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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DIANHYDROGALACTITOL IN PLASMA BY DERIVATIZATION WITH SODIUM DIETHYLDITHIOCARBAMATE

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SUMMARY

A high-performance liquid chromatographic method is described for measuring submicrogram quantities of dianhydrogalactitol, a promising anti-neoplastic agent, in plasma. The drug is derivatized directly in plasma with sodium diethyldithiocarbamate to form a bis(dithiocarbamoyl) ester which absorbs UV light at 254 nm (a_m 17,000). The derivatized product is then extracted quantitatively into chloroform and separated by normal phase chromatography (μBondpak CN column). Dianhydrogalactitol concentration below 50 ng/ml of plasma can be detected in the eluent.

INTRODUCTION

Alkylating agents, consisting specifically of epoxides and nitrogen mustards, form a class of chemotherapeutic agents used in the control of cancer. The relatively high reactivity of such molecules toward nucleophiles and their lack of chromophoric intensity in the UV region limits the analytical methodology available for their determination at theurapeutic levels in biological samples. Capabilities for monitoring drug in various body fluid and tissue samples are necessary to evaluate efficiently new drugs and drug therapy.

One member of this group of anti-tumor agents is 1,2:5,6-dianhydrogalactitol (DAG), a hexitol diepoxide, currently in Phase I and II clinical evaluation. This agent is one of few active drugs capable of crossing the blood—brain barrier [1] and has shown potential utility in treatment of malignancies of the central nervous system.

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Initial pharmacokinetic studies of DAG distribution were carried out in mice [2] and rats [3]. Drug disposition was monitored radiochemically, by measuring total radioactivity in body fluid samples at timed intervals after administration of radio-labeled drug to test species. Although this method provided qualitative indications of drug distribution, its lack of specificity failed to distinguish among parent drug, metabolites and products formed as a result of non-enzymatic reaction of the epoxide ring with tissue nucleophiles.

More recently a gas chromatographic procedure was developed [4] for determination of submicrogram levels (sensitivity about 100 ng/ml) of DAG in biological fluids. It is based on extraction of the parent drug from potassium carbonate-saturated plasma with isopropanol, conversion to the corresponding *n*-butaneboronic ester and chromatography on a SE-30 column. One disadvantage of this method lies in the necessity for extracting the drug with very polar solvents, since undesired tissue components are co-extracted. Secondly, the method is somewhat time-consuming and requires considerable sample manipulation. Finally, the proclivity for DAG to covalently bind to blood components [2, 4] necessitates the rapid centrifugation of whole blood samples and storage of plasma at −40° prior to analysis.

An analytical method was sought in which chemical derivatization of DAG could be carried out directly in biological fluids to (1) stabilize the drug and prevent its loss due to binding with blood components after drawing of samples, (2) form a more hydrophobic derivative extractable into water-immiscible solvents, which are less likely to co-extract potential interfering species, and (3) form a derivative which strongly absorbs UV light at 254 or 280 nm. A high-performance liquid chromatographic (HPLC) method for DAG determination in plasma is described based on prior derivatization of drug with diethyldithiocarbamic acid, which specifically reacts at electrophilic sites.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 600-A solvent delivery system, Model U-6K injector and Model 440 dual channel absorbance detector operated at 254 nm. A 30 cm × 1/4-in. O.D. μBondpak CN column (Waters Assoc.) was used for all separations.

Materials

Crystalline dianhydrogalactitol (m.p. 98—100°) was obtained from the National Cancer Institute (Bethesda, Md., U.S.A.) and used without further purification. Sodium diethyldithiocarbamate (DDTC) was purchased from Sigma (St. Louis, Mo., U.S.A.). Fresh whole blood was obtained from healthy Beagle dogs. Dated human plasma was obtained from the Community Blood Center, Kansas City, Mo., U.S.A.

Derivatization

A 1-ml volume of plasma containing 0.05—50 μg of DAG was placed in a 15-ml centrifuge tube, to which were added 0.5 ml of 0.1 *M* potassium phosphate

buffer (pH 7.0), 0.5 ml of a 5% (w/v) aqueous solution of DDTC (prepared fresh daily), and 2 ml of water. The mixture was sealed with a PTFE-lined screwcap and allowed to stand at room temperature (22—25°) for 1 h.

Extraction

The plasma mixture was then extracted with 10 ml of chloroform for 3 min, and the system centrifuged at 1200 *g* for 5 min. The aqueous layer was discarded and the chloroform layer washed with 2—5-ml portions of a 33% (w/v) sodium chloride solution to remove protein distributed in the organic layer. An 8-ml aliquot of the washed chloroform solution was removed, evaporated to dryness at 40° and the residue dissolved in 200 µl of heptane—chloroform (7:3; v/v) solution.

Chromatography

In all runs, 10 µl of the solution containing derivatized DAG was injected. The mobile phase, heptane—chloroform (7:3) containing 1.2% acetic acid, was pumped through the column at a flow-rate of 2.5 ml/min.

Synthesis of 1,6-bis(diethyldithiocarbamoyl)-2,3,4,5-tetrahydroxyhexane

DAG (400 mg) and DDTC (1.0 g) were dissolved in 25 ml of 0.02 *M* phosphate buffer (pH 7.4). An immediate precipitate formed which was filtered after the mixture had been stirred for 1 h to yield 1 g (82%) of white solid (m.p. 145—147°). The product was established to be the bis adduct based on elemental analysis (calculated for $C_{16}H_{32}N_2O_4S_4$: C 43.24%, H 7.20%, N 6.31%; found: C 43.09%, H 7.15%, N 5.95%).

Synthesis of the bisacetonide derivative of 1,6-bis(diethyldithiocarbamoyl)-2,3,4,5-tetrahydroxyhexane [5]

1,6-Bis(diethyldithiocarbamoyl)-2,3,4,5-tetrahydroxyhexane (0.5 mmole; 222 mg) was dissolved in 5 ml of acetone, to which was added 2,2-dimethoxypropane (4 mmoles; 410 mg) and bis(*p*-nitrophenyl)phosphate (0.6 mmole; 204 mg). The mixture was stirred at room temperature for 1 h. A white precipitate formed which was collected by filtration to yield 260 mg (99%) of white solid (m.p. 119—121°). Analysis calculated for $C_{22}H_{40}N_2O_4S_4$: C 50.38%, H 7.60%, N 5.32%, found: C 50.28%, H 7.61%, N 5.28%.

RESULTS

Derivatization

DAG, like many chemotherapeutic alkylating agents, fails to absorb light above 210 nm. Therefore, development of a sensitive HPLC method for this compound which utilizes spectrophotometric monitoring of the effluent requires chemical derivatization of the drug with a chromophore-producing reagent prior to chromatography. DDTC was selected since it was found to react with DAG in aqueous, hydro-alcoholic or alcoholic solution to yield a product with significant UV absorbance at 254 nm (a_m = 17,000). The reaction was also found to take place directly in plasma containing 0.05 to 50 µg/ml of DAG, and therefore plasma samples could be derivatized without prior extrac-

tion of drug. A 5% solution of DDTC produced maximum reaction with this concentration range of DAG in 60 min, when the reaction was run at room temperature (22–25°) (Fig. 1). A less concentrated solution (1%) of DDTC produced a similar response, but reaction time needed to be increased significantly. Reaction rate could be accelerated significantly by heating the mixture, although maximum yield was not effected (i.e. at 80° and 50° reaction was complete in less than 10 min and 30 min, respectively). For convenience, reactions were carried out at room temperature with 5% reagent for 1 h. Since the DDTC–DAG adduct was stable in plasma for more than 6 h without measurable deterioration of the product, the isolation and work-up of the adduct from plasma samples was not critically time-dependent after reaction was complete.

Extraction

The plasma, containing DAG–DDTC adduct was extracted once with 2.5 volumes of chloroform. Quantitative extraction of adduct was observed. Diethyl ether, ethyl acetate, dichloromethane and 1,2-dichloroethane were also examined as potential extractants but failed to show extraction efficiency comparable to chloroform.

Chromatography

Fig. 2a shows a chromatogram of the extracted DAG–DDTC adduct using the analytical conditions outlined above. Elution time to the UV-detectable peak was 5.0 min, corresponding to an elution volume of 12.5 ml. No background interference was incurred at these retention times with drug-free plasma samples subjected to the assay (Fig. 2b).

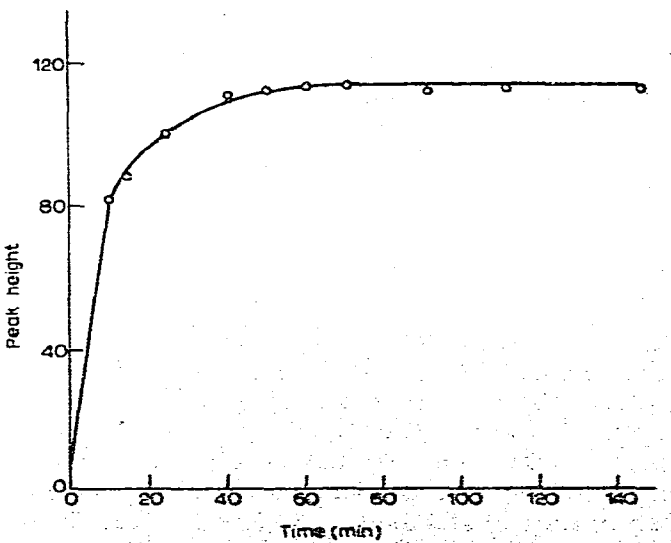


Fig. 1. Effect of reaction time on the conversion of DAG to its bis(diethyldithiocarbamoyl) ester by reaction of drug (10 μg) with 5% DDTC solution at 22°, as described in the text.

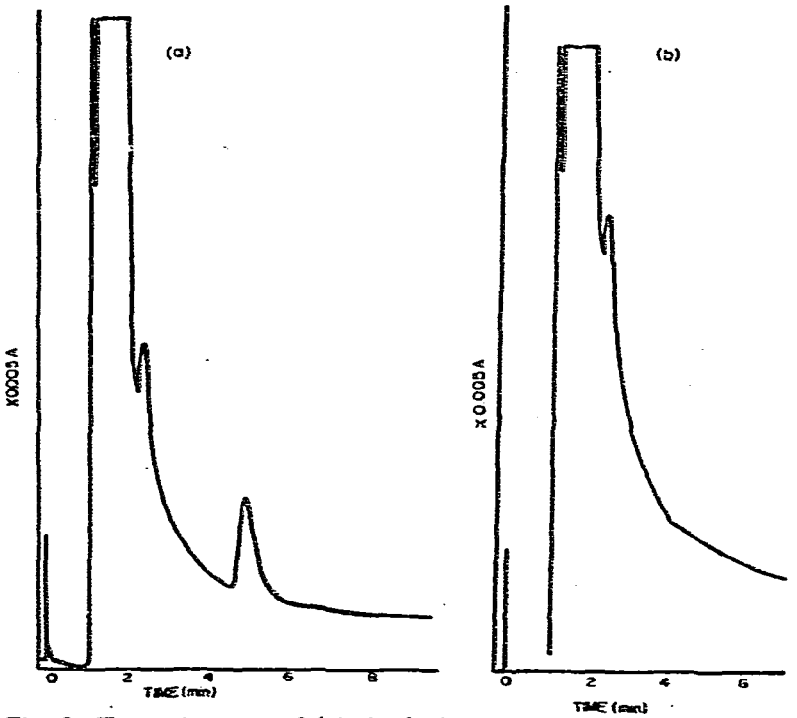


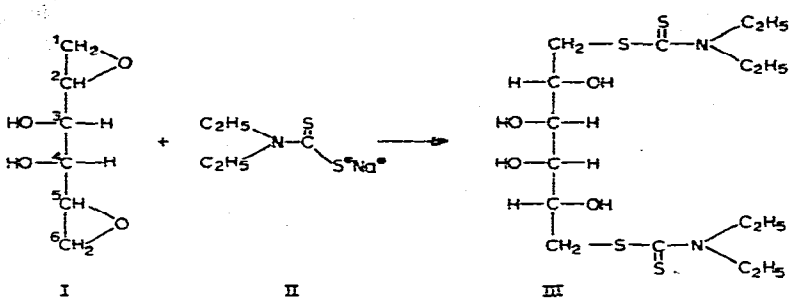
Fig. 2. Chromatograms of (a) the bis(diethyldithiocarbamoyl) ester of DAG obtained by carrying out the methodology described in the text on a 1-ml plasma sample containing 250 ng of DAG and (b) a drug-free plasma sample subjected to the assay.

Standard curves and sensitivity

A standard curve was prepared by carrying out the analysis on fourteen plasma samples to which had been added DAG at different concentrations ranging from 0.05 to 50 μg/ml. Over this concentration range, chromatogram peak heights were related linearly to DAG concentration. Linearity of response was determined by least-squares analysis of data points: slope 0.191, intercept −1.895, correlation coefficient 0.999. Detection limits were approximately 50 ng/ml of plasma, and the precision of the method 1—2% (as determined for duplicate samples).

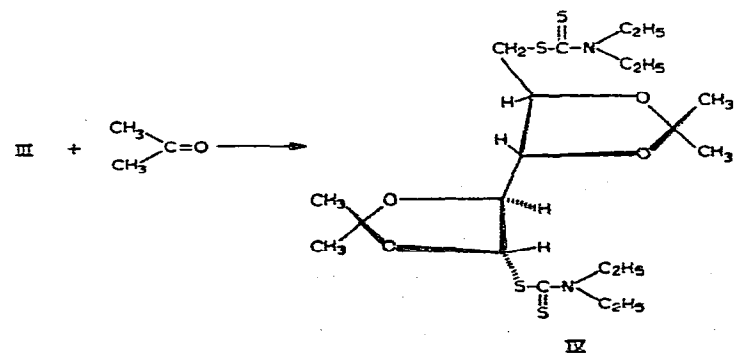
Characterization of the derivatization reaction

The derivatization reaction involved covalent addition of 2 moles of diethyldithiocarbamate (II) to the epoxides of DAG (I) to form an α-hydroxydithiocarbamoyl ester (III). The stoichiometry of the reaction, was confirmed by elemental analysis and from the mass spectrum of the adduct. Reaction can theoretically take place at either carbon of the oxirane skeleton, although in the absence of acid catalysis, it usually occurs at the least hindered carbon. The product was therefore assumed to be 1,6-bis(diethyldithiocarbamoyl)-2,3,4,5-tetrahydroxyhexane, III.



I II III (1)

To verify that reaction of II with DAG occurs at the terminal carbons (C-1 and C-6) and exclude the possibility that nucleophilic attack takes place at C-2 or C-5, III was reacted with acetone. Under the conditions of the reaction, acetone reacts with hydroxyl groups on adjacent carbon atoms in the *threo*-configuration to gove fivemembered cyclic acetals [5]. Thus D-galactitol gives rise to only 2,3:4,5- and 2,3:5,6-isopropylidene derivatives. 1,3-Dihydroxy compounds (such as would be formed by reaction of II with DAG at C-2 or C-5) fail to react under these conditions to form dioxanes. The stoichiometry of the reaction with DAG was determined from elemental analysis to involve condensation of 2 moles of acetone per mole of III. The NMR spectrum of the product revealed the ratio of methyl to methylene and methine proton to be 24:16. The product of the reaction with acetone is therefore IV; thus confirming the structure of the DAG—DDTC adduct proposed in eqn. 1 as III.



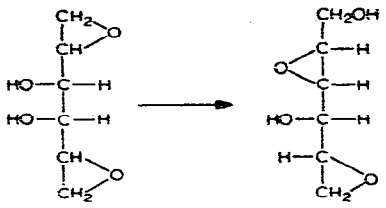
III IV (2)

HPLC analysis of the reaction mixture in which the acetonide is formed failed to reveal any underivatized DAG—DDTC adduct, indicating that the adduct is apparently pure III, and not a mixture of products generated by attack at combinations of C-1, C-2, C-5 and C-6. Also the absence of multiple peaks from the DAG—DDTC adduct, itself, lends support to the formation of the single product, III.

DISCUSSION

DAG can be chemically derivatized by reaction at either the hydroxyl or

epoxide functions. In either case, derivatization should enhance the stability of the molecule by preventing intramolecular rearrangement of the parent (eqn. 3).



(3)

Reaction with hydroxyl groups would involve the use of an electrophilic derivatizing agent. Because of the large number of nucleophile-containing molecules normally present in plasma, derivatization could not be carried out directly in biological fluid. In addition, most of these reagents are sensitive to water. The reagent would be consumed in reaction with many different compounds, introducing potential interferences at all subsequent stages of analysis, as well as requiring the use of very large excesses of reagent. Although this problem might be somewhat circumvented by prior extraction of DAG into an organic solvent, its hydrophilic nature requires the use of highly polar extraction solvents. Such solvents are non-specific, in that co-extraction of undesired materials occurs, and the aforementioned interferences are still encountered.

Expoxides react with nucleophilic compounds. Unlike the electrophilic reagents, relatively few endogenous substances or drugs react rapidly with nucleophiles. Reaction can therefore be carried out directly in plasma without the reagent being consumed in potentially-interfering side reactions with other substances. Accordingly, DAG was derivatized by reacting the epoxide with a suitable nucleophile directly in plasma. In addition, destruction of the epoxide ring in plasma by the derivatizing agent may protect DAG from covalent binding to blood components [2, 4].

Although mercaptides were initially examined as potential derivatizing agents because of their high nucleophilicity, their proclivity for facile oxidation to disulfides makes their use as clinical reagents impractical. Sulfur-containing nucleophiles other than mercaptans have not been exploited as analytical reagents. Dithiocarbamates were selected as derivatizing agents since they retain this high nucleophilicity, are often water soluble and are much less susceptible to oxidation. In addition, the aliphatic derivatives absorb UV light maximally at 254 and 283 nm, which is compatible with HPLC detectors. The reagent should be prepared freshly each day, but is relatively stable above pH 6. Below this pH, the molecule decomposes with evolution of CS_2 [6, 7]. The reagent will form chloroform-extractable chelates with heavy metals [7, 8], but these did not interfere with the assay.

Dithiocarbamic acids appear to be useful reagents for derivatization of alkylating agents prior to their HPLC analysis, derivatization taking place directly in plasma. In the case of DAG, a bis(dithiocarbamoyl) ester is formed, increasing the hydrophobicity of the molecule, so that it can be extracted into chloroform. The parent drug cannot be extracted into water-immiscible solvents, but must be extracted from salt-saturated solution with isopropanol [4], which presents a number of disadvantages as an extractant.

Although reactions of nucleophiles with epoxides potentially yield mixtures of addition products, derivatization of DAG with DDTC gave a single product formed by nucleophilic attack at the terminal carbons of DAG. Therefore, the concentration of DAG could be related to the integrated intensity of a single chromatographic peak, simplifying analysis and enhancing the sensitivity of the method. Early in the investigation, *p*-nitrobenzylpyridine was studied as a potential derivatizing agent. However, reaction with DAG produced a mixture of products, apparently resulting from indiscriminate attack of the pyridine nitrogen at C-1, C-2, C-5 and C-6. Reaction at C-2 or C-5 creates new asymmetric centers in the molecule so that diastereomeric pairs are generated. All of these products are resolved by HPLC, so that DAG appears in the chromatogram as a sequence of 6—8 peaks.

In conclusion, a HPLC method for clinical analysis of DAG is described, based on initial derivatization of the drug directly in plasma with DDTC. The general applicability of this derivatization sequence to the analysis of alkylating agents in biological samples is under investigation.

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REFERENCES

1 R.I. Geran, G.F. Congleton, L.E. Dudeck, B.J. Abbott and J.L. Gargus, Cancer Chemother. Rep., Part 2, 4 (1974) 53.
2 L. Institóris, É. Dzurillay and G. Pethes, Z. Krebsforsch., 79 (1973) 49.
3 V.A. Levin, M.A. Freeman-Dove and C.E. Maroten, J. Nat. Cancer Inst., 56 (1976) 535.
4 T. Kimura, L.A. Sternson and T. Higuchi, Clin. Chem., 22 (1976) 1639.
5 J.A. Mills, Advan. Carbohyd. Chem., 10 (1955) 1.
6 P. Zuman and R. Zahradnik, Z. Phys. Chem., 208 (1957) 135.
7 G.D. Thorn and R.A. Ludwig, The Dithiocarbamates and Related Compounds, Elsevier, Amsterdam, 1962, p. 43.
8 G. Eckert, Z. Anal. Chem., 155 (1957) 23.