Influence of Dimethyl Sulfoxide on the Hemolytic Activity of Antimicrobial Preservatives I

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Abstract □ The influence of dimethyl sulfoxide (DMSO) on the hemolytic activity of various chemical types of antimicrobial preservatives has been investigated *in vitro*, using rabbit erythrocytes. Irrespective of the chemical type of preservative, DMSO causes a depression in the hemolytic response of the erythrocytes to the preservative agents. This depression, which is greatest when the DMSO concentration is between 10 and 20%, is dependent upon preservative concentration and time. The degree of the hemolytic depression caused by the presence of DMSO is decreased with an increase in the concentration of the preservative agent or with an increase in the time of exposure. It was concluded that DMSO affects the rate of preservative-induced hemolysis, probably due to a cellular mechanism rather than to an extracellular preservative–DMSO chemical interaction.

Keyphrases □ Antimicrobial preservatives—hemolytic activity □ Dimethyl sulfoxide effect—hemolytic activity, antimicrobial preservatives □ Erythrocyte protection, hemolysis—dimethyl sulfoxide □ UV spectrophotometry—analysis

In a previous communication (1), it was reported that dimethyl sulfoxide (DMSO) in concentrations up to about 20% increasingly depressed the hemolytic response of rabbit erythrocytes *in vitro* to phenol. At greater DMSO concentrations, hemolysis was increased from its depressed level with an increase in the concentration of DMSO, presumably due to the commencement of the hemolytic effects of the DMSO itself.

The present study is an expansion of the previous work, undertaken to ascertain if the curious hemolytic profile obtained for combinations of DMSO and phenol is peculiar to that pair of agents or whether a similar alteration in the hemolytic response of erythrocytes to other chemical types of antimicrobial preservatives occurs in the presence of DMSO. This information would indicate the specificity or lack of specificity of the action of DMSO toward phenol-induced hemolysis and would help set the direction of subsequent studies aimed at the mechanism of the action, since the data obtained would suggest either a cellular-based, general action of DMSO or a specific extracellular drug–drug interaction.

The group of commonly employed antimicrobial preservatives selected for this study includes members representing the various chemical classes of preservatives and for which the individual hemolytic activities could be readily characterized and quantitated (2).

EXPERIMENTAL

Materials—The dimethyl sulfoxide and the antimicrobial preservatives employed in this investigation were reagent grade and were obtained commercially.

Blood Samples—Rabbit blood, obtained by cardiac puncture and defibrinated in the manner previously described (3), was used throughout this investigation. Each blood sample was collected immediately prior to its use and was verified for osmotic normalcy during the course of each experiment (2).

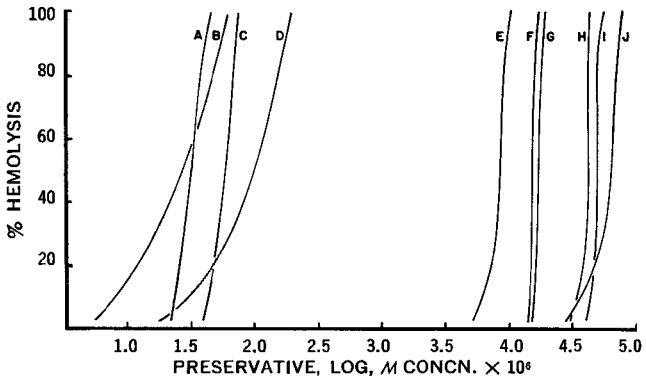

Figure 1—*Hemolytic activities of various preservative agents in 0.6% NaCl. Key:* A, *benzalkonium chloride;* B, *phenylmercuric nitrate;* C, *thimerosal;* D, *chlorhexidine diacetate;* E, p-*chlorophenol;* F, *chlorobutanol;* G, m-*cresol;* H, *phenylethyl alcohol;* I, *phenol; and* J, *benzyl alcohol.*

Quantitative Determination of Percent Hemolysis—The colorimetric method employed for the determination of the degree of hemolysis occurring in each test solution has recently been described (2). In brief, it involved the addition of 0.05 ml. of defibrinated blood to duplicate pairs of colorimeter tubes, each containing 5 ml. of test solution. The test mixtures were incubated in a water bath for 45 min. (except where noted) at 37° after which the unhemolyzed cells were settled by centrifugation and the absorbance readings of the hemolysate determined with a Klett-Summerson photoelectric colorimeter. Each absorbance reading was compared with a total hemolysis reading obtained by laking red cells in distilled water. The degree of hemolysis occurring in each test solution was calculated as a percent of total hemolysis. The data reported represent the average of a minimum of two, but usually four, like experiments. During and after the incubation period, each test mixture was macroscopically observed for color changes, precipitation, and other signs of denaturation.

Experimental Solutions—Aqueous solutions were employed throughout the investigation. In determining the hemolytic activity of each of the test solutions, 0.6% sodium chloride was added as an extracellular agent to protect the erythrocytes from simple osmotic hemolysis (1). Thus, any hemolysis occurring in the test solutions could be attributed to the activity of the antimicrobial preservative and/or DMSO. Appropriate control solutions were processed concurrently with the experimental solutions throughout the investigation (1, 2).

In order to assess the influence of DMSO on the hemolytic activity of the various antimicrobial preservatives, it was initially desirable to use concentration levels of the preservatives in the test solutions which would normally exert an intermediate (approximately 50%) degree of hemolysis in the absence of DMSO. Once the upward or downward influence of DMSO on hemolysis was established, the level of antimicrobial agent in the test solution was adjusted accordingly to permit the collection of the widest range of data.

Kinetic Studies—The general experimental design of the research series is such that the degree of hemolysis is determined after a constant 45-min. incubation period. In the current project the hemolytic activities of certain representative test solutions were also determined at incubation intervals of from 5 to 20 min., during periods up to 120 min.

Spectral Studies—Three preservatives which are diverse in chemical structure, benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol, were subjected to spectral examination

Table I—Molar Concentrations of Preservatives Causing 10%, 50%, and 100% Hemolysis of Rabbit Erythrocytes *In Vitro* in the Presence of 0.6% Sodium Chloride

Preservative	Molar Concn. of Preservative Causing Hemolysis[a] 10%	50%	100%
Phenol	4.3×10^{-2}	4.6×10^{-2}	5.0×10^{-2}
Benzyl alcohol	3.8×10^{-2}	6.3×10^{-2}	7.0×10^{-2}
Phenylethyl alcohol	3.5×10^{-2}	4.6×10^{-2}	4.9×10^{-2}
m-Cresol	1.7×10^{-2}	1.8×10^{-2}	1.9×10^{-2}
Chlorobutanol	1.6×10^{-2}	1.7×10^{-2}	1.8×10^{-2}
p-Chlorophenol	6.2×10^{-3}	8.0×10^{-3}	9.2×10^{-3}
Thimerosal	4.8×10^{-5}	6.1×10^{-5}	6.8×10^{-5}
Chlorhexidine $(Ac)_2$	3.2×10^{-5}	1.1×10^{-4}	1.9×10^{-4}
Benzalkonium Cl	2.2×10^{-5}	3.3×10^{-5}	4.2×10^{-5}
Phenylmercuric NO_3	7.9×10^{-6}	3.1×10^{-5}	4.7×10^{-5}

[a] Data are the average of a minimum of two, but usually four, separate determinations conducted at 37° for 45 min.

alone and in combination with DMSO in order to detect evidence of chemical interaction. Such interaction could account for the decrease in hemolytic activity by the preservatives upon the addition of DMSO.

A Perkin-Elmer 212 and a Beckman DU spectrophotometer were employed in this study, and the peak absorbance for each preservative was determined at 282, 256, and 272 mμ, respectively, for benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol. Dimethyl sulfoxide exhibited no absorbance above 250 mμ and thus in combination with the preservatives was noninterfering with the determinations of preservative concentrations. Reference cells were appropriately prepared for each experiment.

RESULTS AND DISCUSSION

Hemolytic Activity of the Antimicrobial Preservatives—The hemolytic activities of the antimicrobial preservatives employed in this study are depicted in Fig. 1 and summarized in Table I. The data were collected for solutions of preservatives in 0.6% sodium chloride and compare well with the hemolytic data obtained previously for certain of the preservatives in 0.9% sodium chloride (2). By reducing the level of sodium chloride in the present study, dissolution of the various preservative agents was enhanced and yet the tonicity of the solution was maintained at a level sufficient to protect the erythrocytes from simple osmotic hemolysis.

The individual hemolysis curves for the preservatives not only indicate the relative hemolytic potency of these agents, but they also provided the means by which the appropriate (intermediate or near-totally hemolytic) concentrations of the preservatives were selected for study in combination with DMSO to determine the influence of the latter agent. Thus, for each preservative, the strength which caused an intermediate degree of hemolysis (approximately 50%) was combined in initial experiments with various amounts of DMSO to determine whether the latter agent promoted a decrease or an

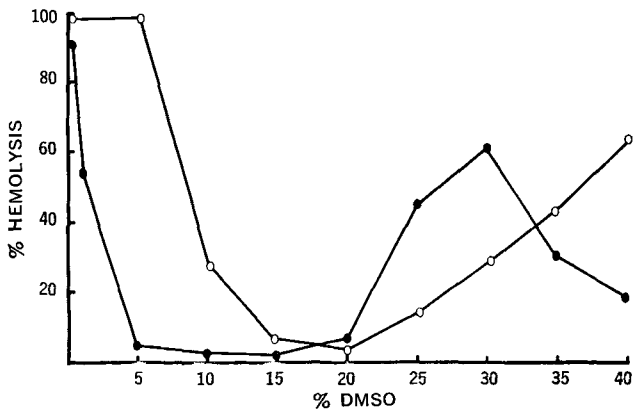

Figure 2—*Influence of DMSO on the hemolytic activity of* m-*cresol and* p-*chlorophenol in the presence of 0.6% NaCl. Key:* ●, *1.8* × 10^{-2} M m-*cresol; and* ○, *8.0* × 10^{-3} M p-*chlorophenol.*

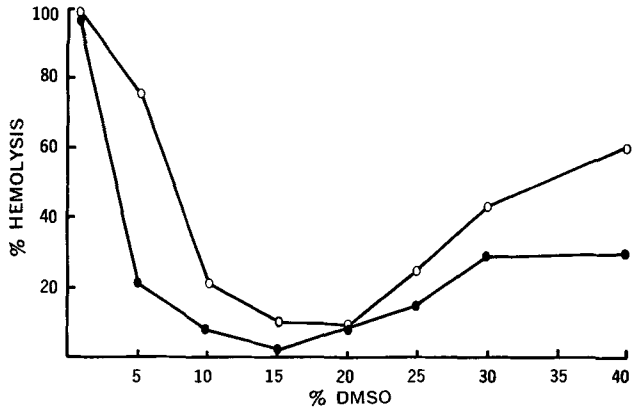

Figure 3—*Influence of DMSO on the hemolytic activity of phenylethyl alcohol and benzyl alcohol in the presence of 0.6% NaCl. Key:* ●, *5.0* × 10^{-2} M *phenylethyl alcohol; and* ○, *7.0* × 10^{-2} M *benzyl alcohol.*

increase in the degree of hemolysis compared to the preservative-saline control solution. It was found, in each instance, that the presence of increasing amounts of DMSO to approximately 10–20% caused a progressive decrease in the degree of hemolysis for each preservative agent within the 45-min. period of each experiment. Thus, the studies were continued using an amount of preservative inducing a greater degree of hemolysis (80–100%) so that the influence of DMSO in reducing hemolysis could be followed over a more extensive course.

Influence of DMSO on Preservative-Induced Hemolysis—Figures 2 through 5 depict the influence of DMSO on the hemolytic activities of various preservative agents. It is interesting to note that in each instance hemolysis is dramatically decreased, and indeed almost prevented, as the DMSO level is increased to between 10 and 20%, irrespective of the chemical type of preservative and its effective hemolytic concentration.

Figures 2 through 5 show that as the level of DMSO is increased beyond that amount which induces optimum retardation of hemolysis, hemolysis is increased and may be quantitated until denaturation of the blood occurs at DMSO concentrations of about 30%. It has been previously shown (1) that DMSO in the presence of 0.6% sodium chloride induces a hemolytic response in erythrocytes at DMSO levels of 25% and causes increased hemolysis with increasing concentrations of DMSO until denaturation occurs at approximately 50% DMSO levels. Thus, it would seem as though the portions of the curves in Figs. 2 through 5 beyond the areas of optimum depression reflect the combined cytotoxic activities of DMSO and the preservatives on the red blood cells, with the high concentrations of DMSO being too destructive to lend further any protective influence to the erythrocytes against preservative-induced hemolysis.

The deleterious effects of high levels of DMSO on blood are observed as amber-brown discoloration of the extracellular liquid,

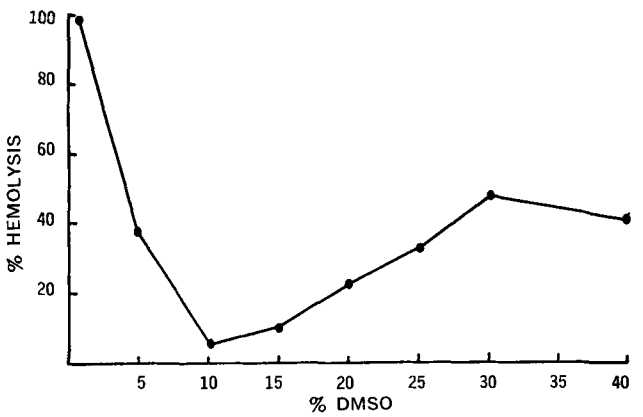

Figure 4—*Influence of DMSO on the hemolytic activity of 1.8* × 10^{-2} M *chlorobutanol in the presence of 0.6% NaCl.*

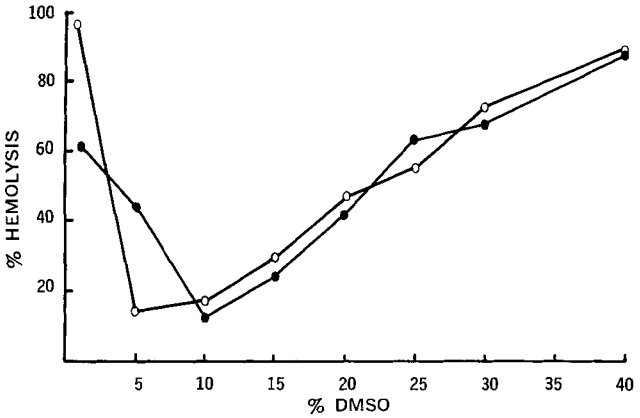

Figure 5—*Influence of DMSO on the hemolytic activity of phenylmercuric nitrate and thimerosal in the presence of 0.6% NaCl. Key:* ●, *3.1* × 10^{-5} M *phenylmercuric nitrate; and* ○, *6.1* × 10^{-5} M *thimerosal.*

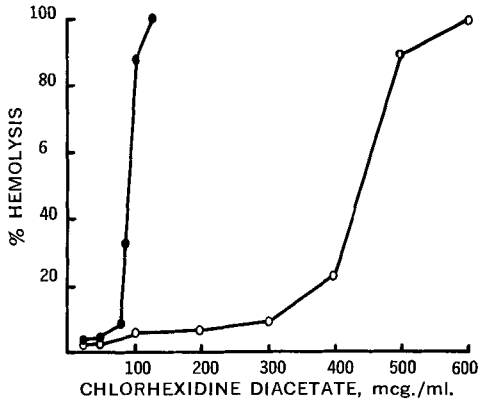

Figure 7—*Influence of DMSO on the hemolytic activity of chlorhexidine diacetate. Key:* ●, *chlorhexidine diacetate in 0.6% NaCl; and* ○, *chlorhexidine diacetate in 0.6% NaCl and 15% DMSO.*

the flocculation of the blood's components, and the precipitation of the hemoglobin from laked erythrocytes. Huggins (4) reported on his observations of the interaction between DMSO and human plasma proteins, noting that true denaturation of plasma protein seems to occur at DMSO concentrations approximating 50%. He stated that precipitation of protein by DMSO occurred with individual plasma fractions—albumin, fibrinogen, gamma globulin—and appears to be a general effect rather than denaturation of a specific plasma component.

At the time it was reported (1) that DMSO interfered with the hemolytic activity of phenol, it was suggested that such an effect might be due to either an extracellular interaction between the two chemical agents, or due to some cellular action of DMSO which retards the ability of phenol to exert its cytotoxic activity.

Since the present work demonstrates that greatly different molar concentrations of the various chemical types of preservative agents are hindered in their effort to destroy the integrity of the erythrocyte by a comparatively large and consistant level of DMSO, it would seem to suggest that the mode of this activity is cellular-based rather than by virtue of extracellular chemical interactions between DMSO and the preservative agents. This hypothesis was supported by the spectral analysis of combinations of DMSO with benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol, which failed to indicate any sign of chemical association between DMSO and the preservatives.

Influence of Preservative Concentration—Figure 6 depicts the pattern generally followed as DMSO is added in increasing amounts to various hemolytic concentrations of a preservative agent. It can be seen that the effectiveness of DMSO in causing a reduction in hemolysis is decreased as the concentration of preservative is increased. This pattern was found for the other preservatives studied, although in instances in which the range of concentration of preservative used was narrow (because of the slight difference in the quantity required to cause trace, intermediate, and total hemolysis) some of the data collected resulted in overlapping and crossing curves when plotted.

Figure 7 shows the influence of 15% DMSO on the hemolytic activity of various concentrations of chlorhexidine diacetate. In the absence of DMSO, chlorhexidine diacetate induces trace hemolysis at 3.2 × 10^{-5} *M* (20 mcg./ml.) concentration and total hemolysis at levels of 1.9 × 10^{-4} *M* (120 mcg./ml.). Dimethyl sulfoxide is effective in keeping the level of hemolysis depressed at chlorhexidine diacetate concentrations below about 6.4 × 10^{-4} *M* (400 mcg./ml.), but is ineffective at higher preservative levels.

The data of Figs. 6 and 7 convincingly show that although DMSO is effective in depressing the level of hemolysis induced by low hemolytic levels of preservative agents, it is ineffective against preservative concentrations of great hemolytic potency. The difference in the concentration of a preservative required to cause total hemolysis compared to that amount needed to cause trace hemolysis in the presence of DMSO does not seem to contribute to the molar strength sufficiently to suggest that the comparatively massive proportion of DMSO present would be any less able to associate in complexation with the greater amount than with the lower preservative level. Thus, the data would seem to suggest that a cellular-based mechanism for the action of DMSO in preventing preservative-induced hemolysis is more likely than an extracellular drug–drug interaction mechanism.

Kinetic Studies—In order to determine if the effect of DMSO in lowering the level of preservative-induced hemolysis was time-related, experiments were conducted using a constant preservative concentration and several levels of DMSO, with hemolytic activity

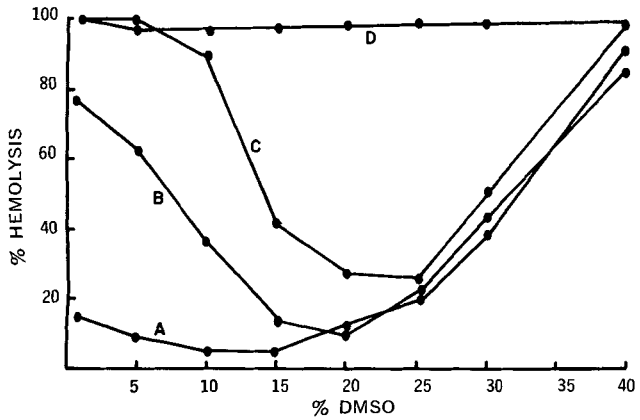

Figure 6—*Influence of DMSO on the hemolytic activity of benzalkonium chloride in the presence of 0.6% NaCl. Key:* A, *2.2* × 10^{-6} M *benzalkonium chloride;* B, *3.3* × 10^{-5} M *benzalkonium chloride;* C, *4.2* × 10^{-5} M *benzalkonium chloride; and* D, *6.5* × 10^{-5} M *benzalkonium chloride.*

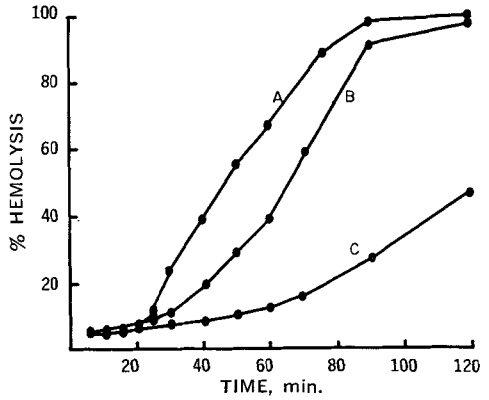

Figure 8—*Influence of time and DMSO on the hemolytic activity of benzalkonium chloride. Key:* A, *3.3* × 10^{-5} M *benzalkonium chloride in 0.6% NaCl;* B, *3.3* × 10^{-5} M *benzalkonium chloride in 0.6% NaCl and 5% DMSO; and* C, *3.3* × 10^{-5} M *benzalkoninm chloride in 0.6% NaCl and 15% DMSO.*

determined at intervals of from 5 to 20 min. during periods up to 120 min.

As can be seen in Fig. 8, the addition of DMSO to solutions of 3.3×10^{-5} *M* benzalkonium chloride in saline slows the rate of hemolysis, with 15% concentrations of DMSO being more effective than 5% concentrations in this effort. Thus, whereas earlier data seemed to indicate that 15% DMSO actually had the ability to reduce or even prevent preservative-induced hemolysis, the present results show that it is a time-related phenomenon. The same pattern of data was obtained in experiments using DMSO–phenol and DMSO–chlorhexidine diacetate systems. This would seem to reinforce the premise that DMSO exerts a cellular effect on the erythrocyte which results in a reduction in the rate of preservative-induced hemolysis.

Work soon to be reported from this laboratory shows that erythrocytes take up DMSO from solution and resist attempts to remove it by washing. Gerhards and Gibian (5) found that in human blood about 30% of injected DMSO is bound to plasma protein and 25% to the formed elements of the blood, the remainder remaining free. Thus, it is not unlikely that the attachment of DMSO to the erythrocyte in some manner affects the vulnerability of the cell toward the preservative agents or alters the permeability barriers of the cell affecting the permeation of the preservative agents into the cell or the exit of hemoglobin from the cell.

Spectral Studies—Preliminary studies of the UV absorption spectra of three preservatives, benzalkonium chloride, chlorhexidine diacetate, and *m*-cresol, alone and in combination with DMSO, were performed to indicate whether or not there is a likelihood of a chemical interaction between DMSO and the preservative agents. The preservatives and DMSO were studied at concentration combinations found to be most inhibitory on preservative-induced hemolysis during the present work. Preliminary data suggest that complexes were not formed. However, more complete studies to rule out complex formation need yet to be made.

In conclusion, the evidence collected more strongly suggests that DMSO interferes with the hemolytic activity of antimicrobial preservatives through a direct action on the erythrocyte or by alteration of its permeability barriers rather than by chemical interaction with the various preservative agents. Further, the degree of this interference is dependent upon the concentrations of both the preservative and DMSO and also upon the length of exposure to these agents.

REFERENCES

(1) H. C. Ansel and W. F. Leake, *J. Pharm. Sci.*, **55**, 685(1966).

(2) H. C. Ansel and D. E. Cadwallader, *ibid.*, **53**, 169(1964).

(3) T. S. Grosicki and W. J. Husa, *J. Amer. Pharm. Ass., Sci. Ed.*, **43**, 632(1954).

(4) C. E. Huggins, *Transfusion*, **3**, 483(1963).

(5) E. Gerhards and H. Gibian, *Ann. N. Y. Acad. Sci.*, **141**, 65 (1967).

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Interaction of Amine Drugs with a Polycarboxylic Acid Ion-Exchange Resin

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Abstract □ The interaction of polacrilin potassium, the salt of a polycarboxylic acid ion-exchange resin, with 11 amine drugs was studied. All drugs showed maximum interaction at pH 4.5–5.5. Tertiary amines exhibited a much greater affinity for the resin than primary, secondary, and quaternary amines. Selectivity coefficients were used to express the degree of interaction, with experiments showing that these values remain constant over wide variations in resin, drug, and alkali metal concentrations. Rate studies demonstrated that both adsorption of drug onto the resin and elution from the resin are very rapid. The rapid elution rates, along with decreasing resin affinity for amine drugs above pH 6.0, indicate that the presence of polacrilin potassium in a dosage form should not affect total drug availability in the gastrointestinal tract.

Keyphrases □ Polacrilin potassium—interaction, amine drugs □ Amine drugs—interaction, polycarboxylic acid ion-exchange resin □ Ion-exchange chromatography—separation □ UV spectrophotometry—analysis

The use of synthetic polycarboxylic ion-exchange resins in pharmacy and medicine has been quite extensive. Their use in congestive heart failure and edema (1), isolation and purification of streptomycin and other drugs (2), and analysis of drugs (3) is well documented. Adsorbates of amine drugs with carboxylic ion-exchange resins for sustained release (4–7) and taste coverage (8) have been prepared, although to a lesser extent than complexes with sulfonic acid resins.

Because of their unusually large swelling capacities, polymethacrylic carboxylic acid ion-exchange resins have found usage in pharmacy as tablet disintegrants. Van Abbe and Rees (9) reported on the effectiveness of polacrilin potassium[1] as a disintegrating agent, while a later patent (10) describes the use of a similar resin in the acidic form for the same purpose. Being cation exchangers, however, these insoluble polymers have the capability of adsorbing amine drugs, thus possibly interfering with drug availability and assay. This potential incompatibility may have limited the use of these resins as tablet disintegrants to some extent.

The present investigation was undertaken to study the interaction between amine drugs and polacrilin potassium. Eleven commonly used drugs were selected to include primary, secondary, tertiary, and quaternary

[1] Amberlite IRP-88, Rohm and Haas Co., Philadelphia, Pa. Previously sold as Amberlite XE-88.