of organic solvents ranging from polar to nonpolar but none of them was found to be effective. These findings support the assumption that cytostasan is a highly hydrophilic drug. We found the distribution ratio to be 0.62 in the n-octanol/buffer pH 7.4 extraction system. However, the distribution coefficient was dramatically enhanced in the presence of DC-H^+ in benzene at pH 5.2.

Table 1 gives the values of the distribution coefficient of ^{14}C-cytostasan upon extraction from 0.5M $HClO_4$ into various organic solvents. In case of benzene-DC-H^+ the value of D is even much higher than that from the phosphate buffer. All other D values are too low or negligible and therefore unsuitable for analytical purposes. Such an unexpectedly high D value as found in the extraction system benzene-DC-H^+

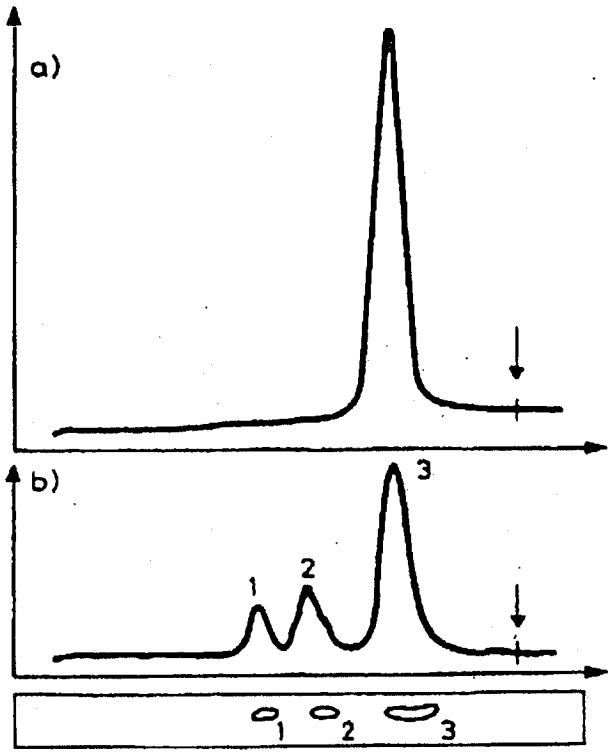

Fig. 3. TLC radiochromatograms showing the effect of drug concentration on the stability of ^{14}C-cytostasan in distilled water; (a) carrier-free ^{14}C-cytostasan, (b) ^{14}C-cytostasan in distilled water saturated with nonradioactive cytostasan. The corresponding nonradioactive spots are shown below. The application point is indicated by an arrow; (1) ^{14}C-cytostasan, (2) ^{14}C-monohydroxycytostasan, (3) ^{14}C-dihydroxycytostasan

Table 1
Values of the distribution coefficient (D) of ^{14}C-cytostosan upon extraction from 0.5M $HClO_4$ into various organic solvents

Organic solvent	D
Chloroform	1.5 ± 0.08
Carbon tetrachloride	0.001
n-Heptane	0.055 ± 0.01
Diethylether	0.040 ± 0.01
Benzene	1.3 ± 0.05
Benzene/dicarbolide*	188 ± 5
Ethyl acetate	8.9 ± 0.1
n-Octanol	2.78 ± 0.08
Toluene	0.21 ± 0.03

* Initial concentration of DC—H^+ in benzene is $c_o = 5.5 \cdot 10^{-3}$ M.

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