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Complexation in Formulation of Parenteral Solutions: Solubilization of the Cytotoxic Agent Hexamethylmelamine by Complexation with Gentisic Acid Species

BO KREILGÅRD *, T. HIGUCHI ‡, and A. J. REPTA ˣ

Abstract □ The apparent solubility of hexamethylmelamine in aqueous solutions suitable for intravenous use was increased by complexation with gentisic acid. Studies were carried out in the pH 0–8 range. Unprotonated hexamethylmelamine did not form complexes with the gentisate ion, while the hexamethylmelammonium ion appeared to form several different complexes with both the gentisate ion and gentisic acid. Two different solid complexes were isolated and characterized. The solubility increases observed at pH 3.5–5.0 are described by mathematical relationships involving the stability constants of some postulated complex species. From these results, suitable formulations for use as parenteral solutions are proposed. The increase in the apparent aqueous solubility of hexamethylmelamine in such formulations may range from five- to 90-fold, depending upon the pH and total gentisate-ion concentrations.

Keyphrases □ Complexation—hexamethylmelamine–gentisic acid species, pH 0–8, effect on solubility □ Solubilization—hexamethylmelamine by complexation with gentisic acid species, pH 0–8, intravenous formulations □ Cytotoxic agents—hexamethylmelamine, solubilization by complexation with gentisic acid species, intravenous formulations □ Hexamethylmelamine—solubilization by complexation with gentisic acid species, intravenous formulations □ Triazine derivatives—hexamethylmelamine, solubilization by complexation with gentisic acid species, intravenous formulations

Hexamethylmelamine[1], a cytotoxic triazine, was evaluated in extensive phase I and II clinical trials (1). Although the efficacy is not particularly striking in any tumor type, clinical interest in hexamethylmelamine continues in view of its consistent, although low, response rate in several tumors including bronchogenic carcinoma (2, 3).

In phase I and II trials, hexamethylmelamine was given by the oral route (1), and GI side effects such as nausea and vomiting were encountered in such studies (4, 5). To avoid such problems, an intravenous dosage form was developed. Because of the weakly basic nature of the drug and its low aqueous solubility (<0.1 mg/ml), the only parenteral formulation used was an aqueous hydrochloric acid solution (pH ~2) of the drug[2]. The clinical use of this solution has been restricted by the occurrence of thrombophlebitis and local irritation. To determine whether these untoward reactions at the injection site are inherently due to hexamethylmelamine or to the very acidic formulation, a less acidic (pH > 3) formulation of hexamethylmelamine at a concentration of 2–5 mg/ml was desired[2].

Previous experience with the complexation tendencies of various hydroxybenzoic acids with substances exhibiting characteristics akin to those of hexamethylmelamine (6–9) suggested that the apparent solubility of this drug probably could be increased through complexation. Such complexation necessitated the identification and use of a suitable ligand. Preliminary studies with gentisic acid as the ligand demonstrated a significant increase in the apparent solubility of hexamethylmelamine.

This report describes the qualitative and quantitative nature of the interactions of hexamethylmelamine with gentisic acid species, with particular attention being paid to those pH and concentration condi-

[1] 2,4,6-Tris(dimethylamino)-*s*-triazine; NSC 13875.

[2] J. P. Davignon, National Cancer Institute, personal communication.

tions that might normally be encountered in the formulation and use of an intravenous solution.

EXPERIMENTAL

Chemicals—Hexamethylmelamine was used as received[3], mp 171–173° [lit. (10) mp 172–174°]. Titrations with perchloric acid in acetic acid showed the molecular weight to be 210 ± 0.5 (theoretical 210.3). TLC [silica gel F-254 and benzene–acetone–formic acid (8:4:1)] indicated that the material was homogeneous when 100 μg was spotted and visualized by iodine vapor and UV light at 254 nm.

Gentisic acid[4], mp 204–205° [lit. (9, 11) mp 205°] was also found to be homogeneous by TLC using the same conditions. Analysis of gentisic acid, using the USP (12) assay for benzoic acid, indicated a purity >99.5%. All other chemicals were of analytical reagent grade and were used without further purification. The water used was distilled from aqueous acidified potassium permanganate solutions and stored in Pyrex glass containers.

Solubility Studies—The experimental method used was described in detail previously (13). In the studies at pH 3.5–5.0, equal volumes of a stock solution of the ligand were pipetted into the 15-ml screw-capped vials containing 210 mg of hexamethylmelamine (1.00×10^{-3} mole). Then different amounts of a solution of either 0.1 or 1 *N* hydrochloric acid were added, and the solution in each vial was brought to a constant final volume of 10 ml with water.

Ligand stock solutions of the desired concentration and pH were prepared by dissolving the necessary quantity of gentisic acid and sodium bicarbonate in water. In the preparation of these stock solutions, ~0.1% sodium bisulfite was included to minimize oxidation of the ligand. The addition of sodium bisulfite did not appear to influence the complexation reaction quantitatively compared to studies in which the solutions were deoxygenated by flushing with nitrogen gas.

Solubility equilibrium was obtained by fixing the sealed vials to a rotating shaft in a water bath thermostated at 25.0 ± 0.1°. The contents of the vials were allowed to equilibrate until no further changes in the concentration of the species in solution were detectable. After 20 hr or more, samples corresponding to the ascending portion of the phase diagram were at equilibrium; samples corresponding to the remaining portion of the phase diagrams achieved equilibrium after 42 hr or more. To avoid oxidation of gentisate, no samples were allowed to equilibrate for more than 72 hr.

Following equilibration, the contents of the vials were filtered through sintered-glass filters. The pH of each solution was measured[5] at 25°, and the total concentrations of the hexamethylmelamine and gentisic acid species were determined.

Since the system was highly pH dependent and since changes in pH occurred when precipitation of the complex took place, it was necessary to run a series of experiments covering the desired pH range (pH 3.5–5) for each concentration of ligand used. As an example, Table I shows experimental results at a gentisate-ion concentration of 0.44 *M*. In Group A samples, the increasing amounts of hydrochloric acid added caused a decrease in pH and an increase in dissolved hexamethylmelamine. This relationship was typical and to be expected when no precipitation of complex took place. However, when a solid complex precipitated, no significant changes in pH occurred with the incremental increase in the volume of hydrochloric acid solution added (Group B). During this precipitation of the complex, the concentration of hexamethylmelamine appeared to be constant while the concentration of gentisic acid species gradually decreased.

With the Group C samples, further addition of hydrochloric acid caused a decrease in pH as well as in the concentrations of substrate and ligand because the supply of excess solid substrate had been exhausted by the precipitation of the complex (in those samples represented by Group B).

The composition of all solutions was plotted against pH, and the concentrations of substrate and ligand at each particular pH value were read. Table I illustrates that no definite value for the concentration of hexamethylmelamine could be obtained at pH 4.5 in this particular experiment. Similar studies were carried out to obtain phase solubility diagrams in the pH 3.5–5.0 range.

Table I—Experimental Results Obtained in Phase Solubility Studies of Hexamethylmelamine at an Initial Gentisate-Ion Concentration of 0.44 *M* at 25°[a]

Group	1 *N* HCl Added, ml	pH	Hexamethylmelamine in Solution, $M \times 10^3$	Gentisic Acid Species in Solution, *M*
A	0.0	5.01	29.0	0.431
	0.1	4.88	39.1	0.439
	0.2	4.76	46.5	0.436
	0.3	4.69	54.8	0.431
	0.4	4.55	68.0	0.439
B	0.5	4.40	47.1	0.421
	0.6	4.38	49.9	0.413
	0.7	4.35	48.5	0.408
	0.8	4.36	46.0	0.377
	0.9	4.33	46.2	0.381
	1.0	4.37	45.4	0.368
	1.1	4.23	39.1	0.353
	1.2	4.41	48.5	0.342
C	1.4	4.28	32.1	0.304
	1.6	4.05	18.9	0.286
	1.8	3.77	11.0	0.269
	2.0	3.54	8.1	0.263
	2.2	3.34	6.5	0.267

[a] Hexamethylmelamine (210 mg, 1.0×10^{-3} mole) was added to each sample, and the total volume of the aqueous phase was 10 ml. See *Experimental* section for detailed explanation.

In studies done at pH > 7, increments of the stock solution of the ligand were pipetted into the vials, sodium bicarbonate (1 *N*) was added to bring the pH in the final solution to pH > 7, and water was added to a final volume of 10 ml. Otherwise, the procedure was as already described.

For studies done in highly acidic solutions, gentisic acid was used as the substrate due to the high solubility of hexamethylmelamine in strongly acidic media. The gentisic acid (231 mg, 1.5×10^{-3} mole) was combined with aliquots of a solution of hexamethylmelamine in 1 *N* hydrochloric acid, and the final liquid volume was adjusted to 10 ml with 1 *N* hydrochloric acid. The remainder of the procedure was as already described.

The influence of ionic strength on the aqueous solubility of hexamethylmelamine was determined at 25°. Distilled water was adjusted to pH 9–10 with aqueous sodium bicarbonate (1 *N*), and the ionic strength was adjusted to the desired value with sodium chloride. Such solutions were equilibrated with excess solid hexamethylmelamine for 20 hr, after which the concentration of dissolved hexamethylmelamine remained constant. Some results are shown in Table II, and the solubility of hexamethylmelamine at zero ionic strength was estimated by extrapolation to be 4.15×10^{-4} *M* at 25.0°.

Analytical Procedure—Hexamethylmelamine and gentisate in aqueous solutions were analyzed by measuring the absorbances[6], at 228 and 322 nm, of solutions diluted to the required extent with sodium borate (10^{-2} *M*) in methanol–water (1:4). Since hexamethylmelamine did not absorb at 322 nm, the concentration of gentisate could be calculated directly from the absorbance of the solution at 322 nm and an independently determined value of $\epsilon_{322} = 3850\ M^{-1}\ cm^{-1}$ for gentisate in the described solvent system.

The calculation of hexamethylmelamine was not quite as straightforward as anticipated due to a small and varying degree of oxidation of gentisate, which occurred during the preparation of the gentisate stock solutions (involving the neutralization of gentisic acid by addition of alkali). This oxidation caused the absorbance values for gentisate at 228 nm to vary by about 2–5% from the value predicted using the molar absorptivity value of $\epsilon_{228} = 7240\ M^{-1}\ cm^{-1}$. This value was obtained using solutions prepared by dissolving gentisic acid directly into the sodium borate–methanol–water system already described.

[3] National Cancer Institute.
[4] 2,5-Dihydroxybenzoic acid, Aldrich Chemical Co.
[5] Radiometer pH-meter, model 26, equipped with Corning glass and calomel electrodes.
[6] Cary model 14 or 15 spectrophotometers.

Table II—Influence of Ionic Strength (μ) on the Acid Dissociation Constants of Hexamethylmelamine, pKa_M, and Gentisic Acid, pKa_G, and on the Solubility of Hexamethylmelamine Base, $[HM]_0$ ($T = 25°$)

μ	pKa_M	pKa_G	$[HM]_0$, $M \times 10^4$
0	4.92[a]	2.95[a]	4.15[b]
0.05	4.97	2.87	4.09
0.10	5.02	2.82	3.94
0.50	5.15	2.66	2.86
1.00	5.20	2.57	1.89

[a] Obtained by calculations as described in the *Experimental* section. [b] Value at zero ionic strength was obtained by extrapolation.

Such oxidation of gentisate did not affect the absorption at 322 nm and, once prepared, the absorbance spectrum of any particular stock solution did not change over a period of more than a week. Therefore, it was possible to obviate the problem by measuring the absorbance values at 322 (A'_{322}) and 228 (A'_{228}) nm for the particular gentisate stock solution used in the samples to be analyzed. A value R ($= A'_{228}/A'_{322}$) was then calculated and used in estimating the concentration of hexamethylmelamine in the samples according to the expression:

$$[\text{hexamethylmelamine}] = [A_{228} - (RA_{322})]/\epsilon_{228,HM} \quad \text{(Eq. 1)}$$

where A_{228} and A_{322} are the values of the absorbances in the sample being analyzed, and $\epsilon_{228,HM}$ (=50,700 M^{-1} cm^{-1}) is the molar absorptivity of hexamethylmelamine at 228 nm.

All reported absorptivity values were obtained from appropriate Beer–Lambert-type plots.

Ionization Constants—The acid dissociation constant, pKa_M, for the hexamethylmelammonium ion was determined at various ionic strengths by a solubility technique which involved measuring the apparent solubility of hexamethylmelamine at several pH values (14) (Table II). When utilizing the Debye–Hückel equation (15), correction of the results obtained at $\mu \leq 0.1$ yielded the value $pKa_M = 4.92$ at zero ionic strength. The corresponding constant for gentisic acid, pKa_G, was determined by potentiometric titrations (14) at 25.0° (Table II). Again using the Debye–Hückel equation (15), the value of pKa_G at zero ionic strength was determined to be 2.95, which agrees with the literature value of 2.97 (16).

Isolation and Characterization of Complexes—Complexes were isolated during the solubility studies at pH 3.5 and 4.0. Immediately after separation by filtration, the crystalline complexes were washed twice with 5 ml of cold water and then dried *in vacuo* at room temperature. Samples of the dry solid were dissolved in methanol, and the ratio of total hexamethylmelamine and gentisate species was determined by UV spectrophotometry as already described. The alkaline equivalent weights of these solids was obtained by nonaqueous potentiometric titration with perchloric acid (0.01 N) in acetic acid. The acidic equivalent weights were determined by titration with aqueous sodium hydroxide (0.5 N) (Table III).

At pH < 0.5, the solid complex was isolated during the solubility studies from solutions with an initial concentration of 0.08 M hexamethylmelammonium chloride. Since the complex dissolved very rapidly when washed with water, it could not be washed free of hydrochloric acid. UV spectrophotometric determination showed it to contain 0.68 mole of hexamethylmelammonium ion/mole of gentisic acid, which closely agrees with a stoichiometry of 3:2.

RESULTS AND DISCUSSION

Phase Solubility Studies—The complexation of hexamethylmelamine and gentisic acid was studied at 25° by the solubility method in the pH 0–8 range. The apparent solubility of hexamethylmelamine at a pH between 7 and 8 did not change measurably with increasing amounts of gentisate ion (0–0.5 M). This finding indicated that the hexamethylmelamine base and gentisate ion did not appreciably associate.

In hydrochloric acid (1 N) solutions at 25°, gentisic acid and hexamethylmelammonium ion did interact appreciably. As shown in Fig. 1, the solubility of the substrate (gentisic acid) was increased by addition of the ligand at concentrations up to about 0.01 M. The slope of the ascending part of the diagram is approximately 2, indicating formation of a soluble 2:1 (or higher order) gentisic acid–hexamethylmelammonium-ion complex (13). At concentrations of ligand >0.01 M, a solid complex precipitated. Based on the length of the plateau region (13), the stoichiometry of the solid gentisic acid–hexamethylmelammonium-ion complex was calculated to be 3:2. This stoichiometric value was supported both by the data obtained from analysis of the isolated solid complex and by calculations utilizing the data for the descending part of the phase solubility diagram (13), which corresponds to the precipitation of the complex.

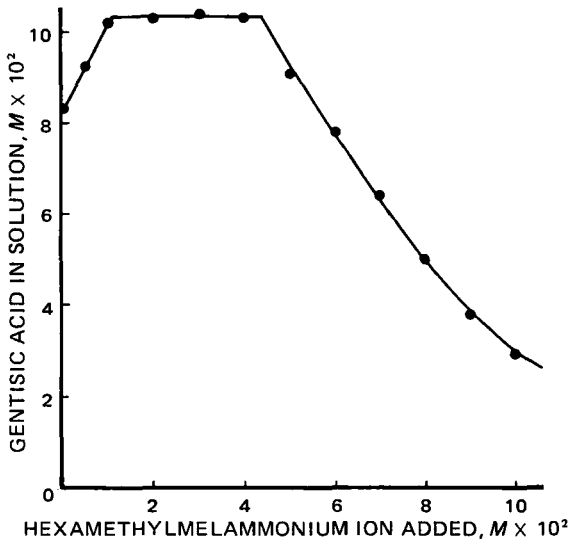

Figure 1—*Solubility of gentisic acid in 1 N hydrochloric acid at 25° as a function of hexamethylmelammonium ion added.*

Similar solubility studies were carried out at 25° and pH values between 3.5 and 5.0, where both the charged and uncharged species of the compounds are present. In this range, control of pH was difficult since the substrate and ligand had electrolytic character and the association as soluble or insoluble complexes caused changes in pH. Therefore, experiments were done covering the whole pH range at each ligand concentration. In such studies, hexamethylmelamine was used as the substrate.

The results at pH 3.5 and 4.0 are shown in Fig. 2, and those obtained at 4.5 and 5.0 are presented in Fig. 3. In all four phase diagrams, the solubility of total hexamethylmelamine increased as increasing amounts of ligand were added. At pH 3.5 and 4.0, a solid complex was precipitated, as indicated by the plateau regions in Fig. 2; at pH 5.0, no complex precipitated. At pH 4.5 and a ligand concentration >0.425 M, a precipitate was also observed, but the concentration data obtained from the solution phase in such samples appeared to be quite scattered and were not reproducible. The cause and nature of these results were not examined further.

Inspection of the data in Fig. 2 demonstrated some unusually sharp changes in concentration of substrate as a function of added ligand at both pH 3.5 and 4.0. To determine whether such abrupt changes were due to supersaturation, 10-ml samples (pH 3.5) of 210 mg of hexamethylmelamine (10^{-3} M) and gentisate concentrations of 0.04–0.07 M were allowed to equilibrate for 1 day. Then, as an aid to nucleation, glass wool and some crystals of the complex isolated at pH 3.5 were introduced and the samples were again kept at 25°.

Supersaturation appeared to be responsible for high substrate concentrations in solution only at ligand concentrations above ~0.065 M. At ligand concentrations below ~0.065 M, the concentration of hexamethylmelamine species did not change as a function of time from ~1 to 7 days, although the substrate concentrations were significantly higher than those at the plateau region. While these studies at pH 3.5 did not elucidate the causes for the sharp changes in substrate concentrations observed at pH 3.5 and 4.0, such variations may be due to small changes in the pH values of the solutions resulting from the precipitation of the complex (as exemplified in Table I and discussed in *Experimental*).

Analytical data for the solid precipitates isolated at pH 3.5 and 4.0 (Table III) strongly indicated that a 1:1:1 gentisic acid–hexamethylmelammonium-ion–gentisate-ion complex was the solid phase in both cases. A plot of the concentration of the ligand added *versus* the ligand in solution (17) verified the 1:2 ratio between gentisic acid species and hexamethylmelamine species.

An interesting observation was made during isolation of this 1:1:1 complex. When the complex was isolated from a sample containing 0.1% sodium bisulfite, the solid complex had a pronounced yellow color. However, if retardation of oxidation was accomplished by expelling the molecular oxygen by bubbling nitrogen gas through the solution, the solid complex obtained was almost white. In both cases, the solutions themselves were colorless. Analysis of the complexes obtained under the different conditions did not show any significant differences in the chemical composition, and the reason for difference in color of the solids has not been explained (Table III).

Estimation of Stability Constants—To use the data obtained for descriptive and predictive purposes, attempts were made to define the formation of complexes and the corresponding increase in the apparent solubility of hexamethylmelamine at different pH values in terms of stability constants.

Since no complexation appeared to occur between free hexamethylmelamine and gentisate ion, as indicated by the studies at pH ≥ 7, only complexes between the hexamethylmelammonium ion and gentisic acid and/or gentisate were considered. At pH 5.0, only a very small fraction of the total gentisate added was present as gentisic acid. The distinct curvature of the solubility diagram indicates formation of complexes of higher order with respect to the gentisate ion. The assumption was made that the increase in apparent solubility of hexamethylmelamine was due mainly to the formation of two complexes, ($HMH^+ \cdot G^-$) and [$HMH^+ \cdot (G^-)_2$][7]. The stability constants for each complex, expressed in terms of the concentration of the various species, are given by Eqs. 2 and 3:

$$K_1 = \frac{[HMH^+ \cdot G^-]}{[HMH^+][G^-]} \qquad \text{(Eq. 2)}$$

$$K_2 = \frac{[HMH^+ \cdot (G^-)_2]}{[HMH^+ \cdot G^-][G^-]} \qquad \text{(Eq. 3)}$$

Calculations (8) made on the basis of these equations did not adequately describe the results obtained in the pH 3.5–5.0 range. Although not isolated at pH 4.5 and 5.0, the solid complex found at pH 3.5 and 4.0 having the composition ($GH \cdot HMH^+ \cdot G^-$) was expected also to be involved in the equilibrium at pH 4.5 and 5.0. Therefore, a stability constant, K_3, was included and may be defined as:

$$K_3 = \frac{[GH \cdot HMH^+ \cdot G^-]}{[HMH^+ \cdot G^-][GH]} \qquad \text{(Eq. 4)}$$

However, calculations based on the involvement of the three complexes defined in terms of K_1, K_2, and K_3 still did not correlate well with the experimental data. Therefore, it was necessary to consider other equilibria to fit the data.

If it is assumed that in highly acidic solvents, the increased solubility seen at low concentrations of ligands (Fig. 1) was primarily due to formation of a 2:1 complex, [$(GH)_2 \cdot HMH^+$], and if it is also assumed that the 2:1 complex forms by association of a 1:1 complex and excess ligand, it is necessary to consider two additional complexes, ($HMH^+ \cdot GH$) and [$HMH^+ \cdot (GH)_2$]. The stability constants for these species may be defined as:

$$K_4 = \frac{[HMH^+ \cdot GH]}{[HMH^+][GH]} \qquad \text{(Eq. 5)}$$

and:

$$K_5 = \frac{[HMH^+ \cdot (GH)_2]}{[HMH^+ \cdot GH][GH]} \qquad \text{(Eq. 6)}$$

The mass balance equations are:

$$[HM]_t = [HM] + [HMH^+] + [HMH^+ \cdot G^-] + [HMH^+ \cdot (G^-)_2] + [GH \cdot HMH^+ \cdot G^-] + [HMH^+ \cdot GH] + [HMH^+ \cdot (GH)_2] \qquad \text{(Eq. 7)}$$

[7] HM = hexamethylmelamine, HMH^+ = hexamethylmelammonium ion, GH = gentisic acid, and G^- = gentisate ion.

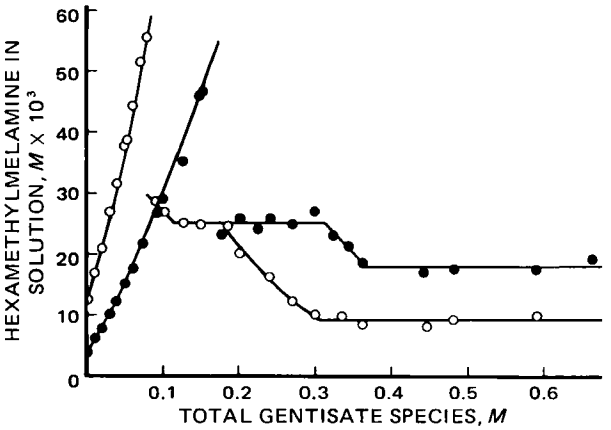

Figure 2—*Solubility of hexamethylmelamine in aqueous solution at pH 3.5 (○) and pH 4.0 (●) at 25° as a function of gentisic acid species added. The ascending part of the curves are calculated from Eq. 7.*

and:

$$[GH]_t = [GH] + [G^-] + [HMH^+ \cdot G^-] + 2[HMH^+ \cdot (G^-)_2] + 2[GH \cdot HMH^+ \cdot G^-] + [HMH^+ \cdot GH] + 2[HMH^+ \cdot (GH)_2] \qquad \text{(Eq. 8)}$$

where $[HM]_t$ is the total concentration of hexamethylmelamine species, and $[GH]_t$ is the total concentration of gentisate species added.

The concentration of uncomplexed hexamethylmelamine species is calculated (18) from:

$$[HM] + [HMH^+] = [HM]_0\left(1 + \frac{(H^+)}{K_{aM}}\right) \qquad \text{(Eq. 9)}$$

where $[HM]_0$ is the solubility of hexamethylmelamine at each particular ionic strength (Table II).

In all calculations, corrections were made for the effect of ionic strength on the solubility of hexamethylmelamine and the values of pK_{aM} and pK_{aG}.

The reportedly (11) weak binding between gentisic acid and gentisate ion was not taken into account, since calculations showed it to have no significant influence (≤5%) on the stability constants reported here.

Estimates of the values of K_1 and K_2 were obtained based on the reasonable assumption that only negligible amounts of gentisic

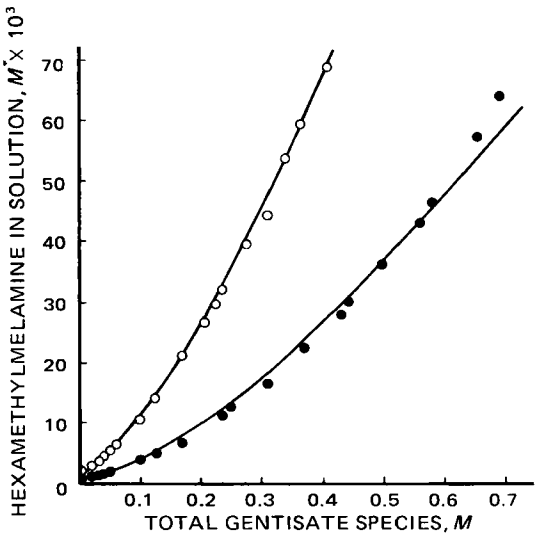

Figure 3—*Solubility of hexamethylmelamine in aqueous solution at pH 4.5 (○) and pH 5.0 (●) at 25° as a function of gentisic acid species added. The solid curves are calculated from Eq. 7.*

Table III—Results of Analysis of Some Solid Hexamethylmelamine–Gentisic Acid Complexes

Complex Isolated at	Melting Point	Ratio HM/GH[a]	Acidic Equivalent Weight[b]	Alkaline Equivalent Weight[c]	Elemental Analysis, %		
					C	H	N
pH 3.5[d]	134–135°	1:2.02	258	515	53.21	5.87	16.36
pH 4.0[d]	134–135°	1:2.09	259	512	53.43	5.77	16.54
pH 4.0[e]	130–134°	1:2.09	257	512	53.63	5.88	16.53
Theoretical[f]	—	1:2.00	259	518	53.58	5.83	16.21

[a] The ratio of hexamethylmelamine (HM) species to total gentisic acid (GH) species was determined by UV spectrophotometry. [b] Determined by titration with 0.5 *N* sodium hydroxide. [c] Determined by titration with 0.01 *N* perchloric acid in acetic acid. [d] Solution contained 0.1% sodium bisulfite. [e] Solution was flushed with nitrogen. [f] Calculated for a 1:1:1 gentisic acid–hexamethylmelammonium ion–gentisate-ion complex.

acid were present at pH 5.0. The values obtained for K_1 and K_2, as calculated by a previously described method (8), were 48 and 7.3 M^{-1}, respectively.

To calculate $[HM]_t$ from Eq. 6, the free ligand concentration, $[G^-]$, must be known. By combining Eqs. 2–6 and 8 and solving for $[G^-]$, Eq. 10 was obtained:

$$[G^-] = [-\alpha + (\alpha^2 + 8[GH]_t\beta)^{1/2}]/4\beta \quad \text{(Eq. 10)}$$

where:

$$\alpha = 1 + K_1[HMH^+] + [H^+](1 + K_4[HMH^+])/K_{aG} \quad \text{(Eq. 11)}$$

and:

$$\beta = K_1K_2[HMH^+] + [H^+][HMH^+](K_1K_3 + K_4K_5[H^+]/K_{aG})/K_{aG} \quad \text{(Eq. 12)}$$

By using the values of K_1 and K_2 already mentioned and varying the values of K_3, K_4, and K_5, values of $[HM]_t$ that best fit the experimental results were calculated[8]. Values of $K_3 = 150\ M^{-1}$, $K_4 = 200\ M^{-1}$, and $K_5 = 50\ M^{-1}$ gave a good correlation between the experimental points and the calculated curve (solid line) shown in Figs. 2 and 3. Only the curve related to pH 5.0 shows a significant deviation between the calculated curve and the experimental results, particularly at ligand concentrations above 0.6 *M*. The deviation probably was due to formation of other soluble complexes of higher order in gentisic acid or gentisate ion than those considered.

The relatively large number of stability constants considered here should be regarded only as empirical parameters that adequately describe the observed increase in the apparent solubility of hexamethylmelamine in the presence of gentisic acid in the pH 3.5–5.0 range. They should not be interpreted as evidence that all such postulated associations actually occur or that other complexes do not exist.

The fact that the hexamethylmelammonium ion associated strongly with the gentisate ion while the corresponding base apparently does not appear to associate is attributed to the difference in resonance structure occurring when the triazine ring is protonated. In the case of gentisic acid and gentisate ion, however, the charge on the gentisate ion is not conjugated with the aromatic ring and no great change in the charge distribution of the aromatic binding site would be expected. Therefore, it seems reasonable that both gentisic acid and gentisate ion would form complexes with hexamethylmelammonium ion as observed.

The importance of a charge transfer reaction between hexamethylmelamine cation and gentisate anion is probably negligible, since such interactions may occur only to a very small extent between organic species in aqueous solution (19).

If plane-to-plane orientation occurs, as has been suggested in the case of 1:1 complexes (19, 20), it seems reasonable to assume that the solid complex isolated at pH 3.5 and 4.0 has a gentisate-ion molecule and a gentisic acid molecule on either side of the hexamethylmelammonium ion. Since this complex is electrically neutral, it is not unreasonable that the solubility of this particular species would be relatively low and precipitation could be expected.

Formulation—From the data discussed, it is apparent that the interactions between the various gentisic acid and hexamethylmelamine species are somewhat complex and highly dependent on pH and system composition. However, the use of gentisic acid species as a ligand clearly resulted in increases in the apparent solubility of hexamethylmelamine. Such increases range from approximately fivefold at pH 3.5 to at least approximately 90-fold at pH 5.0. Thus, physically stable solutions with concentrations of ≥5 mg of hexamethylmelamine/ml can be prepared at pH ≥ 3.5.

Solutions containing 5 mg/ml of drug (pH 3.5; gentisic acid, 6 mg/ml) and 10 mg/ml of drug (pH 4.5; gentisic acid, 54 mg/ml) were prepared and lyophilized[9]. Such preparations were readily reconstituted and appeared to be suitable for use in the clinical testing of hexamethylmelamine. Lyophilization of the solutions was considered desirable to avoid the possible oxidation of gentisic acid species in aqueous solution. In the pH 3.5 preparation, about one-half of the amount of hexamethylmelamine was present as a complex; in the pH 4.5 preparation, more than 95% of the drug was complexed.

The systems described in Figs. 2 and 3 allow for appreciable choice in the composition of a formulation. However, if one chooses a high pH value, the formulation will necessarily require greater concentrations of gentisate species to obtain a given dose of hexamethylmelamine.

Upon administration of such described parenteral solutions, a very rapid release of hexamethylmelamine will occur due to dilution of the sample as well as change in pH to a value of ~7.4 at which no complexation occurs.

SUMMARY AND CONCLUSIONS

The present study demonstrated the practical use of complexation in the formulation of the cytotoxic agent, hexamethylmelamine, as a product potentially suitable for intravenous use. Where the desired objectives of the formulation can be realized through the use of complexation, it is a very useful and attractive method, particularly where a reversible alteration of the physical and/or chemical properties of a drug substance is desired to improve drug delivery. Since complexation is a reversible phenomenon and the equilibrium is shifted by concentration changes, it is obvious that upon dissolution of a solid complex or dilution of a solution containing complexed species, the complexed molecules will dissociate and provide rapid release of the free drug. In view of these rather unusual drug delivery advantages, complexation may well be included when defining prodrug approaches.

However, the application of complexation to the solubilization of drugs is somewhat limited, since complexation between organic molecules generally results in apparent solubility increases of only an order of magnitude or less and this increase is often not sufficient. Another important problem is that it is often difficult to identify suitable ligands which will associate appreciably with the given substrate.

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[8] Hewlett-Packard 9810A calculator.

[9] Virtis model 10-800 lyophilizer attached to a Cenco Hyrac 14 vacuum pump. Precautions had to be taken to avoid sublimation of hexamethylmelamine during the secondary drying.

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ˣ To whom inquiries should be directed.

Amino Acid Analogs III: New Syntheses of Monomethyl- and Monophenylglutamic Acids

HERMAN GERSHON ˣ, RAULO PARMEGIANI, VINCENT R. GIANNASIO, and IRA S. KRULL

Abstract □ Glutamic acid analogs containing 3- and 4-methyl and 2-, 3-, and 4-phenyl substituents were prepared. The 3- and 4-methyl- and 3- and 4-phenylglutamic acids did not inhibit *Plasmodium berghei* and were nontoxic to the host (mice) at 640 mg/kg. The five analogs in addition to 2-methylglutamic acid were inactive against *Lactobacillus casei* at 1000 μg/ml in a defined medium; against *Escherichia coli*, only 2-methylglutamic acid caused 27% inhibition at 10,000 μg/ml. All six analogs failed to inhibit *Aspergillus niger, Aspergillus oryzae, Trichoderma viride*, and *Myrothecium verrucaria* in a defined medium below 10,000 μg/ml.

Keyphrases □ Glutamic acid analogs—syntheses, inhibition of plasmodia, bacteria, and fungi, toxicity in mice, potential antimalarial activity □ Antimalarial agents, potential—syntheses of methyl- and phenylglutamic acids

As a result of an interest in synthesizing potential antimalarial agents, it became necessary to prepare quantities of 3- and 4-methyl- and 3- and 4-phenylglutamic acids. The four glutamic acid analogs were reported previously (1–4).

DISCUSSION

3-Methylglutamic acid (IIIa) was obtained by reacting crotonaldehyde with ethyl acetamidomalonate by means of a Michael condensation, followed by oxidation of the aldehyde function (I) with permanganate and hydrolysis of the oxidation product (II) with acid. The preparation of 4-methylglutamic acid (IIIb) was based on condensing methacrolein with ethyl acetamidomalonate, oxidizing, and hydrolyzing the products, as for 3-methylglutamic acid. 3-Phenylglutamic acid (IIIc) was obtained in a similar manner, by a Michael condensation of cinnamaldehyde with ethyl acetamidomalonate followed by oxidation and hydrolysis.

For the preparation of 4-phenylglutamic acid (V), tropic acid was made into the acid chloride by means of thionyl chloride which, in turn, was esterified with ethanol. Ethyl tropate was subsequently converted to ethyl 3-bromo-2-phenylpropionate with phosphorus tribromide, and the bromo compound was condensed with ethyl acetamidomalonate. Upon hydrolysis of the condensation product with acid, 4-phenylglutamic acid was obtained. Scheme I indicates the preparation of IIIa–IIIc, and Scheme II indicates the preparation of V.

Since it was desired to carry out microbiological and other testing on the three monomethyl- and three monophenylglutamic acids, 2-methyl- and 2-phenylglutamic acids were also required. The synthesis of 2-phenylglutamic acid was reported previously, but it cyclized spontaneously to 2-phenylpyroglutamic acid (5). For the present study, benzoylpropionic acid was treated with ammonium carbonate and potassium cyanide, according to the method of Henze and Speer (6), to form the hydantoin (VI). Compound VI was hydrolyzed to 2-phenylglutamic acid (VII) by successive treatments with sodium hydroxide and hydrochloric acid (Scheme III). This preparation of 2-phenylglutamic acid did not cyclize spontaneously at room temperature. 2-Methylglutamic acid was commercially available. IR spectra of the six glutamic acid analogs appear in Figs. 1 and 2.

3-Methyl-, 4-methyl-, 3-phenyl-, and 4-phenylglutamic acids were screened against *Plasmodium berghei* in mice[1]. These com-

[1] Testing done at the Rane Laboratory, University of Miami, Miami, Fla.