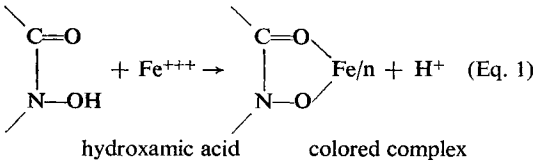
Hydroxamic Acids I: Factors Affecting the Stability of the Hydroxamic Acid-Iron Complex

ROBERT E. NOTARI and JAMES W. MUNSON*

Abstract □ The utility of hydroxamic acid formation and subsequent iron complexation as an analytical tool for succinimide has been investigated. Several factors affecting the stability of the hydroxamic acid-ferric iron complex have been studied and critical factors in the stability of the complex have been established. Conditions for a stable complex are reported. An assay utilizing hydroxamic acid formation from succinimide has been developed and shown to be completely reproducible. Two hydroxamic acids, acetohydroxamic acid and *N*-hydroxysuccinamide, are shown to give stable color complexes under the recommended conditions.

Keyphrases □ Succinimide analysis—hydroxamic acid-iron complex □ Hydroxamic acid-iron complex—stability □ Stability, hydroxamic acid-iron complex—factors affecting □ Colorimetric analysis—spectrophotometer

Hydroxamic acids form highly colored ferric hydroxamates which can be conveniently detected by spectrophotometric methods (Eq. 1).



Hill (1) was the first to employ this property quantitatively for the determination of esters which are capable of forming hydroxamic acids according to the reaction:

$$R—COOR' + NH_2OH \rightarrow R—CO—NHOH + R'OH \quad \text{(Eq. 2)}$$

Subsequently, Bergmann (2) adapted this same principle to a study of the formation of hydroxamic acids from amides (Eq. 3).

$$R—CO—NH_2 + NH_2OH \rightarrow R—CO—NHOH + NH_3 \quad \text{(Eq. 3)}$$

Goddu *et al.* (3) extended these studies to include the analysis of acid chlorides (Eq. 4) and anhydrides (Eq. 5).

$$R—CO—Cl + NH_2OH \rightarrow R—CO—NHOH + HCl \quad \text{(Eq. 4)}$$

$$(R—CO)_2O + NH_2OH \rightarrow R—CO—NHOH + R—COOH \quad \text{(Eq. 5)}$$

The main disadvantage encountered throughout these studies was the instability of the color complex. Goldenberg and Spoerri (4) attempted to resolve this problem by making sequential readings and extrapolating back to zero time for the initial absorbance. This method suffers the disadvantage of requiring several readings and a relatively inaccurate extrapolation technique. Hill (1) reported that 5% water in the final alcoholic solution was optimum for color stability. He also reported that the complex gained intensity for the first 20 min. and that the addition of sodium carbonate would enhance the stability. In a later report, Hill (5) rationalized that the instability of the complex was due to the reduction of the ferric iron by free hydroxylamine. The addition of H_2O_2 to the spectral sample was suggested to oxidize the excess hydroxylamine. No data were offered in support of this suggested technique.

On the other hand, Goddu *et al.* (3) stated that the two important variables in the development of the color are (*a*) the ferric-iron concentration and (*b*) the hydrogen-ion concentration in the final solution. They reported that the color complex was stable for several hours provided that (*a*) the iron concentration was in excess of the ester concentration and (*b*) the acid concentration was adjusted to 0.1 *M*.

Although several suggestions for stabilizing the complex have been presented, no systematic study evaluating all of the possible factors has been reported. A stable complex is a prerequisite for obtaining reproducible results. The present study was undertaken to examine the role of each of the potential factors affecting the stability of the complex. The results of experimentally controlling several factors affecting color stability are presented in this paper. Since there are a number of drugs which can be classified as cyclic imides or esters, succinimide and ethyl acetate were chosen as model compounds in these studies. This report is concerned with the optimum conditions for the formation and stability of the hydroxamic acid-iron complexes. The effect of reaction conditions upon the yields of hydroxamic acid is reported in subsequent papers (6).

EXPERIMENTAL

Hydroxyaminolysis of Succinimide—Succinimide (Aldrich Chemical Co.) was recrystallized from 90% ethanol, m.p. 123°; literature value 126–127° (Chemical Rubber Handbook of Chemistry and Physics).

In order to avoid hydroxylamine degradation, the reaction mixture was freshly prepared by addition of standard $NH_2OH \cdot HCl$ solution to standard NaOH solution to give a solution of 0.395 *N* NH_2OH and 0.36 *N* NaOH. An aliquot of an aqueous stock solution of succinimide was added to this reaction mixture previously equilibrated in a constant temperature bath. The concentration of succinimide in the reaction mixture was 0.0163 *M*. Samples of the reaction were then removed as a function of time and diluted with a given volume of ferric perchlorate reagent solution. Unless otherwise stated, this reagent contained 35.4 g. of ferric perchlorate (ferric perchlorate, nonyellow, G. Frederick Smith Chemical Co.), 100 ml. of 13.3% aqueous perchloric acid and absolute ethanol q.s. 1 l.

Spectrophotometric Determinations—The wavelength of maximum absorbance was established using a spectrophotometer (model 15 Cary Recording). This was found to be 515 mμ in all experimental conditions reported in this study. The single wavelength readings at 515 mμ were done on a spectrophotometer (Beckman D.U.), slit width 0.18 mm. Readings of solutions were corrected by subtracting

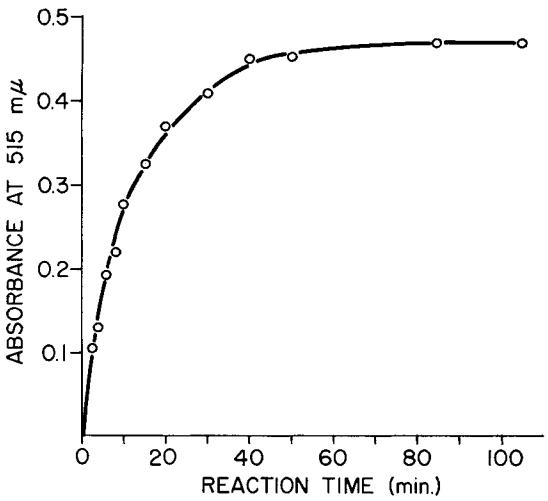


Figure 1—*Typical curve for the hydroxyaminolysis of succinimide (0.0163* M*) in aqueous 0.395* N *NH_2OH and 0.363* N *NaOH at 40°. The reaction was followed by complexing the hydroxamic acid with iron and reading the resulting absorbance at 515 mμ.*

the absorbance values of appropriate blanks. Results for a typical reaction and experimental conditions are shown in Fig. 1.

Absorbance of Acetohydroxamic Acid—Acetohydroxamic acid (*N*-hydroxyacetamide) was synthesized from ethyl acetate by the method of Wise and Brandt (7). The molecular weight was determined as 74.4 (calcd. 75) and the pKa 9.28, lit. 9.40 (7) by potentiometric titration with NaOH using a titrator (Sargent Recording), m.p. 88–90°; 90–91° (8). One-milliliter aliquots of aqueous stock solutions of acetohydroxamic acid were added to 20 ml. of ferric perchlorate reagent solution containing 0.034 *N* NH_2OH. The 515-mμ absorbance of the resulting complex was determined within 24 hr. after mixing and again after storage in the dark for 72 hr.

Absorbance of N-Hydroxysuccinamide—*N*-Hydroxysuccinamide was synthesized from succinimide by the method of Wise and Brandt (7) and treated as described in the previous section, m.p. 118–120° (decomposed); mol. wt. 134, calcd. 132; pKa 8.6 (room temperature).

Effect of H_2O Content on Yield and Stability of the Color Complex—Hydroxyaminolysis of succinimide was continued until a constant absorbance reading at 515 mμ was attained (Fig. 1). One-milliliter aliquots were transferred to six foil-wrapped flasks to give 21 ml. of solution containing 0.0055 *M* $Fe(ClO_4)_3$, 0.112 *N* $HClO_4$, alcohol, and varying amounts of H_2O (1.1–41.1%). After mixing, the absorbance at 515 mμ of each solution was read as a function of time. Each sample was stored in the dark between readings. The final values compared to samples maintained in complete darkness to evaluate the effect of the spectrophotometer light source during readings. See Fig. 2 for experimental conditions.

Effect of Iron Concentration—Hydroxyaminolysis of succinimide was run to a constant absorbance as previously described. One-milliliter aliquots were transferred to six foil-wrapped flasks to give 21 ml. of solution containing 0.575 *N* $HClO_4$, 5.4% water, alcohol, and varying amounts of $Fe(ClO_4)_3$, (3.1×10^{-3} *M* to 62.0×10^{-3} *M*). The absorbance at the wavelength maximum of 515 mμ was read as a function of time for each solution. Samples were kept in the dark between readings. Experimental conditions and yields are given in Fig. 3. The yields here are defined as the highest absorbance values or in this case, the 5-min. readings.

Effect of Acid Concentration—The hydroxyaminolysis of succinimide was effected as previously described. One-milliliter aliquots of the reaction were placed in six foil-wrapped flasks to give 21 ml. of solution containing 3.1×10^{-3} *M* $Fe(ClO_4)_3$, 14.2% water, alcohol, and varying amounts of $HClO_4$. The absorbance at 515 mμ as a function of time was determined as described in previous sections. The $HClO_4$ concentration in each solution was determined by potentiometric titration with a titrator (model D, Sargent Recording), which allowed differentiation between $HClO_4$ and $NH_2OH \cdot HCl$ endpoints.

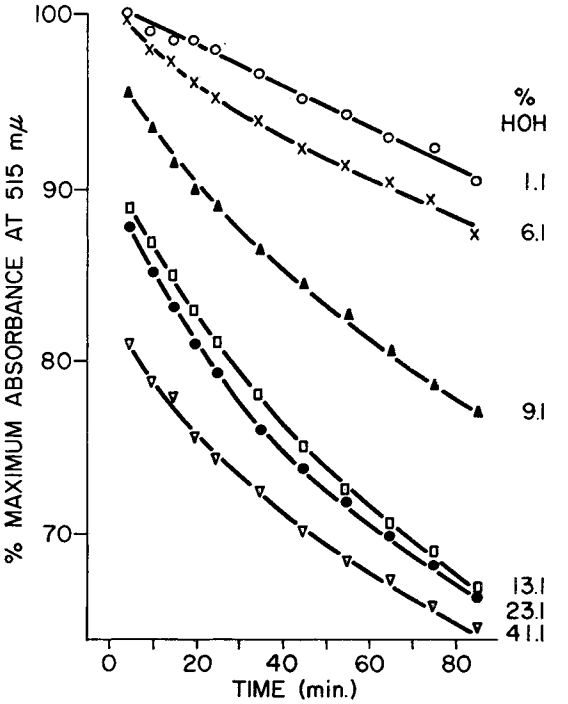


Figure 2—*Decrease in the percent of maximum absorbance at 515 mμ as a function of time in varying amounts of water. Absorbance is due to the ferric complex of the hydroxamic acid from succinimide. The percent of maximum absorbance is based on the highest reading,* $A_\infty = 0.518$.

Effect of Hydroxylamine Concentration—Hydroxyaminolysis of succinimide was run as previously described. One-milliliter aliquots of the reaction were placed in six foil-wrapped flasks to give 21 ml. of solution containing 0.049 *M* $Fe(ClO_4)_3$, 0.141 *N* $HClO_4$, 10.9% water, alcohol, and varying amounts of $NH_2OH \cdot HCl$. The absorbance at 515 mμ was read as a function of time as described in previous sections. The concentrations of $NH_2OH \cdot HCl$ in the final solutions were determined by differential analysis of $NH_2OH \cdot HCl$ and $HClO_4$ on a titrator (model D, Sargent Recording).

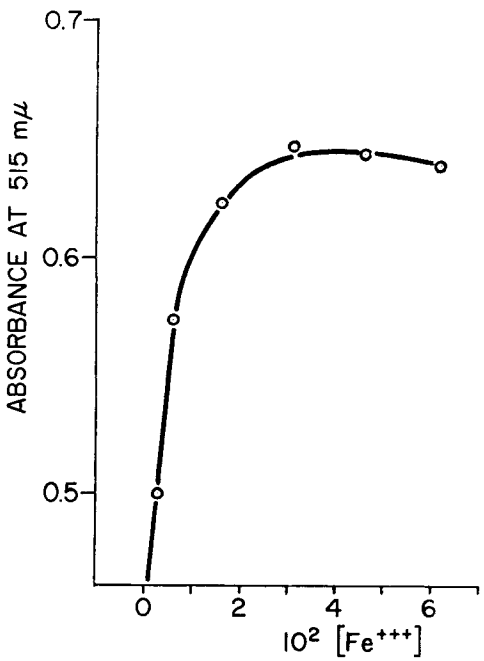


Figure 3—*Increase in yield of the ferric complex of the hydroxamic acid as a function of* $Fe(ClO_4)_3$ *concentration. The yield is defined as the absorbance at 515 mμ 5 min. after mixing the NH_2OH-succinimide reaction aliquot with iron.*

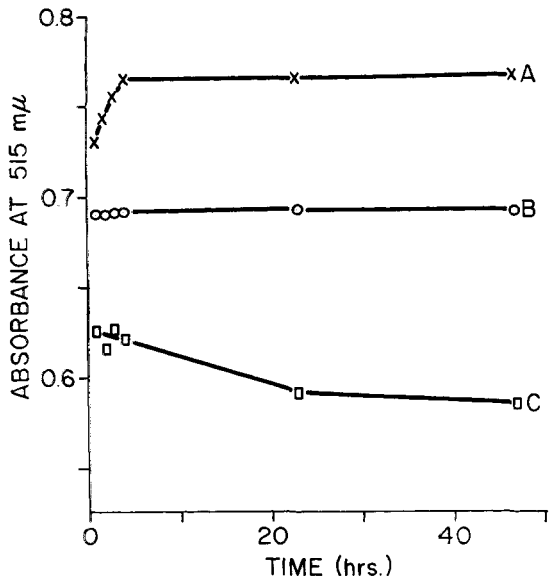


Figure 4—*Stability of the ferric complex of the hydroxamic acid formed from succinimide. B was prepared according to the procedure recommended by the authors. A and C contain 1 and 2% H_2O_2, respectively.*

Comparison with Method of Hill—Hydroxyaminolysis of succinimide was run as previously described. One-milliliter aliquots were transferred to three foil-wrapped flasks to give 21 ml. of solution containing 5.8×10^{-3} *M* $Fe(ClO_4)_3$, 0.31 *N* $HClO_4$, 11.3% water, and alcohol. Sufficient H_2O_2 was added to two of the solutions to make the concentration of H_2O_2, 1% and 2%, respectively. According to Hill (5), 1% H_2O_2 increased the stability of the complex. The absorbance of each solution was read as a function of time after addition of H_2O_2. See Fig. 4 for experimental conditions.

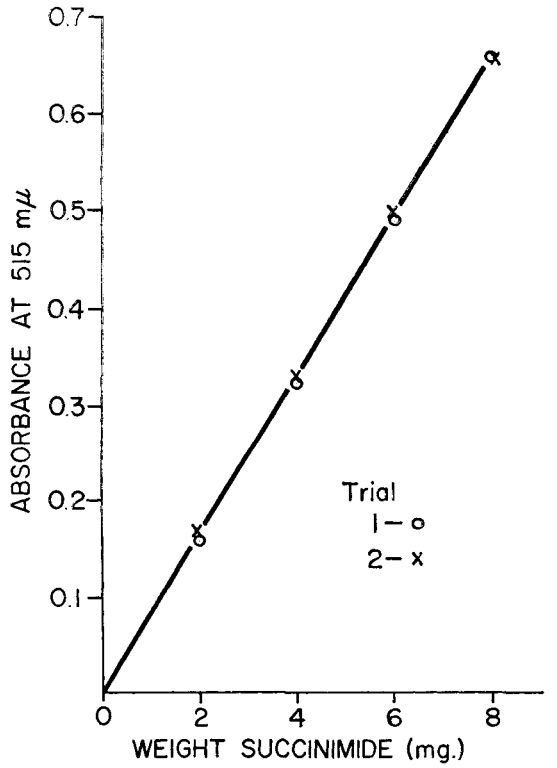


Figure 5—*Assay of succinimide utilizing hydroxyaminolysis. Absorbance at 515 mμ due to the iron complex is plotted* versus *the weight of succinimide.*

Analysis of Succinimide—Hydroxyaminolysis reactions with varying amounts of succinimide were run as previously described. Equal aliquots were removed after 150 min. and placed in foil-wrapped flasks to give solutions containing 0.056 *M* $Fe(ClO_4)_3$, 0.141 *N* $HClO_4$, 7.4% water, and alcohol. This system was shown to be the most stable in previous sections. The absorbance for each solution was read at 515 mμ. The experiment was repeated the following day to determine the degree of reproducibility. A Beer's law plot for both sets of data is shown in Fig. 5.

RESULTS

Spectrophotometric Determination of the Hydroxyaminolysis of Succinimide—Succinimide was shown to form a hydroxamic acid which reacts with ferric iron to form a color complex having a maximum absorbance of 515 mμ. The rate of hydroxamic acid formation was followed by complexation of the acid with ferric iron and spectrophotometric determination of the absorbance at its maximum. Hydroxyaminolysis of succinimide in aqueous 0.395 *N* NH_2OH and 0.363 *M* NaOH solution at 40° was found to approach a constant yield within 90 min. A typical reaction and the resulting data, absorbance at 515 mμ *versus* time, are given in Fig. 1.

Effect of H_2O Content on Yield and Stability of Color Complex—Minimum water concentrations were shown to be best for maximum stability and yield (Fig. 2). It was found that samples maintained in complete darkness were more stable than those exposed to the light source of the spectrophotometer (Beckman DU).

Effect of Iron Concentration—Concentration of $Fe(ClO_4)_3$ was shown to be important in the yield and stability of the complex. It was demonstrated that a minimum concentration of 3×10^{-2} *M* $Fe(ClO_4)_3$ is necessary for complete complex formation in this case (Fig. 3). It was also shown that solutions containing a large excess of $Fe(ClO_4)_3$ as compared to $NH_2OH \cdot HCl$ were more stable than those with little or no excess. The effect of $Fe(ClO_4)_3/NH_2OH$ is discussed in a later section.

Effect of Acid Concentration—It was found that low acid concentrations were most favorable for complex formation (Fig. 6). However, the complex is most unstable at low acid concentrations (Fig. 6). Little difference in stability was found for acid concentrations of 0.11 *N*, 0.17 *N*, and 0.31 *N* (Fig. 7).

Effect of Hydroxylamine Concentration—As previously mentioned, solutions with a large excess of $Fe(ClO_4)_3$ as compared to NH_2OH were found to be more stable than those with little or no

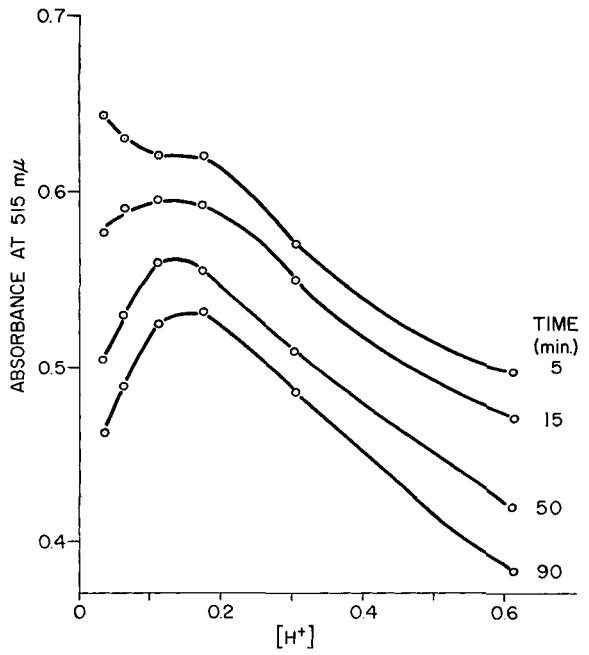


Figure 6—*Effect of $HClO_4$ concentration on yield and stability of the iron complex of the hydroxamic acid formed from succinimide. Absorbance at 515 mμ due to the iron complex is plotted* versus *the acid concentration.*

excess. Data presented in Fig. 8 show that solutions with at least a fourfold excess of iron are most stable.

Comparison with Method of Hill—Solutions containing H_2O_2 were shown to be unstable for 4 hr. before becoming stable for 2 days. The solution developed in this study was immediately stable and remained stable for at least 48 hr. Blanks prepared with H_2O_2 were found to give absorbance values of 0.290 and 0.420 at 515 mμ.

Analysis of Succinimide, Acetohydroxamic Acid, and N-Hydroxysuccinamide—The assay for succinimide based upon conversion to the hydroxamic acid was shown to be completely reproducible on consecutive days without the use of a standard (Fig. 5). Furthermore, there was no problem of color instability in carrying out this assay.

Beer's law plots for the color complexes of acetohydroxamic acid and *N*-hydroxysuccinamide were linear using the 515 mμ absorbance values. The absorptivity of the acetohydroxamic acid complex is 1180 and that of the complex from *N*-hydroxysuccinamide is 1430. Both complexes were stable under the conditions reported here.

DISCUSSION

The hydroxyaminolysis reaction has been successfully applied to the analysis of succinimide. Since the reaction was run in water, the method is of pharmaceutical utility as it can be applied to (*a*) the determination of drug concentration in body fluids; (*b*) release of drug from solid dosage forms; and (*c*) analyses of drugs in pharmaceutical solutions. This assay is of special interest due to its potential application to the assay of therapeutic succinimide derivatives. Previous investigators have carried out this reaction in alcoholic systems. Their assays have thus been limited to analyses of either a powdered drug or its alcoholic solution.

Satisfactory conditions for complex stability have not been previously reported except under nearly anhydrous conditions. Stability of the color complex over a reasonable length of time is imperative in order to obtain reproducible data. Conditions for a stable color complex in the analysis of succinimide, acetohydroxamic acid, and *N*-hydroxysuccinamide have been designed in this study. Several factors influencing stability have been evaluated and the conditions necessary for stability are reported. Several ambiguities in the literature have been clarified.

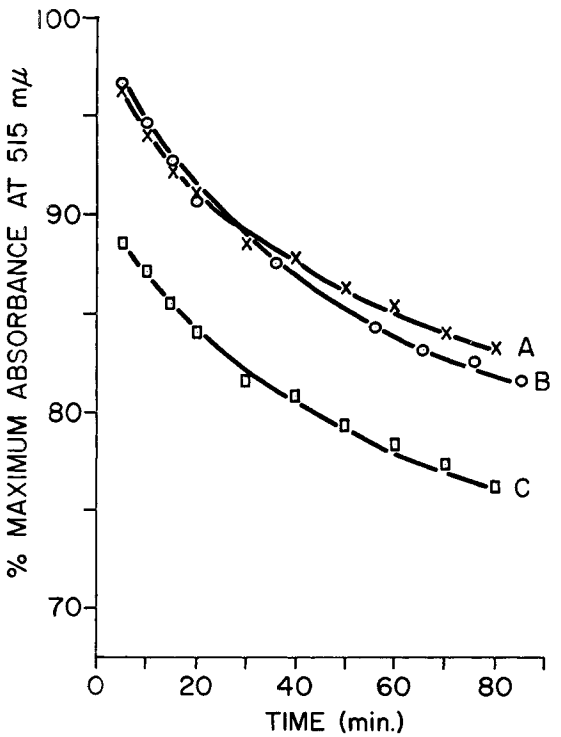


Figure 7—*Comparison of the stability of the ferric complex of the hydroxamic acid from succinimide at various acid concentrations. Absorbance at 515 mμ due to the iron complex is plotted as a function of time. The concentrations of $HClO_4$ are 0.11* N, *0.17* N, *and 0.31* N *for* A, B, *and* C, *respectively.*

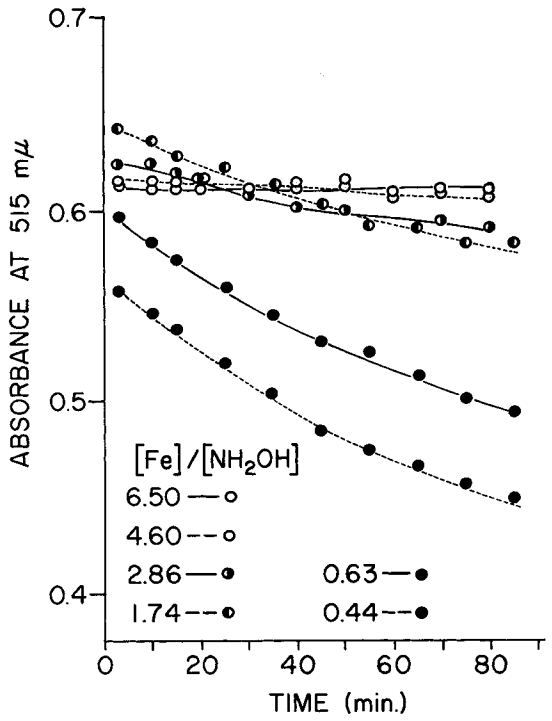


Figure 8—*Decrease in absorbance at 515 mμ of the ferric complex of the hydroxamic acid from succinimide as a function of time for various $Fe(ClO_4)_3/NH_2OH$ ratios.*

The most important factor for complex stability is the ratio of ferric iron to NH_2OH. If the iron is in sufficient excess, the other factors may vary slightly without loss of stability. Hill (1) previously reported that excess NH_2OH reduced the ferric iron and that H_2O_2 could be added to oxidize the excess NH_2OH. It was shown in the present study that addition of H_2O_2 is inferior to the method established herein (Fig. 4). It was also found that the oxidation products of NH_2OH form colored products with the iron. This additional absorbance decreases the sensitivity and accuracy of the assay by resulting in very large blank readings. Hill (1) also suggested that 5% water was optimum for complex stability. It was found in the present study that complex stability is inversely proportional to the water concentration. However, water concentrations as high as 8% (as in the presently suggested succinimide assay) will not affect stability provided there is sufficient iron. Hence, larger aliquots of aqueous hydroxyaminolysis reaction solutions can be used to increase the sensitivity without loss of stability due to increased water content. The acid concentration does not appear to be the prime critical factor as reported by Goddu *et al.* (3). For example, the acid concentration for optimum stability can be increased from 0.14 (reported here) to 0.57 without loss of stability provided sufficient iron is present.

Earlier imide analyses employing the hydroxyaminolysis reaction required the use of a reference standard to compensate for variable yields (4). The method reported here has been demonstrated to be completely reproducible without employing a reference standard (Fig. 5).

Suggested Procedure for Assay of Succinimide—Prepare an aqueous solution of succinimide to contain approximately 8 mg./ml. Prepare a reaction mixture to contain 0.395 *N* NH_2OH and 0.363 *N* NaOH. Transfer a 10-ml. aliquot of the succinimide solution to the reaction mixture and add sufficient water to make 100 ml. Place the reaction mixture in a constant temperature bath at 40°. After 150 min., remove 5 ml. of the reaction mixture and mix with 100 ml. of iron solution to give a final solution containing 0.056 *M* $Fe(ClO_4)_3$, 0.14 *N* $HClO_4$, 7.4% water, and alcohol. A blank is prepared in a similar manner. The absorbance of the final solution is read at 515 mμ. The weight of succinimide can be determined from Fig. 5.

SUMMARY AND CONCLUSIONS

Factors affecting the stability of the hydroxamic acid-iron complex have been studied. It has been shown that the iron concentration is the most important factor for complex stability.

Conditions for a stable complex have been developed and found to be superior to those utilizing H_2O_2.

An assay for succinimide has been designed and found to be reproducible on consecutive days.

The hydroxyaminolysis reaction has been successfully carried out in an aqueous medium. Therefore the assay is potentially useful for pharmaceutical systems.

REFERENCES

(1) U. T. Hill, *Anal. Chem.*, **18,** 317(1946).

(2) F. Bergmann, *ibid.*, **24,** 1367(1952).

(3) R. F. Goddu, N. F. LeBlanc, and C. M. Wright, *ibid.*, **27,** 1251(1958).

(4) V. Goldenberg and P. E. Spoerri, *ibid.*, **30,** 1327(1958).

(5) U. T. Hill, *ibid.*, **19,** 932(1947).

(6) R. E. Notari, *J. Pharm. Sci.*, **58,** 1064.

(7) W. M. Wise and W. W. Brandt, *J. Am. Chem. Soc.*, **77,** 1058(1955).

(8) W. P. Jencks, *ibid.*, **80,** 4585(1958).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 10, 1969, from the *College of Pharmacy, The Ohio State University, Columbus, OH 43210*

Accepted for publication May 16, 1969.

* Undergraduate National Winner, Lunsford Richardson Pharmacy Research Award, 1966.

Hydroxamic Acids II: Kinetics and Mechanisms of Hydroxyaminolysis of Succinimide

ROBERT E. NOTARI

Abstract □ In alkaline pH at 40° succinimide simultaneously hydrolyzes to form succinamic acid and reacts with NH_2OH to form *N*-hydroxysuccinamide which undergoes ring closure to form *N*-hydroxysuccinimide. At pH 9.5 *N*-hydroxysuccinimide is the major product from hydroxyaminolysis of succinimide at 40°. At pH 12, *N*-hydroxysuccinimide undergoes further hydrolysis to form *N*-hydroxysuccinamic acid. Pseudo first-order rate constants for the loss of succinimide as a function of pH and NH_2OH concentration were established by colorimetric determination of the hydroxamic acid-iron complex or UV spectrophotometric determination of the *N*-hydroxysuccinimide. Reaction conditions favoring *N*-hydroxysuccinimide formation were employed to develop a new UV assay for succinimide. This assay is six times more sensitive than the assay based on formation of the hydroxamic acid with subsequent colorimetric determination of its iron complex.

Keyphrases □ Succinimide—hydroxyaminolysis □ Hydroxyaminolysis, succinimide—mechanism □ Kinetics—hydroxamic acid formation, succinimide □ TLC—separation, identity □ Colorimetric analysis—spectrophotometer □ UV spectrophotometry—analysis

Hydroxamic acids form highly colored ferric hydroxamates which can be conveniently detected by spectrophotometric methods. A previous paper has reviewed the potential utility of the hydroxyaminolysis reaction in the quantitative determination of imides, amides, esters, anhydrides, and acid chlorides (1). That report was concerned primarily with the establishment of optimum conditions for the stability of the hydroxamic acid-iron complex. The effect of each variable upon the yield and stability of the complex was determined and conditions were described wherein the complex was stable for more than 48 hr. An assay method was developed for succinimide based on these data. That method has been applied in this kinetic study.

There are three general considerations in the development of optimum conditions for analysis by hydroxamic acid formation. They may be briefly outlined as follows: (*a*) proper reaction conditions for maximum yield (or minimum side reactions such as hydrolysis); (*b*) reaction conditions which are sufficiently fast to achieve the A_∞ reading in a convenient assay time (reaching the plateau of the *A versus t* curve would provide high and constant readings; see Fig. 1); and (*c*) optimum complexing conditions for the most stable complex in highest possible yield. An additional consideration exists in the case of the assay of imides by this method. Since the hydrolysis of imides results in the formation of amides it is necessary to choose conditions which will selectively assay an imide in the presence of its hydrolysis product. The development of an assay which is both selective as well as sensitive requires a knowledge of the kinetics and mechanisms for the hydroxyaminolysis reaction. Since there are a number of drugs with cyclic imide structures, succinimide was chosen as a model compound for kinetic studies. This study serves as an excellent illustration of the validity of the above premise since the appropriate control of reaction conditions has resulted in a new assay with sixfold increase in sensitivity.

EXPERIMENTAL

Kinetics of Hydroxamic Acid Formation from Succinamide—Hydroxyaminolysis of succinimide was carried out under pseudo first-order conditions with pH and temperature maintained constant by use of a pH-stat (Sargent Recording) equipped with a jacketed beaker serviced by a constant temperature bath and circulating pump. The beaker was maintained closed to the atmosphere and samples were withdrawn by syringe through a rubber gasket. The NH_2OH was maintained in sufficient excess of the succinimide concentration to remain essentially constant through-