Effects of Divalent Amino Acids on Iron Absorption

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Abstract □ Solutions of each of 10 amino acids or ascorbic acid were mixed with iron and orally administered to rats. Iron was absorbed to a statistically significantly greater extent ($p < 0.05$) when mixed with asparagine, glycine, serine, or ascorbic acid as compared with a control solution of iron. The largest effects were for asparagine and glycine, which also increased iron absorption to a significantly greater extent ($p < 0.001$) than did serine or ascorbic acid. No statistically significant increase in iron absorption occurred when any of the other amino acids was mixed with iron. The extent of iron absorption from each test solution, as measured by area under the concentration of iron-59 in the blood-time curve ($r^2 = 0.0002$), and the initial rate of iron absorption for each test solution ($r^2 = 0.01$) showed no correlation with the stability constant of the amino acid-iron complex.

Keyphrases □ Absorption—iron, effects of divalent amino acids □ Iron—absorption, effects of divalent amino acids □ Amino acids—divalent, effects on iron absorption □ Complexes—iron-divalent amino acids, effect on iron absorption

Some products are available on the market in which amino acids or protein are combined with iron[1] in the hope of promoting iron absorption. Amino acids chelate with iron (1–3), and under certain circumstances, the resultant chelate enhances iron absorption from the GI tract (4–9). Low molecular weight chelates have been postulated to be essential for iron absorption by keeping iron in solution and preventing hydrolysis or precipitation of iron (10–12).

It has been proposed (8, 9) that amino acids which form the most stable complexes with iron are the most effective in increasing iron absorption from the GI tract, and failure of certain amino acids to promote iron absorption *in vivo* is due to their inability to chelate iron strongly enough to compete with water. Thus, they do not prevent hydrolysis and subsequent precipitation of iron. These data also suggest that amino acids with high stability constants should enhance iron absorption better than amino acids with lower stability constants.

These conclusions were reached based on data obtained for trivalent amino acids only (8). In some previous experiments conducted to study the effects of amino acids on iron absorption (4–9), phosphate buffers were used which have been reported to decrease iron absorption both *in vivo* and *in vitro* (13, 14). The conclusion of these reports that amino acid–iron complexes administered in phosphate buffer increase iron absorption may not be quantitatively correct since iron is known to form insoluble phosphate salts which might result in decreased iron absorption. Also, Monsen and Page (15) have studied some "iron absorption promoting agents," including aspartic acid which is an amino acid that forms a high-affinity iron chelate, and reported no increase in iron absorption.

This study was initiated to define the effect of selected divalent amino acids on iron absorption and to determine whether increased iron absorption with amino acids is related to the stability constant of the amino acid–iron complex. Ascorbic acid, an agent which is reported to enhance iron absorption (16, 17), was also tested to compare its effect on enhancing iron absorption with that of amino acids.

[1] Fe-Plus, a soy-protein iron complex; Miller, Inc.; and Ronium capsules, an amino acid–iron complex; Pasadena, Inc.

EXPERIMENTAL SECTION

Determination of Stability Constants of 10 Amino Acid Complexes with Iron—Glycine, L-proline, D,L-tryptophan, L-asparagine, D,L-alanine, D,L-phenylalanine, D-serine, D,L-methionine, D,L-leucine, and L-isoleucine were obtained commercially. Stability constants were determined by using a standard method for the determination of chelation between amino acids and metals (1–3). The amino acids described above were all dried at 60°C under reduced pressure for 1 h. Each amino acid (50 mL of a 0.01 M aqueous solution) was titrated with 0.1 M KOH first in the absence of metals and then in the presence of one equivalent of the ferrous sulfate salt (0.005 M). Thus, the molar ratio of the complex-forming agent to metal was 2:1 for all bivalent ions. The amount of alkali used in each titration was 5 mL (*i.e.*, the equivalent of the amount of complex-forming agent), and this was added in 10 equal portions of 0.5 mL each.

Experiments were conducted by using a water bath maintained at 37°C, and the amino acid solution was stirred continuously with nitrogen to maintain an inert atmosphere above the solutions. Boiled water was used since air must be removed from the system to avoid change in base concentration. The pH was recorded after each addition.

Preparation of the Buffer—A potassium hydrogen phthalate–sodium hydroxide buffer solution was prepared with a final pH of 6.0. Such a buffer contained 50 mL of 0.1 M potassium hydrogen phthalate and 43.7 mL of 0.1 M NaOH diluted to 100 mL. This buffer system was used because it did not precipitate iron from solution as did the phosphate buffer.

The ferrous ion was the important ion, and the potassium hydrogen phthalate buffer system maintained the iron in the ferrous state. This was determined throughout the study by taking a small portion of the solution, filtering it, and counting the radioactivity (iron-59) in solution. The ferric ion eventually forms and precipitates at pH 6.0 and does not stay in solution. The iron never precipitated in potassium hydrogen phthalate buffer throughout the study. The ferrous ion did not hydrolyze to form the ferric ion in potassium hydrogen phthalate buffer, and the iron was administered as the ferrous ion.

Procedure with Rats—Sprague–Dawley rats were bred and raised at the Oregon State University Animal Resource Center. The rats were housed in

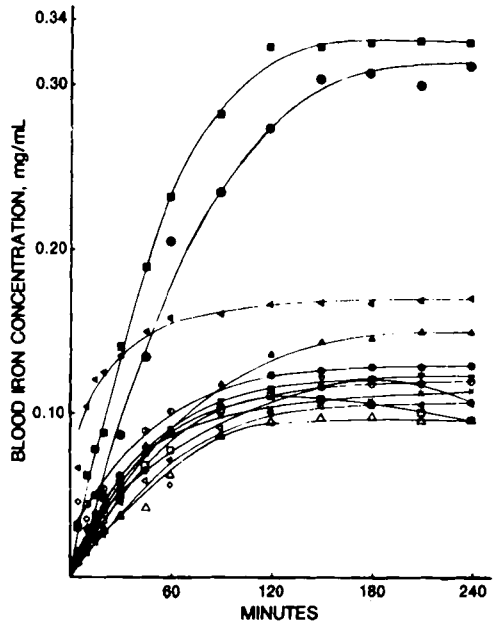

Figure 1—*Comparison of blood iron-59 concentrations-time curves when 0.2 M ferrous sulfate was given as control (△) and with amino acids and ascorbic acid added. Key: (■) asparagine; (●) glycine; (◀) serine; (▲) alanine; (⊖) proline; (▽) isoleucine; (○) leucine; (x) methionine; (◇) ascorbic acid; (◁) phenylalanine; (□) tryptophan; (△) control.*

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Table I—Formula of the Rat Feed

Ingredient	Pounds/Ton of Feed
Alfalfa (ground)	200
Bentonite (as a binder)	50
Corn (ground)	546
Barley (ground)	600
Soybean meal (44% crude protein)	240
Fish meal	100
Feather meal	40
Whey (dried)	200
Trace mineralized salt	10
Tricalcium phosphate	14
Vitamin A (1,000,000 IU/g)	3 g
Vitamin D (36,000 IU/g)	13.6 g

pairs in metal cages (~25.4 × 20.3 cm) for 3 months before use, with a 12-h light/12-h dark cycle. The rats were allowed food and water *ad libitum* prior to fasting. The rat food was a special feed developed at Oregon State University. The formula for the food is presented in Table I.

Solutions of iron (as $^{59}FeSO_4$)[2] as a control solution and complexed with each of 10 amino acids or ascorbic acid were administered orally to groups of six rats each. Male albino rats (weight, 250–270 g each) were used. Rats were fasted for 24 h before the start of the experiment to minimize the occurrence of extraneous complexing agents, present as food constituents in the intestinal lumen, which may affect iron absorption (4). All test solutions were prepared by adding an amino acid and $^{59}FeSO_4$ to aliquots of the buffer so that the resulting iron-59 activity was 10 μCi/mL. The amino acid concentration was 0.4 M. The ascorbic acid concentration was 0.5 M, and the ferrous sulfate concentration was 0.2 M (10 μCi/mL). A control solution was prepared by the same procedure, but it did not contain amino acid or ascorbic acid.

An oral intubation tube[3] was used to deliver 1 mL of test or control solution into the stomach. The tube was then removed, and the rat was kept under anesthesia (ether) during the entire experiment.

At 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min after administration of the solution, 20-μL samples of blood were collected from a toe of the rat by clipping the skin and collecting blood in capillary tubes. The capillary tubes were then placed into counting vials. Each sample was counted for 10 min in a solid scintillation counter chamber[4].

At the end of 4 h, the animals were sacrificed by overdose of ether. Liver, kidney, heart, brain, and muscle samples were collected from the animals, weighed, and then counted for 10 min[4].

RESULTS AND DISCUSSION

The concentration of iron-59 in blood *versus* time curves of iron-59 absorbed from all amino acid–iron, ascorbic acid–iron, and control solutions are shown in Fig. 1. All test solutions of amino acid and ascorbic acid with iron produced concentrations of iron-59 in blood higher than that in the control solution. It is known that blood or serum iron *versus* time curves cannot be used alone for quantitative and reliable iron absorption measurements (15–17). Other tissue iron concentrations should be included with concentrations of iron-59 in blood so that the total iron absorbed can be measured or estimated from the tissues sampled. The concentration and amount of iron-59 in tissues sampled 4 h after administration of all the amino acid–iron solutions, the ascorbic acid solution, and the control solution are shown in Table II.

The concentration of iron-59 was highest in tissues of animals receiving asparagine–iron and glycine–iron solutions, whereas it was lower in the tissues of animals receiving the other amino acid–iron solutions or the ascorbic acid–iron solution. The fact that the concentrations of iron-59 in blood and tissue at the end of the experiment were higher in the animals receiving amino acid–iron solutions than control solutions indicates that the elevated concentrations of iron-59 in blood in these animals is an accurate reflection of increased absorption from the GI tract.

In the tissues, the iron-59 concentration was highest in the heart, and the largest amount was found in muscle (due to large muscle mass in the body). High concentrations of iron-59 were also found in the liver and kidney, and generally, there were very small concentrations in the muscle and brain. This was consistent for the control experiments as well as for all 10 amino acid–iron complexes and the ascorbic acid–iron solution. Stores of iron are mainly in reticuloendothelial cells and, thus, are found in organs rich in such cells, especially the liver (18, 19). The heart is also an organ that has been reported

[2] New England Nuclear Corp., Boston, Mass.
[3] Popper and Sons, Inc., New York.
[4] Tracer Northern T-1705 NCA 40 mL NaI (Tl) detector.

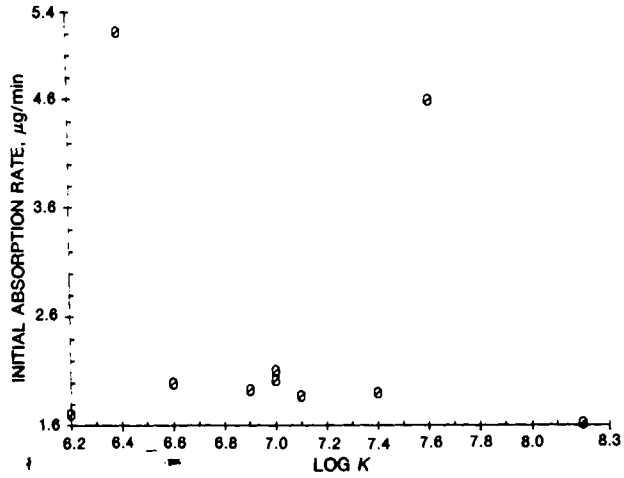

Figure 2—*Initial rate of absorption* versus *log* K *for each of the amino acids.*

(20, 21) to be high in iron content, which is most likely due to the heart being an organ high in iron sulfur proteins and cytochrome enzymes. Cytochrome *c*, for example, contains 1 g of iron/mol (20). The amount of stored iron varies with sources and also with demands made on it for hemoglobin formation. Glycine and asparagine increased iron-59 absorption to the greatest extent ($p < 0.001$) as compared with the control solution. Although the increase of iron-59 absorption over that of the control by serine and ascorbic acid was statistically significant (by ANOVA, $p < 0.05$), the increase was minimal, only 72.7 and 54.5%, respectively, compared with 309% for glycine and 345% for asparagine. These data are consistent with the data of Forth and Rummel, who observed a 300% increase in the amount of iron absorbed from the gut *in situ* in rats with glycine–phosphate buffer compared with iron–phosphate buffer (9) alone. Diets for chicks that were low in glycine but adequate in iron caused a marked reduction in the amount of iron absorbed *versus* diets in which glycine and iron were present in adequate amounts (22).

The concentration of iron-59 in tissues of animals receiving amino acid–iron solutions, control solution, or the ascorbic acid–iron solution was consistent with concentrations of iron-59 in the blood obtained from each test or control solution. Therefore, the concentration of iron-59 in blood *versus* time curves were used to compare the chelating affinity of the amino acid to iron (stability constant) with the ability of the amino acid to increase the rate or an approximated relative extent of iron absorption. This was done by comparing the initial rate of absorption with the stability constant of the amino acid–iron complex or by comparing the area under the concentration of iron-59 in blood *versus* time curve (0–4 h) with the stability constant of the amino acid complex. For all amino acid–iron complexes, the concentration of iron-59 in the blood *versus* time (Fig. 1) was fit to a trinomial equation (r^2 range, 0.9857–0.9991). Figure 1 shows the plot of the experimentally observed *versus* calculated concentrations of iron-59 in blood for each test solution. An empirical equation which describes the concentrations of iron-59 in blood curves, therefore, is:

$$y = B_0 + B_1 t + B_2 t^2 + B_3 t^3 \qquad \text{(Eq. 1)}$$

where t is time. This equation was used to fit the data so that the initial rate of absorption could be estimated.

The initial rate of absorption is approximated by the tangent to the concentration of iron-59 in blood curve at time zero and is equal to B_1 after differentiating dy/dt. The initial rates of iron-59 absorption, in decreasing order starting with the amino acid that increased the initial rate of absorption most, were: asparagine, glycine, serine, leucine, methionine, isoleucine, tryptophan, alanine, phenylalanine, and proline (Table III).

The effects of different amino acids on iron-59 absorption from time zero to 4 h were compared with the control by comparing the area under the concentration of iron-59 in blood *versus* time curve (AUC) for each amino acid–iron complex from time zero to 4 h. When integrating the above equation over the experimental period of 4 h, the area under the concentrations of iron-59 in blood time curve ($AUC_{0\text{-}240\ min}$) for each amino acid iron complex can be obtained as:

$$\int_{y_0}^{y_{240}} y\,dt = \int_{t=0}^{t=240} (B_0 + B_1 t + B_2 t^2 + B_3 t^3)\,dt \qquad \text{(Eq. 2)}$$

$$= 240\,B_0 + 28{,}800 B_1 + 4608 \times 10^3 B_2 + 82{,}944 \times 10^4 B_3$$

The values of B_0, B_1, B_2 and B_3 for each amino acid were obtained by curve

Table II—Iron in Tissues Sampled 4 h after Oral Administration of Test Solution to Rats

Test Solution	Average Concentration of Iron, μg/g of tissue					Conc. of Iron Remaining in GI Tract, μg/g of tissue[a]	Total Amount of Iron Absorbed, mg[b]
	Liver	Heart	Kidney	Muscle	Brain		
Iron alone	3.4 ± 0.3	4.5 ± 1.0	0.81 ± 0.15	0.81 ± 0.06	0.29 ± 0.05	- -	1.1 ± 0.1
Glycine-iron	16.4 ± 2.0	14.0 ± 2.4	3.58 ± 1.45	2.02 ± 0.44	0.68 ± 0.10	—	3.4 ± 0.2[c]
Leucine-iron	3.7 ± 0.1	5.2 ± 0.1	0.59 ± 0.11	0.96 ± 0.08	0.21 ± 0.17	—	1.3 ± 0.1
Tryptophan-iron	4.5 ± 1.2	5.2 ± 0.6	1.41 ± 0.38	1.01 ± 0.17	0.33 ± 0.04	–	1.1 ± 0.1
Asparagine-iron	21.7 ± 2.1	23.1 ± 0.9	13.19 ± 1.66	2.54 ± 0.22	0.36 ± 0.15	1.02 ± 2.6	3.8 ± 0.2[c]
Serine-iron	4.9 ± 0.3	6.9 ± 0.2	1.44 ± 0.17	1.25 ± 0.18	0.45 ± 0.08	—	1.9 ± 0.1[d]
Proline-iron	3.8 ± 0.3	5.6 ± 0.3	0.94 ± 0.19	0.98 ± 0.16	0.30 ± 0.07	—	1.4 ± 0.1
Methionine-iron	3.6 ± 0.2	5.1 ± 0.2	0.90 ± 0.12	0.84 ± 0.11	0.32 ± 0.04	45.2 ± 2.4	1.2 ± 0.1
Alanine-iron	3.9 ± 0.3	6.3 ± 0.8	0.93 ± 0.11	1.01 ± 0.17	0.33 ± 0.06	—	1.6 ± 0.1
Isoleucine-iron	3.8 ± 0.1	5.2 ± 0.4	0.78 ± 0.08	0.94 ± 0.10	0.37 ± 0.07	—	1.3 ± 0.1
Phenylalanine-iron	3.6 ± 0.2	4.9 ± 0.2	0.85 ± 0.13	0.84 ± 0.15	0.32 ± 0.02	46.8 ± 5.1	1.1 ± 0.1
Ascorbic Acid-iron	4.3 ± 0.2	5.2 ± 0.2	1.27 ± 0.18	1.08 ± 0.18	0.40 ± 0.07	—	1.7 ± 0.1[d]

[a] After 4 h. [b] After 4 h. Calculations assume homogenous iron content throughout the tissues sampled. Also, the skeletal and adipose tissues are assumed to have the same iron content as the muscle tissue. Tissue mass was not determined in this study, but published (23) averages values for specific tissue masses, corrected for rat weights, were used in these estimations. Although these assumptions may not be totally accurate for estimating total iron absorbed, they are adequate because the actual values of iron absorbed would only be all scaled-up or -down in proportion to the relative values listed. The dose of iron administered was 11.2 mg. [c] $p < 0.001$. [d] $p < 0.005$.

fitting[5], and the area (Table III) under the concentrations of iron-59 in blood–time curve (0–4 h) was then calculated for each test solution.

Table III lists stability constants determined experimentally for each amino acid–iron complex, as well as the initial rate of iron absorption and the area under the concentration of iron-59 in blood *versus* time curves.

Figure 2 is a plot of the initial rate of iron absorption *versus* stability constant for each amino acid–iron complex, and Fig. 3 is a plot of AUC *versus* stability constant for each amino acid–iron complex. No useful correlation between either the initial rate of absorption ($r^2 = 0.01$) or area under the concentration of iron-59 in blood *versus* time curve ($r^2 = 0.000208$) (Fig. 2) and the stability constants of the appropriate amino acid–iron complex was observed (Fig. 3). This is contrary to the earlier hypothesis that amino acids which have larger amino acid–iron complex stability constants should increase iron absorption (8, 9). Although the amino acids reported to increase iron absorption had high stability constants, neither glycine nor asparagine have high stability constants, but both were effective in increasing iron absorption from the GI tract. What may have been measured in the earlier experiments was the ability of those amino acids that have large iron-complex stability constants to protect iron from precipitating with phosphates. It is interesting to note that EDTA forms a more stable complex than any of the amino acids tested, yet it does not facilitate increased iron absorption (9, 16, 17) and is even reported to negate any iron absorption enhancement produced by an iron absorption-promoting agent.

As another measure of the ability of the amino acid to increase iron absorption, the amount of iron-59 in gut tissue was determined after administration of phenylalanine–iron and methionine–iron complexes, in which the increase in iron absorption was lowest, and asparagine–iron complexes, in which it was highest. The amount of iron-59 in gut tissue (Table II) was much higher in the case of the phenylalanine–iron and methionine–iron complexes. This, too, is an indication that the elevated concentration of iron-59 in blood in the rats is a reflection of increased absorption from the GI tract. Conversely, when iron absorption is lower, the iron is retained in the gut tissue.

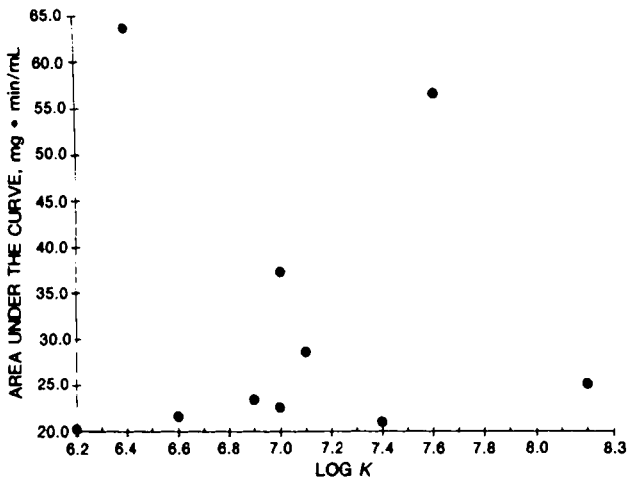

Figure 3—*AUC from time zero to 4 h of blood iron-59 concentration–time profile* versus *log K for each of the amino acids tested.*

Protein repeatedly has been reported to promote iron absorption (4–8, 24–26). This effect may account for the high fraction of iron in meat that is absorbed and for the enhancement of iron absorption from vegetables with simultaneous ingestion of meat (24–27). However, protein has also been reported to have a negative effect on iron absorption. Egg protein retards the absorption of iron (23, 27); it is not known whether the effect is related to its content of conalbumin. Conalbumin forms a stable complex with iron *in vitro* that drastically reduces the transfer of iron in jejunal segments of anemic rats (10). Egg yolk also contains phosphoproteins that form stable complexes with iron and prevent iron absorption (13, 23). The differential effects of proteins on absorption of iron may be attributed to the degree of proteolysis that occurs in the different experiments (23). All the data suggest that iron chelation does not always promote iron absorption.

Several products on the market have iron-chelating agents that are reported to promote iron absorption. These products, which contain agents reported to promote iron absorption, vary from protein hydrolysate to combinations of amino acids to a single amino acid. The iron absorption-promoting ability of these mixtures of agents needs to be investigated and compared with the ability of each one to promote iron absorption. It may be that only certain amino acids are responsible for enhanced iron absorption, and others can be omitted from iron product formulations; the result may be further increased iron absorption. A product containing only glycine and/or asparagine with iron may be sufficient to produce significantly increased iron absorption compared with other mixtures.

The amount of amino acids used in this study (or what would be used for an iron preparation for adult human use) is far too little to maintain a good nitrogen balance. Also, use of a single or a few selected amino acids as dietary supplements has caused significant shifts toward a negative nitrogen balance

Table III—Stability Constants, Initial Absorption Rates, and AUC for Each Amino Acid Tested

	log *K* Stability		Initial Absorption Rate, μg/min[b]	AUC, mg·min/mL[c]
	Experimental	Literature[a]		
Asparagine	6.4	6.5	5.21	63.59
Glycine	7.6	7.8	4.57	56.51
Serine	7.0	7.0	2.10	37.31
Leucine	7.0	6.9	2.02	22.60
Methionine	6.6	6.7	1.99	21.71
Isoleucine	6.9	7.1	1.93	23.48
Tryptophan	7.4	7.6	1.9	21.05
Alanine	7.1	7.3	1.87	28.64
Phenylalanine	6.2	6.3	1.70	20.24
Proline	8.2	8.3	1.62	25.22

[a] Values are from Refs. 1–3. [b] Calculated from Eq. 1. [c] Calculated from Eq. 2; time, 0–4 h.

[5] The organization and analysis of the data base associated with this investigation were carried out in part using the PROPHET system, a unique national resource sponsored by the National Institutes of Health. Information about PROPHET, including how to apply for access, can be obtained from the Director, Chemical/Biological Information-Handling Program, Division of Research Resources, National Institutes of Health, Bethesda, MD 20205.

in individuals (29). Therefore, individuals taking amino acid–iron complexes should be made aware that such products do not have value as protein supplements or replace the need for dietary protein. In addition, depending on the quantity and essentiality of the amino acids ingested from the supplement, protein metabolism may be adversely affected.

A consensus has been reached that amino acids considered to be essential are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine for children and adults. Histidine is an essential amino acid for infants, but it is not considered essential for children or adults (30). It is important to note that the amino acids that appear to increase the absorption of iron are not considered to be essential amino acids. Nitrogen balance is maintained by an adequate diet of protein and not by an iron supplement containing one or two amino acids in very low quantities (29, 30). Likewise, increasing the dosage of the amino acid by taking more tablets of an amino acid–iron preparation could cause iron toxicity.

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Evaluation of High-Performance Liquid Chromatography and Gas Chromatography for Quantitation of Dextromethorphan Hydrobromide in Cough–Cold Syrup Preparations

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Abstract □ A rapid and sensitive high-performance liquid chromatographic (HPLC) procedure is described for the analysis of the antitussive dextromethorphan hydrobromide in several cough–cold syrup preparations and compared with a gas chromatographic (GC) procedure. In the HPLC procedure, the active ingredient is analyzed as the hydrobromide salt by dilution in the mobile phase and separation on a reverse-phase cyano column. In the GC method, the active ingredient is analyzed as the free base, in which an aqueous solution of the antitussive is made alkaline and extracted with dichloromethane before injection onto the GC column. Excellent resolution of the antitussive agent was obtained by both systems; however, the HPLC assay is preferred for routine analysis (*RSD* 1%), as compared with the GC assay (*RSD* 4%).

Keyphrases □ Dextromethorphan hydrobromide—HPLC, GC □ Antitussive agents—dextromethorphan hydrobromide, HPLC, GC □ Formulations—cough–cold syrup, analyses by HPLC and GC

Dextromethorphan hydrobromide [(+)-3-methoxy-17 methyl-9α,13α,14α-morphinan hydrobromide monohydrate (I)] is commonly used an an antitussive agent in many commercial cough–cold syrup preparations. There are several clinically active compounds classified as morphinans. All have the drawbacks of morphine: its dependence liability and respiratory-depressant characteristics (1). However, the side effects of some morphinans are not as severe as those of morphine. As a result, dextromethorphan hydrobromide has gained wide acceptance as a nonaddictive antitussive agent because it is nearly devoid of any analgesic activity (2).

Dextromethorphan hydrobromide has been separated by

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