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Stoichiometric analysis by chromatographic techniques I. Analysis of some antioxidants and penicillins by HPLC methods *

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Summary

An HPLC method has been utilized to follow a titration by monitoring either the reactant(s), product(s) or both. Such techniques produce linear titration curve(s) and the end point is determined from extrapolation to zero reactant concentration and/or maximum product concentration. This procedure has been successfully applied to the stoichiometric analysis of several antioxidants and antioxidant mixtures. This technique has also been used to obtain useful information about the stoichiometry, kinetics and mechanism of the hydroxylamine reactions of ampicillin, cyclacillin and 6-aminopenicillanic acid. This method is superior in specificity, sensitivity and scope over conventional titration stoichiometric methods. This study shows that the HPLC method is a powerful tool for conducting and exploring stoichiometric analysis, the basis of analytical chemistry.

Introduction

Stoichiometric analysis is of fundamental importance to the establishment of absolute purity of a chemical substance. Physical methods such as phase solubility and DSC determinations are often used to study absolute purity; however, only chemical methods relate molecular structure to absolute purity.

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Presently, chemical methods are usually carried out by volumetric titrations. A conventional analysis determines the end-point of a quantitative and stoichiometric reaction. Only a few general reactions, such as acid–base, oxidation–reduction and complexation reactions have been utilized extensively for such analysis. The end-point employed for volumetric titrations is usually based on potentiometry, spectrophotometry, and/or color indicators. Since both the titration reactions and the end-point detection methods are often poor in specificity, most volumetric titration methods are also deficient in this respect.

In order to utilize conventional stoichiometric analysis, the basic reaction must have appropriate properties. Specifically, it must reach completion rapidly even at very low concentrations (at the end-point) and provide a sharp change in potential, absorbance, or color of an indicator at the end-point. These difficult conditions have limited the conventional methods to the few classes of reactions mentioned above for the analysis of a relatively small number of functional groups. As a result, the conventional methods have been limited to the analysis of only a few classes of bulk materials.

Modern chromatographic methods are highly specific, sensitive, and simple and should be ideal to perform stoichiometric analysis. This possibility is strongly suggested by the fact that chromatographic methods have been the most widely used techniques to study organic chemical reactions.

The present study is one of a series designed to explore and demonstrate the applications of chromatographic techniques to the stoichiometric analysis of compounds and reactions.

Specifically, two types of reactions were evaluated for stoichiometric analysis by HPLC methods. The iodine–antioxidant reaction was utilized to demonstrate the stoichiometric analysis of antioxidants by HPLC methods. This type of reaction is widely used by conventional methods. In addition, the penicillin–hydroxylamine reactions were studied for the possible application to the stoichiometric analysis of penicillins. This latter reaction is not amenable to conventional analysis due to the rate and the lack of end-point determination method.

Materials and methods

Apparatus

A liquid chromatograph equipped with a Constametric IIG pump (LDC), a model 70-10 loop-injector attached with Model 70-11 loop-filler (Rheodyne), a Model SF 770 spectroflow monitor (Schoeffel), and a 250 mm × 4 mm i.d. stainless steel column prepacked with 10 μm diameter silica having either octadecyltrichlorosilane (μ-C18) (C 18HL Alltech) or quarternary ammonium (μ-SAX) (Chromegabond SAX, E.S. Industry) chemically bonded to its surface, was used.

Experimental conditions

Ascorbic acid. The μ-C18 column was used. The mobile phase composition was methanol 100 ml, distilled water 500 ml, tetrabutylammonium phosphate solution 10

ml, and sodium phosphate monohydrate 0.6 g. Detection was made at a wavelength of 248 nm, injection volume 10 μl, and flow rate 1.5 ml/min. The tetrabutylammonium phosphate solution was prepared by adjusting the pH of 20.0 ml 10% tetrabutylammonium hydroxide in water to 7.5 using a 10% phosphoric acid solution. The final volume was brought to 200 ml with distilled water.

Sodium formaldehyde sulfoxylate. The μ-C18 column was used. The mobile phase composition was methanol 200 ml, distilled water 700 ml, and tetrabutylammonium phosphate solution 10 ml. The detection was made at a wavelength of 210 nm, injection volume 10 μl, and flow rate 1.5 ml/min.

Ampicillin and cyclacillin. The μ-C18 column was used. The mobile phase was prepared by adding 3.0 ml concentrated ammonium hydroxide (29.2% NH_3), 3.0 ml concentrated phosphoric acid (85%), and 150 ml of acetonitrile to 900 ml of distilled water. The detection was made at a wavelength of 205 nm, injection volume 20 μl, and flow rate 1.0 ml/min.

6-Aminopenicillanic acid (6-APA). The μ-SAX column was used. The mobile phase consisted of 500 ml 0.1% NaH_2PO_4 aqueous solution and 50 ml acetonitrile. Detection was made at a wavelength of 205 nm, injection volume 20 μl and flow rate 1.5 ml/min.

Reagents and solvents

USP reference standard ascorbic acid, in-house reference standards of ampicillin, cyclacillin and 6-APA, USP grade sodium formaldehyde sulfoxylate (Diamond Sharmrock), certified grade 0.100 N iodine solution, tetrabutylammonium hydroxide 10% in water (Fisher), reference standard arsenic oxide (NBS), hydroxylamine sulfate (99% min.) (Eastman Kodak), hydroxylamine hydrochloride (96% min.) (Mallinckrodt), and distilled in glass grade methanol (B and J) were used. All other chemicals used were analytical reagents.

Sample and standard solutions

All solutions were prepared fresh. Sample solutions of ascorbic acid were prepared in methanol. The sample solutions of sodium formaldehyde sulfoxylate were prepared in N_2-purged distilled water with or without 2% by volume formaldehyde, T.S. The sodium metabisulfite solutions were obtained from direct addition of accurately weighed sodium metabisulfite into a known volume of sodium formaldehyde sulfoxylate sample solutions.

Both penicillin and hydroxylamine solutions were prepared in a pH 7 buffer solution. The pH 7 buffer solution was obtained by diluting the mixture of 10.0 ml 0.5 M NaH_2PO_4 and 15.6 ml 0.5 M Na_2HPO_4 to 1 liter.

HPLC titration procedures

An aliquot sample solution (2.0–5.0 ml) was transferred to a 50 ml conical flask with stopper and an aliquot of iodine or hydroxylamine titrant was added through a precision syringe. The resulting solutions were intermittently swirled. The iodine-titrated solutions were allowed to stand until the solutions turned colorless and then injected on HPLC. The treatment of hydroxylamine-titrated solutions before HPLC

analysis are described in the following for the individual samples.

An HPLC titration was accomplished by repeating the above procedure with different volumes of titrant added to a fixed volume of sample solutions.

Official methods

The procedures of USP assay method for ascorbic acid, the NF methods for sodium formaldehyde sulfoxylate and sodium metabisulfite, and the standardization for iodine titrant are given in USP XIX or NF XIV, respectively.

Results and discussion

Iodine-antioxidant titrations

Typical HPLC chromatograms obtained from HPLC procedures are shown in Fig. 1. HPLC titration plots, plot of peak height of antioxidant $\times (V + v)/V$ vs the volume of iodine titrant (v) added to a fixed volume (V) of sample solutions, are depicted in Figs. 2 and 3. The end-point was obtained by extrapolation to zero peak

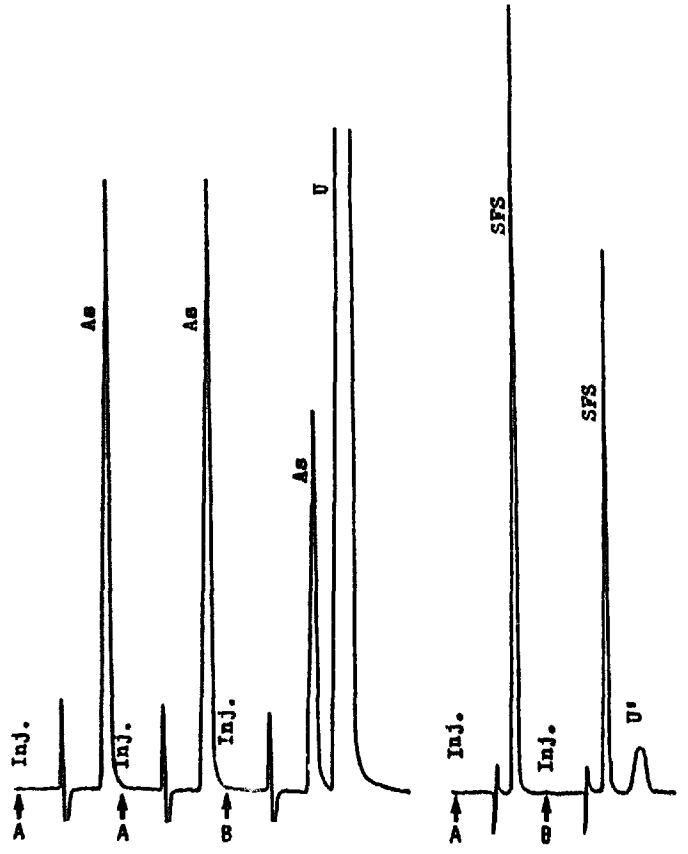


Fig. 1. Typical HPLC chromatograms of antioxidants (A) and their iodine-titrated solutions (B). As, ascorbic acid ($t_R = 3$ min); U and U', unknown titration products; SFS, sodium formaldehyde sulfoxylate ($t_R = 2$ min).

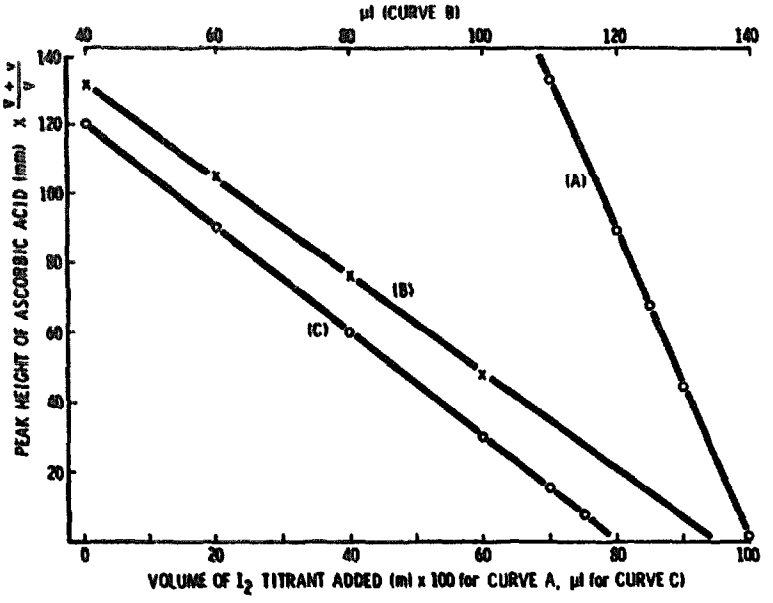


Fig. 2. HPLC-titration curves of ascorbic acid. The fixed volumes and the concentrations of sample solutions are: A, 5.0 ml 1.773 mg/ml; B, 3.0 ml 0.400 mg/ml; C, 3.0 ml 0.243 mg/ml.

height and HPLC titration results were calculated by Equation 1.

$$\% \text{ purity} = \frac{K \times N \times V_e}{V \times C} \tag{1}$$

where V_e = the end-point volume of iodine titrant (μl); V = the fixed volume of sample solutions (ml); C = the concentration of sample solution (mg/ml); K = 8.806 for ascorbic acid (i.e. mg ascorbic acid/ml 0.1N I_2), K = 2.952 for sodium formaldehyde sulfoxylate, and K = 3.203 for sodium metabisulfite (for the calculation

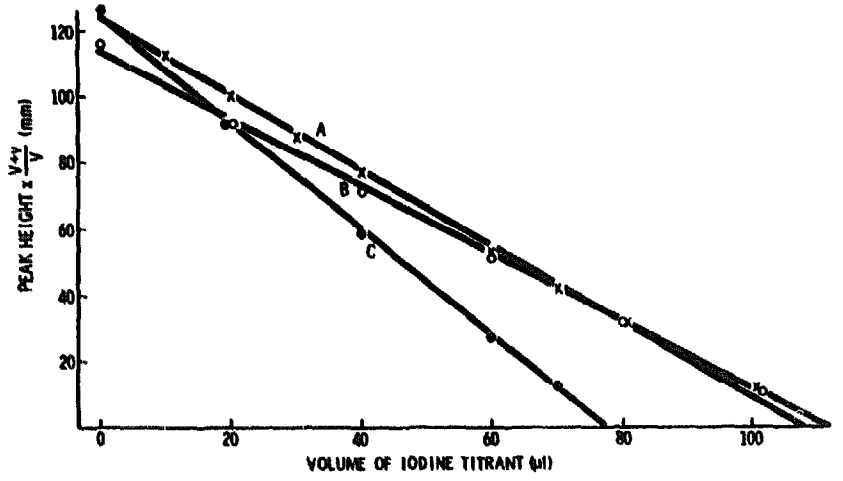


Fig. 3. HPLC-titration curves of sodium formaldehyde sulfoxylate (C) and mixtures of sodium formaldehyde sulfoxylate and sodium metabisulfite (A and B).

of % SO_2 content); and N = the normality of iodine titrant.

The end-point for a solution containing a mixture of sodium formaldehyde sulfoxylate and sodium metabisulfite was treated as an overall end point of both antioxidants. The end-point of a pure sodium formaldehyde sulfoxylate solution was determined independently. The amount of sodium metabisulfite was calculated from the difference between the overall end-point and the theoretical end-point of the sodium formaldehyde sulfoxylate in the mixture. The antioxidant samples analyzed by HPLC titration methods were also assayed by iodometric titration methods described either in USP XIX or NF XIV using the same iodine titrant which was standardized to be 0.100 N. Results are summarized in Table 1.

The sample preparation procedure used for the HPLC methods for sodium formaldehyde sulfoxylate was similar to that of the NF titration procedure. Therefore, it provides a good comparison to the conventional method. In the case of ascorbic acid, this was not possible. Aqueous solutions of ascorbic acid show rapid degradation as demonstrated by continuous decrease of peak height from repetitive injections and accurate HPLC titration results could not be obtained. However, use of methanol as the solvent significantly improved the stability of ascorbic acid solution. The USP method includes addition of sulfuric acid prior to idometric titration. However, it was found by HPLC that addition of sulfuric acid or the milder acetic acid to a solution of ascorbic acid results in instantaneous degradation of the antioxidant. Therefore, acid was not used in the HPLC titration for ascorbic acid. In methanol solution it took less than 15 min for the iodine–ascorbic acid reaction to reach completion.

TABLE I

SUMMARY OF STOICHIOMETRIC ANALYSIS ON ANTIOXIDANTS

Sample	Conc. (mg/ml) [a]	Percent purity	
		HPLC methods	USP or NF methods
Ascorbic acid	0.243	97.1	100.5, 100.7
	0.400	99.4	
	1.773	99.6	
SFS with 2% F	0.1006	76.3	76.0, 76.1
without 2% F	0.1005	75.8	78.5
Mixture of SFS with 2% F and 0.0526 mg/ml SM	0.1006	76.3	
SM			66.1, 66.2 (%SO_2)
Mixture of SFS	0.10045	76.3	
and SM	0.0510	64.1 (%SO_2)	
Mixture of SFS	0.08036		
and SM	0.0822	65.1 (%SO_2)	

SFS = sodium formaldehyde sulfoxylate; SM = sodium metabisulfite; F = formaldehyde. [a] The concentrations of samples used only in HPLC methods.

The NF iodometric titration method for sodium formaldehyde sulfoxylate requires the addition of formaldehyde T.S. to the sample solution. The HPLC titration results suggest that the addition of formaldehyde T.S. can effectively eliminate the interference of sodium metabisulfite to the determination of sodium formaldehyde sulfoxylate. However, as would be expected, without formaldehyde, the HPLC titration of a sodium formaldehyde sulfoxylate solution containing sodium metabisulfite gave an end-point which reflected the overall potency of both antioxidants. The linear relationships given in Fig. 3 suggest that both antioxidants are proportionally consumed by iodine titrant and, therefore, will be simultaneously depleted to give an overall end-point. Since a pure sodium formaldehyde sulfoxylate solution can be titrated independently, its end-point volume in the mixture can be calculated. Accordingly, the end-point of sodium metabisulfite can be calculated by taking the difference between the overall end-point volume and the calculated end-point of sodium formaldehyde sulfoxylate in the mixture. The SO_2 content of a sodium metabisulfite sample so determined from two solutions containing the above two antioxidants at different concentration ratios were in good agreement (Table 1). The HPLC result is somewhat lower than that obtained by the official iodometric titration method, probably due to the instability of dilute sodium metabisulfite solutions. These results demonstrate that an antioxidant can be analyzed indirectly even though no HPLC method is available for its analysis.

The HPLC methods require considerably less sample than the official methods for antioxidants analysis. Since one of the antioxidants, sodium formaldehyde sulfoxylate, is only a weak UV absorber, for compounds with comparable or stronger UV absorption, the HPLC methods would be much more sensitive than the conventional methods. However, handling sample solutions at low concentration levels introduces certain analytical problems. Fresh, high purity, and preservative free solvents should be used for sample preparation. A control HPLC calibration curve must be obtained using sample solutions carried through the same reaction condition as the titration samples. If the calibration curve obtained is linear and passes through the origin, the solvent is qualified for use in sample preparation in the HPLC procedure.

Hydroxylamine titration on penicillins

Fig. 4 shows typical chromatograms for HPLC-titration of cyclacillin, ampicillin and 6-APA. Since the completion of a penicillin–hydroxylamine reaction can not be observed visually, it had to be determined experimentally. The HPLC-titration curves were plotted for different reaction conditions. When linear titration curves are obtained, the completion of the titration reaction is indicated.

At room temperature, the time to reach completion for both cyclacillin and ampicillin at concentration ranges from 0.5 to 1.0 mg/ml, respectively, was greater than 3 h. However, both reactions reached completion within 2 h at 45°C. At lower concentration, 0.1 mg/ml, the reactions were not complete after 2.5 h at 45°C and 20 min at 65°C. Surprisingly, it was also found that the reaction rates were reduced when the concentrations of both reactants were proportionally increased. At 5 mg/ml, the hydroxylamine reaction rates appeared to be significantly slower than

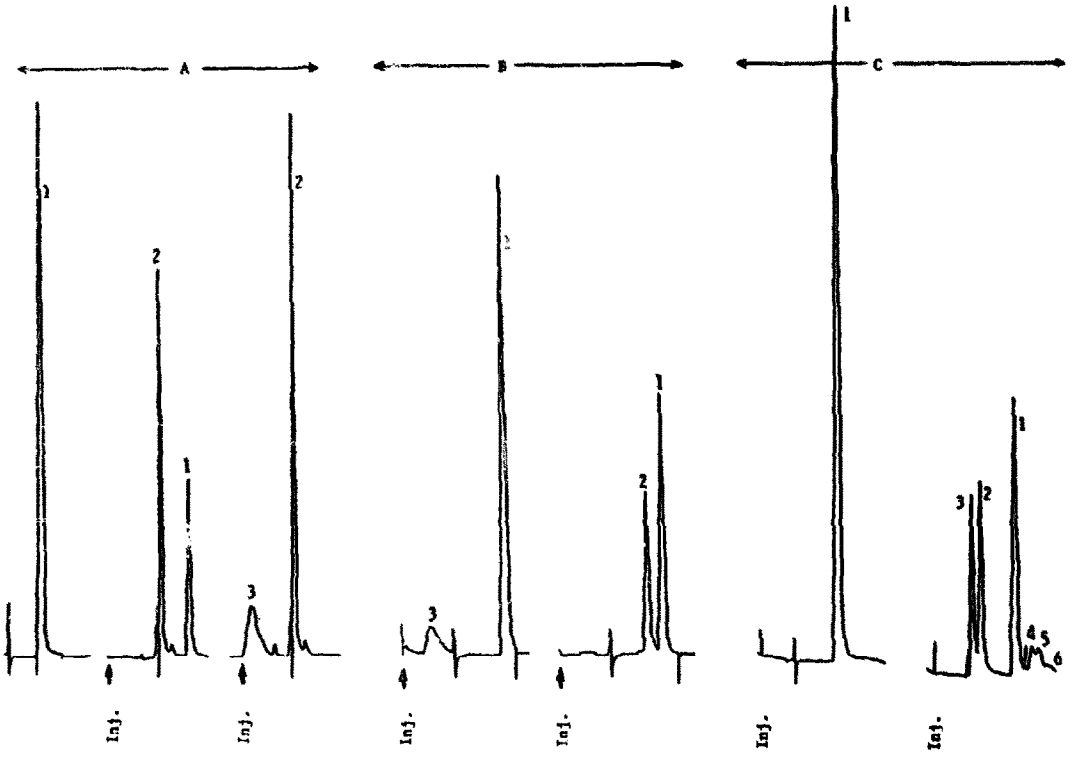


Fig. 4. The HPLC chromatograms of penicillins and their hydroxylamine-titrated solutions. A: (1) cyclacillin ($t_R = 3$ min), (2) short-retention product ($t_R = 2$ min), (3) long-retention product ($t_R = 15$ min). B: (1) ampicillin ($t_R = 4$ min), (2) short-retention product ($t_R = 3.4$ min), (3) long-retention product ($t_R = 18$ min). C: (1) 6-APA ($t_R = 4$ min), (2–6) titration products.

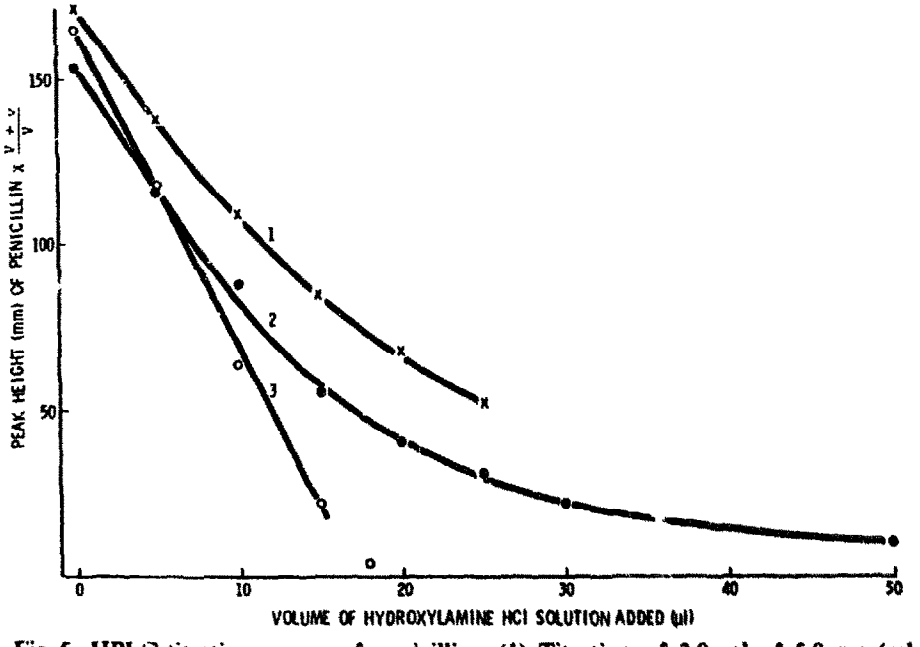


Fig. 5. HPLC-titration curves of penicillins. (1) Titration of 3.0 ml of 5.0 mg/ml cyclacillin by 80.0 mg/ml hydroxylamine-HCl with reaction time of 3.5 h at RT. (2) Titration of 3.0 ml of 5.0 mg/ml ampicillin by 80.0 mg/ml hydroxylamine-HCl with reaction time of 2.5 h at 45°C. (3) Titration of 4.0 ml of 1.0 mg/ml cyclacillin by 40.0 mg/ml hydroxylamine-HCl with reaction time of 3.5 h at RT.

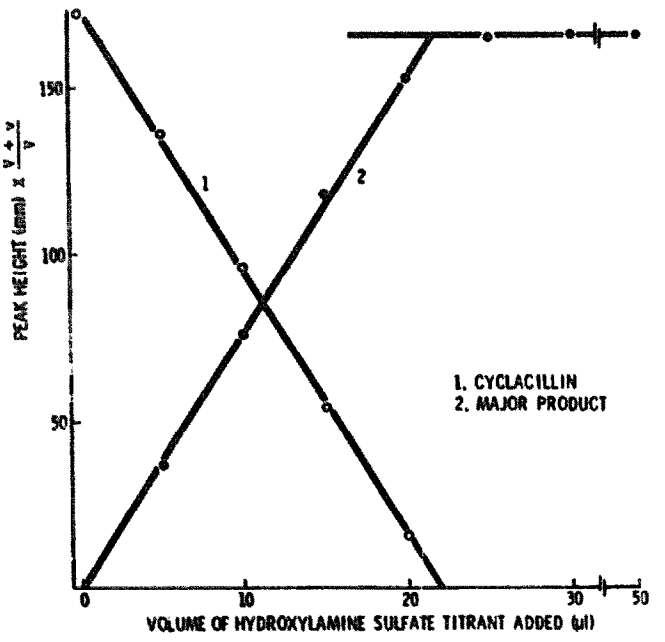


Fig. 6. HPLC-titration curves obtained from 1.0 mg/ml cyclacillin solutions at pH 7 with reaction at 45°C for 3 h. (1) cyclacillin, (2) short-retention product.

those at 1 mg/ml and 0.5 mg/ml for both cyclacillin and ampicillin. An example of this is shown in Fig. 5. This finding strongly suggests that penicillins interact to form dimer or micelles at high concentrations and such self-association interactions hinder their hydroxylamine reactions. The presence of self-association interactions in solutions was also indicated in other penicillin studies (Bundgaard, 1976, 1977).

It is clear from Fig. 4 that the reaction between a penicillin and hydroxylamine

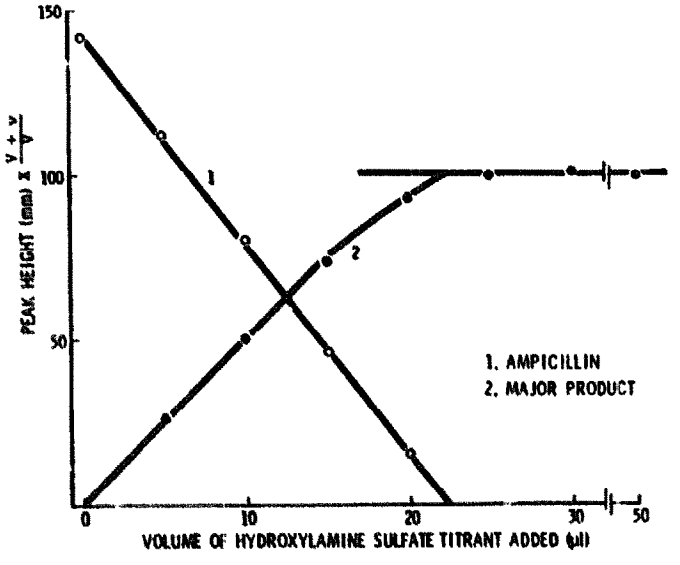


Fig. 7. HPLC-titration curves obtained from 1.0 mg/ml ampicillin solutions at pH 7 with reaction at 45°C for 3 h. (1) ampicillin, (2) short-retention product.

results in more than one reaction product. Theoretically, the monitoring of products formation can also be utilized to determine the end-point of an HPLC titration provided that the titration is carried out beyond the end-point. Figs. 6 and 7 compare the titration curve of a product with that of its parent penicillin to demonstrate such an application.

The stoichiometric relation of the penicillin–hydroxylamine reaction was calculated by Equation 2.

$$\text{Stoichiometric ratio}\left(\frac{\text{penicillin}}{\text{hydroxylamine}}\right) = \frac{1000 \times C_p \times V \times M_h}{V_e \times M_p \times C_h} \quad (2)$$

where V_e = the end-point volume of hydroxylamine titrant (μl); V = the fixed volume of penicillin sample solutions (ml); C_h = the concentration of hydroxylamine salt solution (mg/ml); C_p = the concentration of penicillin sample solution (mg/ml); M_h = the molecular weight of hydroxylamine salt; and M_p = the molecular weight of penicillin.

Table 2 summarizes the results obtained with the HPLC-titration procedures.

For all 3 penicillins studied the hydroxylamine titrants appeared to be more potent (16–30%) than expected even though two different analytical grade hydroxylamine salts were used. The heat treatment, 2.5 h at 45°C, was found to introduce 2–4% thermal degradation on the penicillins used. However, this relatively small degradation is not expected to introduce any significant error on the end-point determination.

The hydroxylamine reactions of all 3 penicillins studied give more than one product. It is possible that hydroxylamine not only attacks the β-lactam ring to form

TABLE II

STOICHIOMETRIC RELATIONSHIP BETWEEN PENICILLIN AND HYDROXYLAMINE

Sample	Concentration (mg/ml)	Titrant	Reaction condition	Stoichiometric ratio $\left(\frac{\text{penicillin}}{\text{hydroxylamine}}\right)$
Cyclacillin	0.102	A	2.5 h at 45°C and 20 min at 65°C	0.74
	0.502	A	3 h at 45°C	0.70
	0.504	B	2 h at 45°C	0.72
	1.00	B	3.5 h at RT	0.84
Ampicillin	0.100	A	25 h at 45°C and 20 min at 65°C	0.84
	0.503	A	3 h	0.71
	0.504	B	2 h at 45°C	0.77
	1.00	B	2.5 h	0.73
6-APA	0.50	A	2.5 h at 60°C	0.84

Where A = hydroxylamine sulfate, minimum content 99%; B = hydroxylamine hydrochloride, minimum content 96%.

hydroxamic acid (Stevenson et al., 1969) but also attacks other functional groups to induce parallel reactions. However, such parallel reactions should also follow the 1 : 1 stoichiometry for both reactants.

Following are two possible explanations for the hydroxylamine titrant appearing more potent than expected and the HPLC titration curves to remain linear. In the first case one or more of the penicillin–hydroxylamine products can react quantitatively with the remaining penicillin. In the second situation the hydroxylamine not only reacts with but also catalyzes the degradation of penicillins.

One possible hydroxylamine-catalyzed degradation of penicillin is the formation of penicilloic acid analogous to the OH^--catalyzed degradation (Hou and Poole, 1969). This hypothesis was tested by HPLC methods. It was found that although the retention time of one of the hydroxylamine reaction products of cyclacillin is similar to that of the expected penicilloic acid, no penicilloic acid was found with the ampicillin–hydroxylamine reaction.

A kinetic study on the penicillin–hydroxylamine reactions at room temperature was performed for both ampicillin and cyclacillin using HPLC method. The rates of disappearance of penicillins were compared with the formation rates of products, as shown in Fig. 8, for cyclacillin in terms of peak height vs time plots. For both penicillins, two major products were formed with one at shorter retention time and one at longer retention time than their parent penicillin. In both cases, the short retention product forms, without delay, at a comparable rate to the disappearance of its penicillin and should be a direct reaction product of the penicillin–hydroxylamine reaction. However, the long retention product, which shows an initial delay in its formation, is likely a secondary product. The kinetic curves clearly show that the formation of both products stops when penicillin is exhausted. Therefore, it is likely that the long retention product is a secondary reaction product of the penicillin and the direct penicillin–hydroxylamine product. These data favor the explanation that the penicillin reacts further with the reaction product of hydroxylamine to make

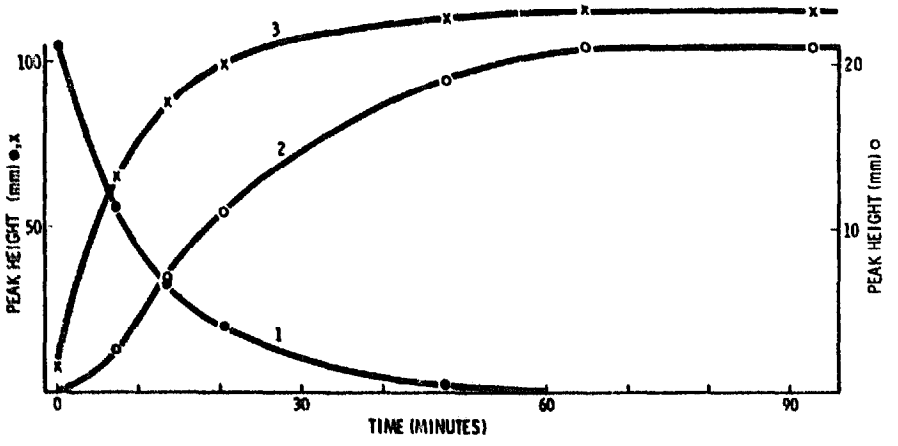
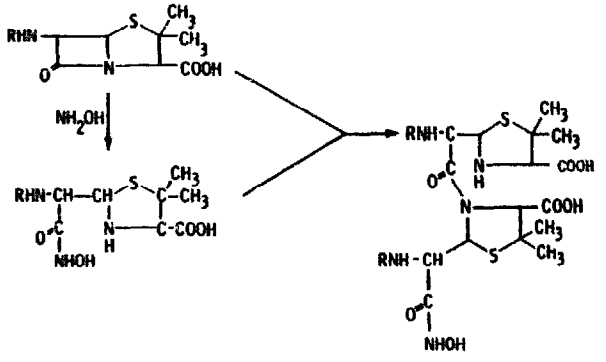


Fig. 8. The reaction kinetics of 10.0 ml of 0.5 mg/ml cyclacillin and 50 µl of 40.0 mg/ml hydroxylamine sulfate at room temperature (24± 1°C). (1) cyclacillin, (2) short-retention product, (3) long-retention product.

POSSIBLE REACTIONS AMONG PENICILLIN, HYDROXYLAMINE, AND THEIR PRODUCT



SHORT RETENTION PRODUCT LONG-RETENTION PRODUCT

SCHEME I

hydroxylamine solutions appear more potent than expected. A possible mechanism of such a series of reactions is given in Scheme I.

The fact that penicillin–hydroxylamine reaction does not follow simple 1:1 stoichiometry precludes its application as a universal HPLC titration method for the stoichiometric analysis of penicillin. However, it has value for limited analytical applications.

Conventional titration methods require sufficient titration points before and after the end-point and as many data as possible in the region of the end-point in order to provide an accurate determination. However, the linear titration curves of the HPLC analysis allows a minimum of two HPLC determinations for the estimation of end-point. Additional data can be used in the linear plots to increase the precision for the end-point determination.

This study demonstrated that either sample or reaction product or both can be monitored by HPLC methods to determine the end-point. When sample concentration is monitored, only those titration points before the end-point are needed. However, when a titration product is used, data points beyond the end-point are also needed for the determination of the end-point. It should also be possible to monitor a titrant by an HPLC method to determine the end-point. In this case, only those points beyond the end-point are needed.

The reactions chosen for stoichiometric analysis by HPLC methods in this study are irreversible. The mobile phases of the reverse-phase and the ion-pair HPLC methods used are essentially aqueous solutions. The HPLC methods described are not applicable to acid–base type of reactions, since they are reversible reactions in aqueous solutions. However, it should be possible to carry out such a study in a non-aqueous system using a normal phase HPLC method. The mobility of the acid–base equilibrium will be hindered or halted in many non-aqueous solutions such as aprotic solvents (Davis, 1968). We plan to explore this possibility in future work.

When a linear plot is obtained from the HPLC titration data, the completion of the titration reaction is indicated. Sometimes only higher sample concentration points are linear while those close to end-point deviate. In this case, the end-point may be estimated from the extrapolation of the linear portion of the curve. One of the advantages of the HPLC procedure is that each titration point is a separately titrated sample and all samples can be appropriately exposed to a suitable accelerated condition for their titration reaction to reach completion simultaneously. In addition, with the HPLC procedure, it is not necessary to wait for titration reaction to reach completion for points close to and at end-point. These advantages allow slow reactions, which are not suitable for conventional titration methods to be used for stoichiometric analysis by HPLC methods.

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