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ANALYSIS OF D-PENICILLAMINE BY GAS CHROMATOGRAPHY UTILIZING NITROGEN—PHOSPHORUS DETECTION

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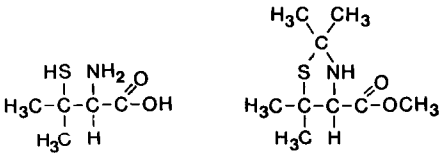
SUMMARY

A method is presented for the analysis of the "orphan" drug D-penicillamine (D-Pa), which is used for the treatment of the inherited rare copper metabolism dysfunction known as Wilson's disease, by assaying a derivative of the compound by gas chromatography employing a rubidium sensitized nitrogen—phosphorus detector. Analytical procedures are described for the analyses of residues of D-Pa • HCl salt in animal feed and for the analyses of the salt or free base from aqueous solutions by utilizing a single-step double derivatization with diazomethane—acetone. Stability data for D-Pa • HCl in animal feed and for the free base in water are presented. An ancillary fluorescence derivatization procedure for the analysis of D-Pa in water is also reported.

INTRODUCTION

Wilson's disease is a rare inherited metabolic disorder resulting in the accumulation of excess copper, first in the liver, then in the cornea, brain and kidneys and finally overflowing into the urine. The untreated course of the disease is cirrhosis of the liver, destruction of motor coordination, eclipsing of the intellect, renal failure and eventual death of the patient.

The idea that the disease could be arrested by the removal of copper from patients suffering from Wilson's disease was credited to Cumings [1] of London, who suggested in 1948 that the chelating agent 2,3-dimercapto-propanol (BAL) developed to detoxify trivalent arsenic be used for the elimination of copper as the chelated copper complex. However, the BAL treatments were not successful due to most patients developing tachyphylaxis while each successive painful intramuscular injection of BAL exhibited diminishing efficacious results. In 1956, Walshe [1] introduced the oral administration of penicillamine (Fig. 1, I), as a copper-chelating agent for the treatment of



I II

Fig. 1. Structures of D-penicillamine (I) and of a diazomethane—acetone derivative (II).

Wilson's disease and obtained only limited success due to its toxicity. In 1957, the toxicity observed for penicillamine was attributed to impurities. Further testing with purified D(—)-penicillamine (D-Pa) proved it to be a remarkably effective therapeutic agent for the elimination of copper. In addition to the disease being arrested, the patients were eventually rendered essentially symptom-free with real prospects of leading an otherwise normal life when they maintained daily dosages of D-Pa [1]. Unfortunately, a small percentage of Wilson's disease patients developed an intolerance to D-Pa [2] and the drug therapy had to be withdrawn (a life-threatening situation).

Studies have been proposed to be conducted at the National Center for Toxicological Research (NCTR) for the evaluation of the toxicology of D-Pa. Prerequisite for these toxicological studies was development of analytical methodology for chemical characterization, dose certifications and stability evaluations of the drug in the dosage forms. A search of the chemical literature for analytical techniques for characterization and analysis of D-Pa yielded a few limited analytical procedures which were inadequate for our proposed toxicological studies [3—5]. We, therefore, developed new analytical methodology for the analysis of D-Pa which incorporated a single-step double derivatization with detection by gas chromatography (GC) utilizing a rubidium sensitized nitrogen—phosphorus detector.

This paper, therefore, describes new analytical procedures for the analysis of D-Pa and residues of D-Pa · HCl in animal feed at 1.6% and 0.016% by weight. Stability data for D-Pa in water, D-Pa · HCl in animal feed, and an ancillary fluorescence derivatization procedure for the analysis of D-Pa in water are also included. Structures of D-Pa and its diazomethane—acetone derivative are shown in Fig. 1.

EXPERIMENTAL

Reagents

D-Pa was obtained from Merck Sharp & Dohme (Rahway, NJ, U.S.A.). The Diazald™ and carbitol [2-(2-ethoxyethoxy)ethanol] were from Aldrich (Milwaukee, WI, U.S.A.) and the diethyl ether from Fisher Scientific (Pittsburgh, PA, U.S.A.). The N,N-diethylaniline was from Chem Service (West Chester, PA, U.S.A.), the Fluram™ from Roche (Nutley, NJ, U.S.A.), and the animal feed Type 5010M from Ralston Purina Company (St. Louis, MO, U.S.A.). The acetone and hexane were grades suitable for GC. All other chemicals were CP grade.

Preparation of D-Pa · HCl from D-Pa

Approx. 30 g of D-Pa were added to 200 ml of methanol in a 500-ml round bottom flask. The bulk of the material was insoluble. Anhydrous hydrochloric acid was bubbled through a sintered glass sparge into the methanol with frequent swirling of the solvent to facilitate mixing. The D-Pa base was converted rapidly to the soluble D-Pa · HCl resulting in a clear solution. The excess hydrochloric acid and most of the methanol solvent were removed by rotary evaporation at room temperature by using water pump vacuum. The flask was chilled in a refrigerator (ca. 5°C) overnight and the resulting crystals were collected by decanting the excess solvent. The flask was placed back on the rotary evaporator to remove additional methanol from the crystalline matrix. This material was then placed in a small flat-bottomed dish and enclosed in a vacuum oven at 55°C at a pressure of 250 mmHg for 1 h. The resulting white crystalline cake of D-Pa · HCl was ground using a mortar and pestle and placed back in the vacuum oven at room temperature for an additional two days. The D-Pa · HCl salt (approx. 30 g) was sealed in an amber bottle and stored in a dessicator for subsequent use.

Preparation of diazomethane

Diazomethane was produced in a diazomethane generator consisting of a round glass tube, 12 cm × 2 cm I.D. fitted with a 19/22 Wheaton two-arm adapter with one arm extending within approx. 2 cm of the bottom of the tube and its upper end capped or connected to a nitrogen purge source. The other arm was joined by a 2-cm PTFE tube to a Pasteur pipette. All joints of the apparatus were constructed of clear glass fittings as diazomethane has been reported to occasionally explode when distilled in ground-glass apparatus [6]. For safety precautions diazomethane was generated in an efficient fume hood behind a shield.

To the generator 2 ml of 60% potassium hydroxide, 1 ml of carbitol and 1 ml of diethyl ether were added. Approx. 1 g of Diazald was added and the apparatus was quickly capped. Sufficient diazomethane was generated to methylate several samples. Additional diazomethane was expelled from the apparatus using a gentle nitrogen purge.

Purity and structural characterization

Purity and structual characterization of D-Pa and the D-Pa · HCl were assessed by the following analytical techniques: polarimetry, high-resolution gas chromatography with flame-ionization detection (GC—FID), high-resolution gas chromatography with mass spectroscopy (GC—MS) and nuclear magnetic resonance (NMR) spectrometry.

A Rudolph and Son (Caldwell, NJ, U.S.A.) Model 80 polarimeter was used to obtain the specific rotation $[\alpha]_D^{25}$ of a 5% solution of D-Pa (free base) in 1 *M* sodium hydroxide. A Finnigan (San Jose, CA, U.S.A.) Model 4023 mass spectrometer equipped with a Finnigan Model 9610 gas chromatograph and a J & W (Rancho Cordova, CA, U.S.A.) 30 m × 0.25 mm DB-1 bonded fused-silica column (0.25 μm film thickness) was used to obtain electron impact (EI) and ammonia chemical ionization (CI) spectra of D-Pa derivatized with diazomethane—acetone. The D-Pa free base in 1 ml of water was diluted with 9 ml of

acetone containing 0.1 ml of concentrated hydrochloric acid and a 1-ml aliquot was derivatized with diazomethane, extracted into hexane (9.4 ml) and reserved for subsequent injection of an aliquot into the GC—MS system. Purity analysis of the free base was performed by 500-MHz ^1H NMR in ^2H_2O on a Bruker (Fallanden, Switzerland) WM-500 NMR spectrometer.

Purity of the D-Pa · HCl salt was assessed by diazomethane—acetone derivatization followed by high-resolution GC—FID analysis and by ^{13}C NMR analysis of the salt itself. A Tracor (Austin, TX, U.S.A.) Model 560 gas chromatograph equipped with a flame-ionization detector, a J & W on-column injector and a J & W 30 m × 0.25 mm DB-1 capillary column was used to determine the purity of the D-Pa · HCl salt. A 1-μl aliquot of 1 mg/ml concentration of D-Pa · HCl derivatized with diazomethane—acetone was injected directly on column for analysis. The column was held at 60°C for 1 min and then temperature-programmed at 20°C/min to 220°C and maintained there for 10 min. The helium carrier flow-rate was 1.8 ml/min and the resulting retention time (t_R) for the derivative of D-Pa · HCl was 6.37 min. The purity of the D-Pa · HCl salt was also determined in ^2H_2O from the 67.9 MHz ^{13}C NMR spectrum obtained on a Bruker WH270 NMR spectrometer.

Derivatization of D-Pa · HCl in animal feed and D-Pa base in water

Animal feed. Triplicate samples of 1.6, 0.16 and 0.016% by weight of D-Pa · HCl in animal feed were prepared by adding 160, 16 and 1.6 mg of the salt, respectively to 10 g of animal feed contained in 250-ml screw topped Erlenmeyer flasks. The flasks were sealed with PTFE-lined screw caps, shaken to facilitate mixing and placed in the dark for two days. Acetone (100 ml) was added to each flask and the flasks were then shaken on an Eberbach (Ann Arbor, MI, U.S.A.) mechanical shaker for 1 h at 200 excursions per min. Aliquots (1 ml) of the 1.6% samples were diluted to 10 ml with acetone and 1-ml portions of these dilutions were transferred to 12-ml PTFE-lined screw cap tubes. Aliquots of 1 ml of the 0.16% samples were directly transferred to 12-ml tubes. Aliquots of 10 ml of the acetone extract of the 0.016% samples were rotary evaporated at room temperature in a 50-ml round-bottom flask and quantitatively transferred to 12-ml tubes by using 4 × 0.25-ml washes. These latter samples were evaporated to dryness using a stream of nitrogen and reconstituted in exactly 1-ml vols. of acetone. A 1-ml aliquot of a standard of 160 μg/ml of D-Pa · HCl in acetone was used for quantitation purposes. All 1-ml samples and the standard were derivatized by bubbling diazomethane into the samples which immediately turned the solvent yellow. The samples were capped and allowed to stand for 5 min, whereupon 1 ml of water was added to each to stop the derivatization. The addition of 1 ml of water caused the expulsion of the gaseous diazomethane with considerable effervescence. Hexane (9.4 ml) was added, the tubes were capped, shaken for 2 min by hand and allowed to stand approx. 2 min to allow the immiscible layers to separate. The upper layer now consisted of 10.0 ml in volume due to the partitioning of the acetone between the hexane and water layers. The hexane, upper layer, containing the diazomethane—acetone derivative of D-Pa · HCl was then reserved for analysis by GC. For GC analyses utilizing a nitrogen—phosphorus detector, an appropriate amount of N,N-diethylaniline was added in this hexane layer as an internal injection standard.

Water. The derivatization of D-Pa base in water was accomplished in a similar manner with minor modifications. A 1-ml aliquot of a 1 mg/ml water solution of the D-Pa free base was diluted with 9 ml of acetone containing 0.1 ml of concentrated hydrochloric acid thus converting the base to the hydrochloride salt. A 1-ml aliquot was then derivatized as described above and reserved for GC analysis. Quantitation was by comparison to a freshly prepared D-Pa free base standard prepared in water and derivatized as described above.

Gas chromatography

A Tracor Model 560 gas chromatograph was equipped with a nitrogen—phosphorus detector and a 90 cm × 2 mm I.D. glass column containing 5% SP2100 (Supelco, Bellefonte, PA, U.S.A.) on Supelcoport 100—120 mesh. The injection port, column oven and nitrogen—phosphorus detector temperatures were 140°C, 110°C and 280°C, respectively. The helium carrier gas flow-rate was 25 ml/min and the hydrogen and air flow-rates to the nitrogen—phosphorus detector were 2.0 and 110 ml/min, respectively. Samples of diazomethane—acetone derivatized D-Pa · HCl extracts from animal feed or derivatized D-Pa free base from water were injected in 2 μl of hexane. The t_R of the derivative was 2.0 min. Samples of diazomethane—acetone derivatized D-Pa · HCl extracts from animal feed were also analyzed by GC using flame-photometric detection (GC—FPD) on a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 3920B GC equipped with a linearizer for the sulfur mode. The 180 cm × 2 mm I.D. glass column contained 10% SP 2100 on Supelcoport 100—120 mesh. The injection port, column and detector temperatures were 180°C, 160°C and 200°C, respectively. The helium carrier flow-rate was 25 ml/min and the hydrogen and air flow-rates for the flame-photometric detector were 70 and 100 ml/min, respectively. All injections were in 2 μl of hexane. The t_R of the derivative was also 2.0 min.

Stability experiments

Tests were performed to determine the stability of the test chemical at the highest and lowest proposed dose levels under simulated animal test conditions (i.e., opened container, in light) and under storage conditions (i.e., closed container, in dark). In animal feed, D-Pa · HCl was blended at levels of 0.016% and 1.6% by weight by the addition of 160 mg of the salt to 1 kg of feed and 16 g to 984 g of feed, respectively. These were blended for 30 min in a Model LV Twin-Shell Lab Blender (Patterson-Kelly, East Stroudsburg, PA, U.S.A.) operated at 20 rpm with the intensifier bar turned off. Each dosage level was then divided in half. One portion of each was then placed in an open container under incandescent lighting and the other portion stored in a sealed container in the dark. Extraction and analyses of D-Pa · HCl in animal feed were determined as previously described with the exception that the 0.016% D-Pa · HCl in animal feed samples were not concentrated ten-fold by rotary evaporation prior to the diazomethane—acetone derivatization of 1 ml of the extract.

The stability of D-Pa in distilled deionized water was determined for 100, 10 and 1 mg/ml concentrations, respectively, at ambient temperature under laboratory lighting conditions. The D-Pa solutions were sampled at 6, 24 and

48 h after preparation and analyzed as previously described. Samples were quantitated by comparison to a freshly prepared standard of D-Pa in water.

Preparation of fluorescent derivative of D-Pa in water

Triplicate 1-ml samples of 100, 50, 20, 10, 5, 2 and 1 μg/ml of D-Pa in water were placed in 8-ml screw-top tubes containing 1 ml of 0.2 *M* borate buffer, pH 9. Acetonitrile (3 ml) containing 150 μg/ml Fluram was added, the tube capped, and immediately vortexed before proceeding to the next tube. After allowing the samples to stand for 20 min, their fluorescence was read on an Aminco-Bowman (Silver Spring, MD, U.S.A.) spectrofluorometer. The excitation wavelength (λ_{ex}) was 350 nm and the emission wavelength (λ_{em}) was 490 nm. The Aminco-Bowman spectrofluorometer was calibrated to yield a response of 50% at 10× when using a quinine sulfate standard of 0.3 μg/ml in 0.05 *M* sulfuric acid with λ_{ex} = 350 nm and λ_{em} = 450 nm.

RESULTS AND DISCUSSION

Upon receipt of the D-Pa (base, MW 149) from Merck Sharp & Dohme, chemical characterization and purity of the material were assessed. The D-Pa was found to have a specific rotation $[\alpha]_D^{25}$ of −54° which compared well with the Merck Index listed value of −55°. When reacted with ninhydrin or phosphotungstic acid, D-Pa also produced the characteristic blue color described under the identification section for D-Pa in The United States Pharmacopeia Twentieth Revision [7]. As previously described, the number of analytical techniques reported in the chemical literature for the analysis of D-Pa were extremely limited, were non-specific for the chemical or did not meet our specific needs. We, therefore, developed a simple, rapid analytical procedure applicable to the analysis of D-Pa in water or D-Pa · HCl in animal feed or water. D-Pa is not amenable to GC analysis per se, but can be analyzed by GC after methylation with diazomethane in acetone under acidic conditions. The derivative was chromatographed on a fused-silica capillary column and the EI and ammonia CI spectra subsequently obtained on a Finnigan mass spectrometer. EI data (Fig. 2) indicated the molecular ion of the derivative to be at *m/z* 203 and CI data indicated a corresponding M+1 ion at *m/z* 204. Mass spectral data were consistent with the interpretation that D-Pa not only reacted with the diazomethane but also reacted with the solvent acetone. Proton NMR spectral data of this derivative indicated that acetone caused ring closure to a five-membered ring (thiazolidine); structure shown in Fig. 1. Proton NMR data of the original D-Pa free base in ^2H_2O indicated a purity of 99%. The purity of the D-Pa · HCl by high-resolution GC—FID and ^{13}C NMR analyses also indicated a purity in excess of 99%. The purities of the D-Pa and D-Pa · HCl were judged sufficient for the proposed toxicology studies.

The highest and lowest proposed levels for penicillamine as the hydrochloride salt in animal feed were 1.6% and 0.016% by weight, respectively. In Fig. 3, GC chromatograms of the D-Pa · HCl extracted from animal feed and derivatized with diazomethane—acetone are shown for the 0.016% level. Triplicate assays at the two levels by both FPD and nitrogen—phosphorus

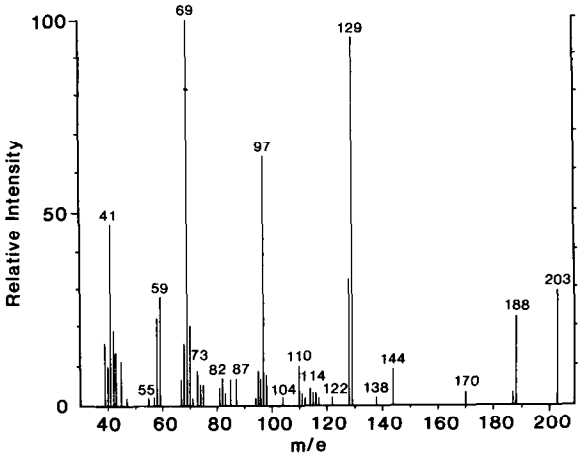


Fig. 2. Mass spectrum (EI) of the diazomethane—acetone derivative of D-penicillamine.

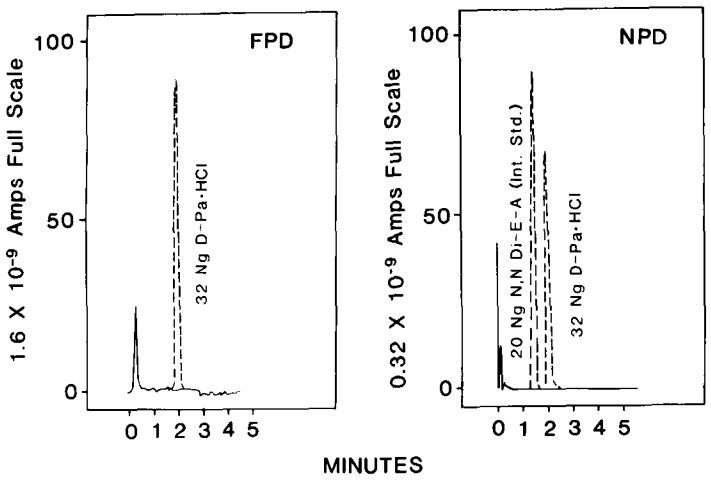


Fig. 3. Chromatograms of D-Pa • HCl as the diazomethane—acetone derivative by GC with flame-photometric detection (FPD) and with nitrogen—phosphorus detection (NPD); (—) responses from derivatized control animal feed samples, (- - -) response from 0.016% by weight of D-Pa • HCl in animal feed, N,N-diethylaniline (N,N-Di-E-A) included as an internal injection standard for nitrogen—phosphorus detector as indicated.

detection gave similar results and showed no interfering peaks. The flame-photometric detector output signal was electronically linearized because the sulfur mode is a non-linear square root function while the nitrogen—phosphorus detector output signal is inherently linear. Also, the flame-photometric detector baseline was found to be considerably noisier than the baseline of the nitrogen—phosphorus detector as shown in Fig. 3. Therefore, analysis by GC with nitrogen—phosphorus detection was the procedure of choice.

Recovery experiments were also performed in which (1) D-Pa free base and (2) D-Pa • HCl in methanol were added to animal feed. However, these experiments were unsuccessful in recovering the original material. The D-Pa free base was found to be essentially insoluble in the extraction solvent (acetone) as opposed to the salt which is very soluble indicating that the base

does not significantly react with acetone. Recovery of D-Pa · HCl in methanol spiked into animal feed was also unsuccessful (ca. 0% recovery). This was probably due to the intimate contact between the D-Pa · HCl and the animal feed. The D-Pa · HCl salt as well as the D-Pa free base are not chemically inert. In fact, they have several reactive functional groups within the molecule that could react with animal feed components. Therefore, by blending the D-Pa · HCl dry with the animal feed, intimate contact with the feed was reduced resulting in much improved recoveries of 75% and 95% from the 0.016% and 1.6% by weight of the D-Pa · HCl in the animal feed, respectively.

The proposed levels for penicillamine as the base in water were 100, 10 and 1 mg/ml to be administered by gavage. Analysis of D-Pa in water was similar to that previously described for analysis of D-Pa · HCl in feed with minor modifications. An aliquot of the D-Pa in water was diluted with acetone—hydrochloric acid before derivatization with diazomethane. The acetone serves to dilute the water therefore allowing the diazomethane to dissolve in solution and the D-Pa free base was converted to the salt form by the hydrochloric acid thereby allowing the diazomethane—acetone derivatization to proceed smoothly. GC data of diazomethane—acetone derivatized D-Pa from water, however, did show a minor secondary peak that eluted after the main peak and was completely separated from it. Ammonia CI data of this peak indicated a M+1 ion of *m/z* 218 as opposed to the main peak at *m/z* 204. Since this is a difference of 14 MU it is proposed that the diazomethane reacted to add a second CH_2 group into the molecule. In addition, the diazomethane—acetone derivatization reaction was found to be linear for various quantities (0.05—10

TABLE I

STABILITY OF D-Pa · HCl IN ANIMAL FEED SPIKED AT TWO LEVELS

Sampling interval		D-Pa · HCl recovered* (%)	
		Spiked at 1.6% by weight	Spiked at 0.016% by weight
*Short-term study***			
Days:	0	94.9 ± 1.1	74.6 ± 0.4
	1	97.9 ± 2.2	70.0 ± 1.2
	4	96.5 ± 1.2	62.2 ± 8.3
	8	93.3 ± 1.2	48.3 ± 1.1
	14	90.1 ± 0.1	N.D.***
Long-term study §			
Weeks:	0	94.9 ± 1.1	74.6 ± 0.4
	1	94.5 ± 3.0	49.6 ± 1.8
	2	91.1 ± 2.5	N.D.
	4	89.1 ± 2.6	N.D.
	8	84.6 ± 1.6	N.D.

*Mean ± standard error from triplicate assays.
**Open container, incandescent lighting, and ambient temperature.
***N.D. = not determined.
§ Sealed container, light-tight cabinet, and ambient temperature.

TABLE II

STABILITY OF D-Pa IN WATER SPIKED AT THREE LEVELS

Sampling interval (h)	D-Pa recovered* (%)		
	Spiked at 100 mg/ml	Spiked at 10 mg/ml	Spiked at 1 mg/ml
6	100	102	101
24	100	97.7	94.0
48	102	96.4	83.5
pH	5.2	5.1	4.5

*Sealed container, incandescent lighting, and ambient temperature.

mg) of D-Pa · HCl even in the presence of 1 g equivalent of feed extract. A similar linear response was also found for the diazomethane—acetone derivatization of the D-Pa free base from water.

Tables I and II indicate the stability of D-Pa · HCl in animal feed and the stability of D-Pa free base in water. The D-Pa · HCl concentrations in animal feed were found to decrease with time as would be expected for the chemically active D-Pa · HCl as it reacts with the animal feed components. The data also indicate that this decrease was independent of whether the samples were in the light in an open container or in a closed container in the dark. A decrease in the concentration of D-Pa free base in water with respect to time was also noted. Therefore, both dosage forms must be prepared and used immediately to provide assurance that the requisite dosages are administered to the test animals.

Fig. 4 represents data from an ancillary procedure for the spectrofluorometric analysis of D-Pa from water after derivatization with Fluram

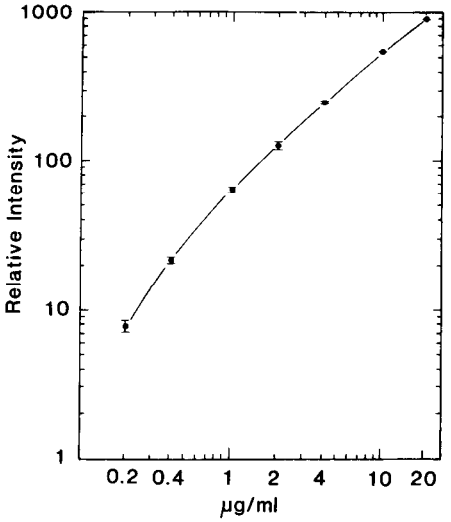


Fig. 4. Fluorescence response for the derivatization of D-penicillamine in water using Fluram; triplicate samples.

(a non-fluorescent chemical that hydrolyzes in aqueous media to non-fluorescent products) [8]. Initially, a high fluorescence reading resulted which subsided to an almost constant response. Therefore, for accurate quantitation, timing of the fluorescence readings was a critical factor.

In summary, this paper describes a new, rapid GC procedure for the analysis of D-Pa · HCl in animal feed and for the analysis of D-Pa · HCl or D-Pa free base in water.

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