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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE ANALYSIS OF A SYNTHETIC COPOLYMER WITH ANTITUMOR ACTIVITY (COPOVITHANE) AND METHYLAMINE IN HUMAN BLOOD PLASMA AND URINE*

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SUMMARY

A method for the determination of a synthetic polymeric compound with antitumor activity (copovithane) and methylamine in blood plasma and urine is described. Copovithane is prepared by radical polymerisation of a diurethane with N-vinylpyrrolidone.

The method is based on high-performance liquid chromatography of the methylamine hydrochloride which arises during the hydrochloric acid hydrolysis of the parent substance. The methylamine hydrochloride is converted to the trinitrobenzenesulphonyl derivative for the purpose of chromatographic detection.

The limit of determination for copovithane in blood plasma is 1.2 mg/l and in urine 1.5 mg/day. The determination limit for methylamine in blood plasma is 0.2 mg/l and in urine 0.3 mg/day. The imprecision is dependent on the sample, and amounts to ± 6.8% for blood plasma and ± 6.4% for urine.

INTRODUCTION

A variety of synthetic polymeric compounds with antitumor activity have been discovered. The majority of them have toxic side-effects. Recently, a group of water-soluble homo- and copolymers which contain 2-methylene-1,3-propanediol as the essential chemical unit have been found [1]. These copolymers significantly inhibit the growth of experimental murine tumors in a wide therapeutic dose range.

The compounds are prepared by radical polymerisation of diurethanes or diesters of 2-methylene-1,3-propanediol alone, or with copolymerisable ionic or non-ionic monomers such as maleic acid, acrylic acid, acrylamide, and N-vinylpyrrolidone.

*Dedicated "in memoriam" to Prof. Dr. Otto Bayer.

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Comparative investigations revealed that a non-ionic copolymer consisting of 30% by weight of 2-methylene-1,3-propanediyl-bis(methylcarbamate) (Fig. 1, I) and 70% by weight of N-vinylpyrrolidone (Fig, 1, II) was prominent by its excellent tolerability.

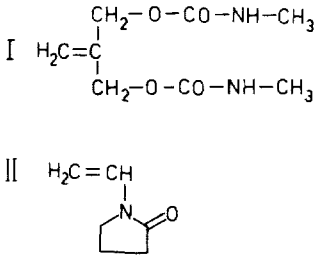


Fig. 1. Chemical structures of 2-methylene-1,3-propanediyl-bis(methylcarbamate) (I), and N-vinylpyrrolidone (II).

The copolymer with an average molecular mass of about 5000—6000 was selected for detailed investigations.

The assay method presented here is based on high-performance liquid chromatography (HPLC) of the methylamine which is generated by hydrolysis and on its detection by conversion to the trinitrobenzenesulphonyl derivative.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 1084 B liquid chromatograph was used, equipped with a HP 79875 UV detector. Ready-to-use columns (250 × 4 mm I.D., Hibar; E. Merck, Darmstadt, G.F.R.) filled with LiChrosorb RP-18 (10 μm) were employed. UV monitoring was performed at 340 nm.

Reagents

All reagents used were of guaranteed reagent grade (Merck), and were used without further purification.

Copovithane (BAY i 7433) standards were obtained by preparative gel permeation chromatography on Fractogel PVA 20,000 (E. Merck) with methanol as the eluent*. Determination by membrane osmosis of the average molecular weight proved that the preparation has a narrow distribution of its molecular weight and that the preparation contains less than 0.5% by weight of molecules with a molecular weight greater than 25,000.

Methylamine hydrochloride standards were purchased from EGA-Chemie (Steinheim, G.F.R.).

Method

The method consists of four principal steps: protein precipitation, acid hydrolysis, derivatisation and HPLC analysis. To blood plasma samples (2 ml each) are added drop-wise 8 ml of acetone over a period of 2 min while under

*Copovithane standards were prepared by Dr. B. Boemer, Bayer AG, Leverkusen, G.F.R.

vigorous homogenisation with an Ultra-Turrax in order to precipitate the proteins. After centrifugation (2000 *g*, 5 min) the supernatant is then shaken for 15 min with 12 ml of dichloroethane to remove the acetone, and then centrifuged again. The aqueous phase (1 ml) is diluted with 1 ml of water and, by means of a 2-ml disposable syringe, layered onto a Sep-Pak C_{18} cartridge (Waters No. 51910; Waters Assoc., Milford, MA, U.S.A.) which has been preconditioned by washing with 20 ml of water and 20 ml of methanol. A further 2 ml of water are added to the column and then it is eluted with 10 ml of 80% methanol. The eluate is reduced to dryness at 70° C under a stream of nitrogen. The residue is taken up in 1 ml of 5 *N* hydrochloric acid and hydrolysed for 16 h at 160°C. The hydrolysate is cooled in an ice-bath and adjusted to pH 8.00 ± 0.01 with 2 ml of standard buffer pH 8.00 (E. Merck) and 0.5 ml of 10 *N* sodium hydroxide. The hydrolysate is then derivatized by the addition of 0.75 ml of 0.5% of 2,4,6-trinitrobenzenesulphonic acid solution (TNBS). The reaction is allowed to proceed for 2.5 h at room temperature in complete darkness and then the reaction product is extracted by shaking (15 min) with 6 ml of toluene. The phases are allowed to separate (2 min) and 3 ml of the toluene phase are reduced to dryness by heating at 50° C under a stream of nitrogen. The residue is taken up in 250 µl of the mobile phase acetonitrile—water (45:55) and transferred to a micro sample vessel.

The analysis of the urine samples follows the same procedure. The protein precipitation step is not required.

Chromatographic conditions

The chromatographic system consists of a mobile phase of acetonitrile—water (45:55) percolating at a flow-rate of 2 ml/min through a ready-to-use column packed with LiChrosorb RP-18 (10 µm). The detector wavelength was set at 340 nm, which corresponded to the largest difference between the methylamine-TNBS derivative and mobile phase absorptions. The column temperature was maintained at 35° C. In all analytical studies constant volumes of 50 µl were injected onto the column at each operation.

RESULTS

Concentrations of copovithane in biological material

Copovithane (BAY i 7433) is poorly soluble in organic solvents that are immiscible with water. This excludes the possibility of direct extraction of the substance from blood plasma and urine.

In order to concentrate the substance present in the blood into a smaller volume, the plasma proteins are precipitated with acetone. The acetone is then removed by shaking with dichloroethane and the aqueous phase is used for further analysis.

Quantitative analysis of copovithane in urine does not require the protein precipitation step.

Removal of interfering substance

The methylamine that arises from the hydrochloric acid hydrolysis of control plasma or control urine is mostly derived from creatine and creatinine.

A considerable reduction in the blank value is achieved by passing the sample over a conditioned Sep-Pak C_{18} cartridge. This results in a reduction of more than 90% in the amount of methylamine formed by hydrolysis.

Optimal hydrolysis conditions

On average a 100% yield of methylamine hydrochloride is achieved by a 16-h hydrolysis of copovithane in 5 *N* hydrochloric acid at 160° C.

Sham hydrolysis, carried out with methylamine hydrochloride under the above-mentioned conditions, did not cause any reduction in yield.

Derivatisation with 2,4,6-trinitrobenzenesulphonic acid

Methylamine hydrochloride does not display a characteristic absorption spectrum in the UV range, so it is necessary to introduce a chromophore for detection purposes.

2,4,6-Trinitrobenzenesulphonic acid was found to be optimal for the derivatisation of methylamine hydrochloride. Maximal formation of the derivative occurred during a period of 2.5 h at pH 8.0 at room temperature in the absence of light. The methylamine-TNBS derivative can easily be separated from excess TNBS by extraction with toluene.

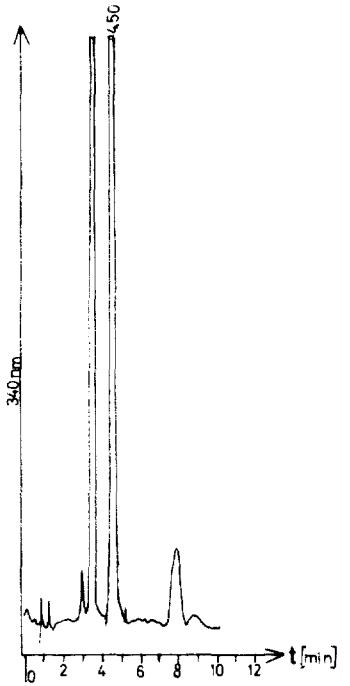
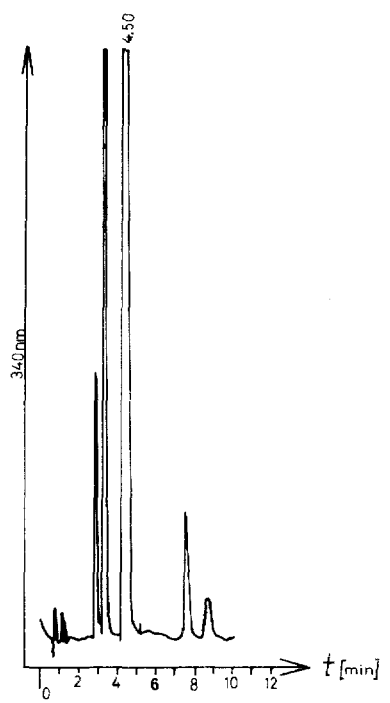


Fig. 2. Chromatogram of methylamine-TNBS derivative standard, monitored with a UV detector (340 nm). RP-18 column; mobile phase acetonitrile—water (45:55); retention time 4.50 ± 0.01 min.

High-performance liquid chromatography

The methylamine-TNBS derivative is chromatographed on an RP-18 column using the solvent system acetonitrile—water (45:55). The retention time is 4.50 ± 0.01 min. A typical chromatogram is shown in Fig. 2. Chromatograms of samples collected from blood plasma and urine are demonstrated in Figs. 3 and 4, respectively.



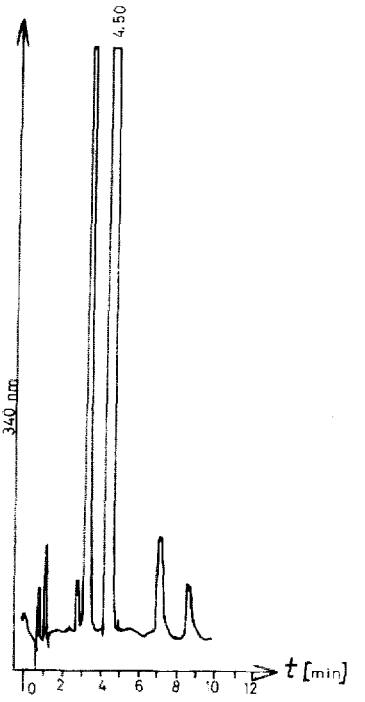


Fig. 3. Chromatogram of methylamine-TNBS derivative after HCl hydrolysis of copovithane in blood plasma; chromatographic conditions as in Fig. 2.

Fig. 4. Chromatogram of methylamine-TNBS derivative after HCl hydrolysis of copovithane in urine; chromatographic conditions as in Fig. 2.

Standard curve

The peak areas derived from HPLC of the methylamine-TNBS derivative were proportional to its concentration within the range 10 ng/ml to 100 μg/ml.

Reagent blank

The average reagent blank has a peak area associated with the retention time of 4.50 min, which corresponds to an apparent copovithane concentration of 0.8 ± 0.06 μg/ml of blood plasma and 1.47 ± 0.49 μg/ml of urine.

The corresponding values for methylamine hydrochloride are 0.2 ± 0.015 μg/ml of blood plasma and 0.47 ± 0.16 μg/ml of urine.

Biological blank

Samples of blood plasma from nineteen untreated volunteers were processed as described in the Method section. The analysis resulted in a mean apparent biological content of copovithane (after subtraction of the reagent blank) of 0.3 ± 0.53 μg/ml of blood plasma.

Processing the urine collected over a period of 24 h from ten untreated volunteers resulted in a mean biological blank value of 1.7 ± 0.65 mg/day.

The biological blank for methylamine was 0.05 ± 0.09 μg/ml of blood plasma and 0.3 ± 0.115 mg/day for urine.

Limit of determination

The limit of determination is calculated from the standard deviation of the biological blank values with 95% confidence limits. The resulting values for copovithane are: 1.2 μg/ml of blood plasma and 1.5 mg/day for urine; and for methylamine, 0.2 μg/ml of blood plasma and 0.3 mg/day for urine.

Yield

The yield of the procedure was determined by adding 10, 25, 50 and 100 μg of copovithane to each millilitre of ten blood plasma samples obtained from ten untreated volunteers and then processing the samples as described in the Method section. The mean overall yield was found to be 64.6 ± 4.5%. The correction factor for the yield is therefore 1.55.

The yield in urine was determined by adding various amounts of copovithane to each of ten urine mixtures. The final concentrations of copovithane were set at 10, 25, 50 and 100 μg/ml, and then all samples were processed as described in the Method section. The mean yield obtained from these assays was 55.9 ± 3.5%. The yield correction factor for urine is therefore 1.78.

Imprecision

The imprecision of the determination is a correlate of the relative standard deviation of the yield. This amounts to ± 6.8% for the blood plasma determination and ± 6.4% for the urine determination.

Standardisation

Each analysis series is quantified by chromatography of three external standards (methylamine-TNBS derivatives). A standard curve is then constructed by means of linear regression analysis of the peak areas of these standards.

Calculation

$$\frac{(A_T - A_B) \cdot S \cdot 5.621 \cdot f}{A_S} = \mu g \text{ copovithane per ml of plasma or urine}$$

where A_T = area test sample, A_B = area blank, A_S = area standard, S = methylamine hydrochloride standard, 5.621 = conversion factor and f = yield correction factor.

DISCUSSION

The instrumental chromatographic analysis of polymeric compounds did not develop with the same speed as that of low-molecular-weight compounds. High-performance size-exclusion chromatography offers the versatility of providing information on both low- and high-molecular-weight components of polymers. However, this technique is not suitable to detect trace quantities. For this purpose chemical cleavage or degradation of various individual polymers has been described [2, 3]. The polymer reactants produced by cleavage frequently contain highly polar groups, i.e. carboxyl, hydroxyl, and amino groups, which exhibit poor chromatographic behaviour. Therefore, simple derivatisation must be carried out before chromatographic analysis.

Chemical cleavage has been applied to linear polyurethane materials using acidic or alkaline hydrolysis or by aminolysis [4]. It frequently requires prolonged reaction periods and becomes less satisfactory as the complexity or cross-linking is increased.

The amines generated after hydrolysis of polyurethanes cause special analytical problems because of the similar chemical nature of these compounds. The most sensitive techniques to analyze amines are spectrofluorimetry and gas chromatography. The use of HPLC for the analysis of trace quantities of amines becomes more important since amine derivatives can be detected by UV absorption. Wellons and Carey [5] described an HPLC method for amine analysis by forming the *m*-toluoyl derivatives. The method was modified by Chen and Farquharson [6]. The analytical method described in this paper proved also to be a valid procedure for determining trace amounts of methylamine in biological fluids.

The determination of copovithane in blood plasma and urine is a relatively time-consuming method, because the substance cannot be extracted from blood plasma and urine by means of organic solvents and because the acid hydrolysis takes 16 h.

Due to the chromatographic background signal of methylamine, which generates creatinine and creatine during sample work-up by acid hydrolysis, also in untreated volunteers, the method presented is not specific for copovithane.

Nevertheless, the imprecision of measurement of copovithane in cancer patients during patient monitoring will not be further complicated because the creatinine and creatine levels in blood plasma and urine do not vary significantly between cancer patients and healthy persons [7—9].

The relative coefficient of variation of this method does not exceed the normal physiological variation of creatinine content in blood plasma and urine [10].

Furthermore, the assay method possibly is not specific for unchanged copovithane since potential metabolites that yield methylamine hydrochloride on hydrolysis with hydrochloric acid will also be detected.

The sensitivity of the method is good. Preliminary studies have shown that the maximal blood plasma concentration following intravenous administration of 10 mg of copovithane per kg body mass (probably the lowest therapeutic

dose) reached values of ca. 100 mg/l. These values are approximately 100 times greater than the limit of detection. The blood plasma level of copovithane could be easily followed over six half-lives.

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