Journal of Chromatography, 222 (1981) 249—255
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 736

DETERMINATION OF D-PENICILLAMINE IN SERUM BY FLUORESCENCE DERIVATIZATION AND LIQUID COLUMN CHROMATOGRAPHY

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(Received July 14th, 1980)

SUMMARY

A simple and fast method for the determination of D-penicillamine in serum is described. The analysis is based on a fluorescence derivatization of the sulfhydryl group combined with a reversed-phase liquid chromatographic separation and fluorescence detection. Before derivatization the serum proteins are precipitated with ethanol and removed by centrifugation. As derivatizing agent 5-dimethylaminonaphthaline-1-sulfonylaziridine is used which reacts selectively with thiols under defined reaction conditions. The detection limit is in the pmol range; 50—300 ng of D-penicillamine can be determined with a relative standard deviation of 7—8%. Thus the method permits a simple determination of D-penicillamine in serum at therapeutic levels.

INTRODUCTION

D-Penicillamine (D-PA) is used for the treatment of polyarthritis [1], cystinuria [2], poisoning caused by heavy metals [3] and Wilson's disease [4]. Additionally, synergistic effects in the metabolism of organic mercury compounds [5] and in the treatment of other pathogenic conditions have been

0378-4347/81/0000—0000/$02.50

described [6—8]. Despite its frequent use the pharmacological effects of D-PA have not been sufficiently investigated. This is partly due to the lack of selective and sensitive methods for its analysis.

For determination of D-PA the properties of its functional groups are used. Colorimetric analysis can be carried out after complexation with $FeCl_3$ [9] or with 5,5'-dithiobis-2-(nitrobenzoic acid) [10]. Polarographic determination [11] and separations using automatic amino acid analyzers [12—14] have been described. For detection, reaction with H_2PtCl_2—KI [12] or ninhydrin [13, 14] is used. Faster analysis is possible by high-performance liquid chromatographic (HPLC) separations on cation resins combined with electrochemical detection [15].

HPLC has proved to be very useful for the determination of many substances in biological fluids due to the speed and selectivity of separation. Sometimes detection is a problem; the required detection limit can in some cases only be achieved by chemical derivatization. For D-PA selective derivatization of the thiol group seemed to be most promising. Recently, the use of 5-dimethylaminonaphthaline-1-sulfonylaziridine (dansylaziridine) for pre-column derivatization of thiols was described [16]. The derivatives are stable and can be separated by reversed-phase liquid chromatography within a short time. The fluorescence is linear over a wide range and permits trace determination of D-PA.

EXPERIMENTAL

Materials

All solvents used were of analytical grade quality (Merck, Darmstadt, G.F.R.). 5-Dimethylaminonaphthaline-1-sulfonylaziridine puriss. biochim. was purchased from Fluka (Buchs, Switzerland). For ion-exchange separations Dowex 50W-X2, 200—400 mesh (Serva, Heidelberg, G.F.R.) packed in polystyrol columns (Sarstedt, Nümbrecht-Rommelsdorf, G.F.R.) was used. The stationary phase for HPLC was LiChrosorb RP-18, 7 μm particle size (Merck), packed in 150 × 3.2 mm I.D. steel columns.

Apparatus

A Waters Assoc. 6000A pump in combination with a Perkin-Elmer LC 1000 fluorescence detector (excitation filter 338 nm) was used for HPLC determinations. The fluorescence emission was measured at 540 nm or by means of a 430 nm cut-off filter. For sample injection a Waters U6K injection system (injection volume 100 μl) was used. All separations were carried out isocratically at room temperature, thermostating being unnecessary. Peak area calculations were done with a Hewlett-Packard 3380A integrator.

Procedure

As a result of optimization studies the following procedure for sample clean-up and derivatization is recommended. One millilitre of ethanol is dispensed into a 10-ml vial that can be tightly closed; then 0.5 ml of serum stabilized with a 0.3 *M* aqueous solution of sodium EDTA (10:1) is added. The vial is closed and shaken well for 2 min. After centrifugation for 3 min at 2700 *g*, 1 ml of the clear supernatant is pipetted into another vial, and 2 ml of phosphate

buffer (pH 8.2, 0.067 *M*) and 0.2 ml of dansylaziridine solution (10 mg of dansylaziridine per ml of methanol) are added. The closed vial is placed in a thermostated water-bath at 60°C for 1 h. After this reaction time the solution can be directly injected into the liquid chromatograph. The slight opalescence observed in some solutions can be removed by centrifugation (5 min at 2700 *g*).

RESULTS AND DISCUSSION

Sample preparation

Since many compounds with sulfhydryl groups are present in biological fluids such as serum, direct determination without previous clean-up is not possible. Pre-separation of the proteins from the low molecular weight compounds is necessary. One possibility is the use of short ion-exchange columns such as those described for the determination of iodinated amino acids in serum [17]. Serum (0.2 ml) is pipetted onto the top of the cation-exchange column. The resin (Dowex 50W-X2, 200—400 mesh, H^+) is filled to a height of 15 mm in a polystyrol column, 6 mm I.D. The proteins are eluted with 10 ml of 0.02 *M* NaOH, the amino acids being concentrated at the bottom of the column by this procedure. The amino acids can then be eluted with a mixture of ethanol—5% ammonia (1:1). The elution is carried out by applying two 0.5-ml portions of this solvent. The first 0.5-ml portion is discarded; the second 0.5 ml contains the D-PA and can be directly used for derivatization. The recovery for a D-PA concentration of 12 μg/ml serum was 42.4 ± 8.3%. As the ion-exchange columns are used only once, cross-contamination of different samples is avoided. The method gives reproducible results but for routine analysis the amount of time and material is not negligible. Therefore we tried to work out a simpler and faster alternative.

This second method is a combination of precipitation and extraction. The high molecular weight proteins are precipitated by an organic solvent which serves at the same time as an extracting agent for the amino acids present in the serum. The procedure is described under Experimental. Two solvents were compared: ethanol and tetrahydrofuran (THF). The results are given in Table I. With THF the extraction yields are higher, but a serious disadvantage is the low precision found with this solvent. The reason for the poor reproducibility using THF could be difficulties in the derivatization reaction or the presence of traces of peroxides. Ethanol was therefore preferred in the subsequent investigations.

Thiols can be easily oxidized or complexed [18]. Therefore tests of the stability of free penicillamine in serum were made. Saetre and Rabenstein [15] described a stabilizing effect of EDTA. EDTA has the ability to complex traces of heavy metals which catalyze the oxidation of free thiol groups. The influence of this agent was checked in order to determine suitable storage conditions for samples that cannot be analyzed immediately. The results of the study are summarized in Fig. 1. Freshly centrifuged pooled serum was used as a matrix and 20 μg of D-PA per ml were added. For the stabilized solutions, 0.1 ml of aqueous sodium EDTA solution (0.3 *M*) was pipetted into 1 ml of serum. The stability of stabilized and non-stabilized solutions was compared at 4°C

TABLE I

RELATIVE PEAK HEIGHTS FOR PENICILLAMINE AFTER EXTRACTION WITH ETHANOL AND THF FROM SERUM

The standard deviations are calculated from eight trials each. For conditions of extraction and derivatization see Experimental. For chromatographic conditions see Fig. 3.

D-Penicillamine (μg/ml serum)	Relative peak height	
	Ethanol	THF
2	7 ± 0.4	8 ± 1.0
10	34 ± 1.7	37 ± 3.9
25	89 ± 4.6	97 ± 9.2
50	168 ± 8.7	177 ± 19.1

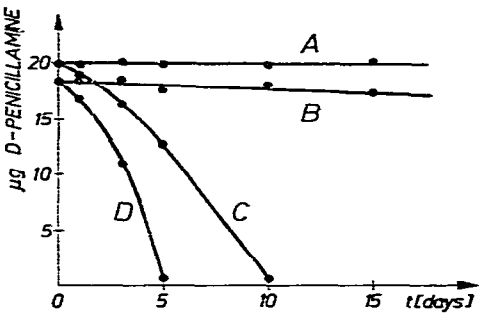

Fig. 1. Stability of D-penicillamine in serum (20 μg/ml) under various conditions. (A) Serum stabilized with sodium EDTA, stored at −18°C. (B) Serum without sodium EDTA, stored at −18°C. (C) Serum stabilized with sodium EDTA, stored at +4°C. (D) Serum without sodium EDTA, stored at +4°C.

and −18°C. Storage in the refrigerator (4°C) is not sufficient, whereas at −18°C the stabilized solution shows no loss of D-PA over a period of 15 days.

Derivatization

An account of the derivatization reaction and optimization of the conditions was published recently [16]. The maximum yield is reached at pH 8.2 with a minimum of 2.7-fold molar reagent excess using a reaction time of 1 h at 60°C. Under these conditions only free sulfhydryl groups are derivatized; weaker nucleophils such as amines or alcohols do not react. This finding is an important presupposition for the selective determination of D-PA in the complex matrix of serum. The derivatives are stable; no degradation could be observed in a week.

As all thiols present in serum are derivatized, the minimum amount of reagent required had to be found empirically. The results for a study in which pooled serum containing 29 μg/ml D-PA was used is summarized in Fig. 2. This figure shows the peak heights gained for the derivative of D-PA by HPLC determination. A minimum of 0.2 ml of reagent (corresponding to 2 mg of dansylaziridine) is necessary for complete derivatization. It is interesting to note that

the method for sample clean-up (ion exchange or precipitation) has no influence on the amount of reagent. Removal of the excess reagent is not necessary; the separation is done by the following HPLC determination.

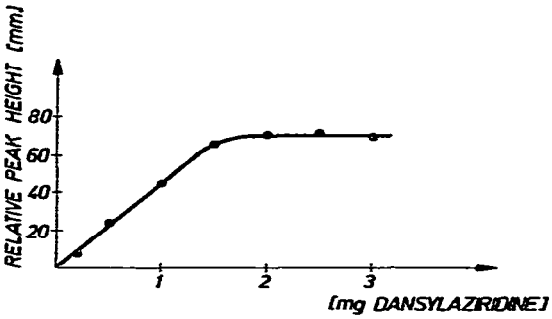

Fig. 2. Derivatization of D-penicillamine after extraction from pooled serum (29 μg D-PA per ml serum) with various amounts of dansylaziridine (10 mg of reagent per ml of methanol).

Chromatography of the derivatives

One of the most serious disadvantages of D-PA analysis by amino acid analyzers is the time required, which can be several hours [12, 13]. Faster separations are possible by reversed-phase liquid chromatography. The derivatives formed from amino acids containing thiol groups and dansylaziridine show an amphoteric character: both acidic and basic functional groups are present that can be used for ion-pairing. Good separations were achieved with a 1:2 mixture of acetonitrile and phosphate buffer (pH 8.2, 0.033 *M*) with the addition of 0.05% ethylenediamine as mobile phase and LiChrosorb RP-18 as stationary phase. Typical chromatograms are shown in Fig. 3. The two chromatograms show the results for a pure solution of D-PA and for serum from a polyarthritic patient treated with D-PA. The patient was given 250 mg of D-PA orally 2 h before the sample was taken. The derivatives of the amino acids are eluted before the reagent peak within a few minutes.

The derivatization has two advantages: first the selectivity of the reaction, second the selectivity and sensitivity of the fluorescence detection. UV and fluorescence spectra of dansylaziridine, which are nearly identical with the spectra of the derivatives [16], are given in Fig. 4. The UV absorption at 254 nm or 345 nm could also be used for detection, but in this region many other compounds present in serum show strong UV absorption and interfere in the chromatogram. In addition to higher selectivity, measurement of the fluorescence is more sensitive than UV detection. For excitation of fluorescence a 338-nm filter can be used (the optimum wavelength is 345 nm), the emission is measured at the maximum of 540 nm. The detection limit under these conditions is 85 pmol of penicillamine (signal-to-noise ratio of 3:1). Using a 430 nm cut-off filter for measuring emission, the detection limit can even be lowered to 12 pmol of penicillamine. The cut-off filter causes no loss of detection selectivity because no fluorogenic substances other than the dansylated derivatives are present.

Linear calibration curves were achieved by the addition of known amounts of D-PA to pooled serum. In Table II the results for the biologically relevant concentration range are summarized. The calibration curve passes through the origin of the coordinates and is linear up to 16 pmol penicillamine (correlation coefficient = 0.999). The relative standard deviation in the concentration range 50—300 ng per 100 µl (= injection volume) is 7—8%.

Besides the analysis of penicillamine, this method offers a possibility for the determination of other thiol compounds such as cysteine and glutathione. Investigations into the analysis of the reduced and oxidized forms of these thiols in biological fluids are in progress.

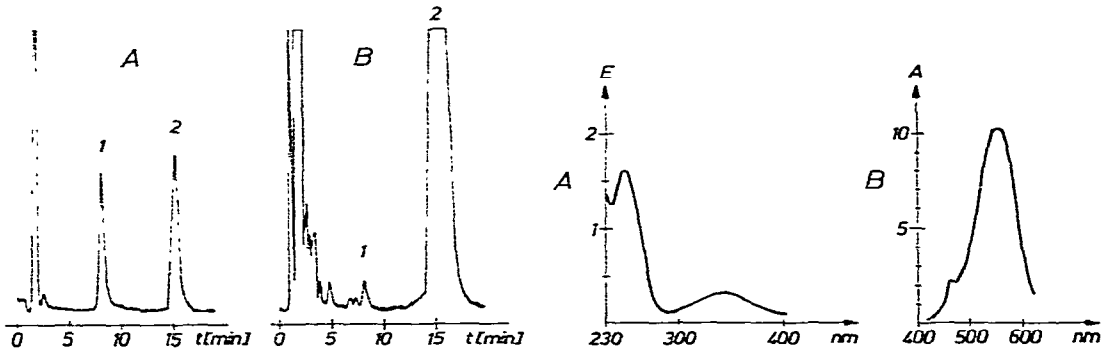

Fig. 3. Chromatographic determination of D-penicillamine after derivatization with dansylaziridine. (A) D-PA standard solution. (B) Serum from a patient treated with D-PA. Peaks: 1 = S-(2-dansylaminoethyl)-penicillamine; 2 = dansylaziridine. Solvent: acetonitrile—phosphate buffer (pH 8.2, 0.033 *M*) (1 : 2) + 0.05% ethylenediamine. Column: LiChrosorb RP-18. Flow-rate: 1 ml/min at 1700 p.s.i. Injection volume: 100 µl. Detection: λ_{exc} 338 nm; λ_{em} cut-off filter 430 nm.

Fig. 4. UV (A) and fluorescence emission (B) spectra of dansylaziridine (2.57×10^{-5} *M*) in methanol.

TABLE II

DETERMINATION OF D-PENICILLAMINE FROM SERUM AFTER DERIVATIZATION WITH DANSYLAZIRIDINE

Calculations of peak areas were made with a Hewlett-Packard 3380 A integrator.

D-Penicillamine (ng per 100 µl*)	Digits	S_{rel}** (%)
12.8	5,830	15.6
25.6	11,580	12.2
48.0	21,910	7.9
96.0	43,640	6.8
152.0	69,320	7.5
304.0	138,290	6.7

*100 µl = injection volume.

**S_{rel} = relative standard deviation (n = 7).

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