A CHEMICAL METHOD FOR THE DETERMINATION OF PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS

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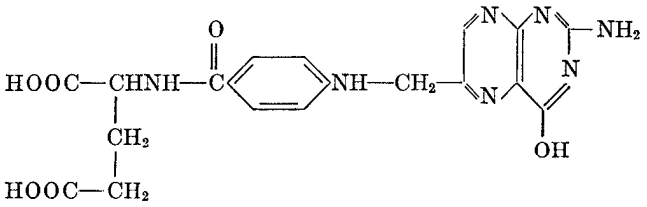
Pteroylglutamic acid, N-[4-{[(2-amino-4-hydroxy-6-pteridyl)methyl]-amino}benzoyl]glutamic acid, on either chemical or catalytic reduction at



an acid pH is cleaved to yield a pteridine and *p*-aminobenzoylglutamic acid.[1] The reaction seems to be general for this group of compounds and is suggested as a basis for a chemical method for the determination of pteroylglutamic acid, its derivatives, and analogues.

The method consists of reducing the compound with zinc dust in 0.5 N hydrochloric acid. The aromatic amine is measured by the method of Bratton and Marshall (1) before and after reduction. The difference between the two values multiplied by the appropriate factor is a measure of the pteroyl derivative present.

Method

Apparatus—A photoelectric colorimeter is necessary to measure accurately the color formed in the Bratton and Marshall procedure. An Evelyn photoelectric colorimeter and a Beckman spectrophotometer have been used.

Reagents—5.0 N hydrochloric acid.

Reagent grade of zinc dust.

[1] Hutchings, B. L., Stokstad, E. L. R., Mowat, J. H., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., unpublished data.

0.5 per cent solution of gelatin containing 0.1 per cent benzoic acid as a preservative.

0.1 per cent solution of sodium nitrite.

0.5 per cent solution of ammonium sulfamate.

0.1 per cent solution of N-(1-naphthyl)-ethylenediamine dihydrochloride. This reagent is light-sensitive and should be kept in a brown bottle.

0.1 per cent solution of *p*-aminobenzoic acid in 50 per cent ethanol.

Procedure

A general outline of the method, with pteroylglutamic acid as an example, follows. A solution containing from 0.5 to 3.0 mg. of pteroylglutamic acid is placed in a 100 ml. volumetric flask. To this flask are added approximately 80 ml. of water, 10 ml. of 5.0 N hydrochloric acid, 1.0 ml. of a 0.5 per cent solution of gelatin, and sufficient water to make the total volume 100 ml. A sample of this unreduced solution, usually 2 ml., is removed and used for determining the free amine. If the original compound or crude product contains less than 2 per cent of free amine, an aliquot of the original solution must be used in order to obtain a readable color.

The remainder of the solution is transferred to a 250 ml. Erlenmeyer flask and reduced by the addition of from 0.5 to 1.0 gm. of zinc dust. The amount of zinc dust is not critical and can be conveniently estimated once a weighed amount has been used. After 10 minutes reduction, during which time the flask is shaken intermittently, the zinc dust is filtered off and the amine is determined on 2 ml. of the filtrate by the following procedure: The sample is diluted to 6.6 ml. with water. To this solution are added, with thorough mixing, 0.4 ml. of 5.0 N hydrochloric acid and 1.0 ml. of sodium nitrite solution. After 3 minutes, 1.0 ml. of ammonium sulfamate solution is added and the decomposition of the excess nitrous acid is allowed to proceed for 2 minutes. At the end of this period, 1 ml. of N-(1-naphthyl)-ethylenediamine solution is added. The color reaches a maximum in 5 minutes and is stable for several hours. The color may be measured at 550 mμ in a Beckman or Coleman spectrophotometer or in a photoelectric colorimeter with a 550 mμ filter. The use of specially cleaned cuvettes decreases the tendency of bubbles of nitrogen gas to adhere to the sides of the cuvettes.

Since *p*-aminobenzoic acid, *p*-aminobenzoylglutamic acid, and *p*-aminobenzoylglycine give the same molal color, it is reasonable to suppose that other peptides of *p*-aminobenzoic acid would give similar results. On the basis of this assumption *p*-aminobenzoic acid has been used as a standard in the Bratton and Marshall method. A response curve is constructed with from 5 to 20 γ of *p*-aminobenzoic acid. This is necessary since the color intensity does not exactly follow Beer's law.

Calculations—The difference between the amine obtained after reduction

and the amine obtained on the unreduced solution yields the combined amine present. This value multiplied by the factor, (molecular weight of pteroylglutamic acid)/(molecular weight of *p*-aminobenzoic acid), gives the micrograms of pteroylglutamic acid in the diluted sample.

EXPERIMENTAL

Extent of Reduction—The magnesium salt of pteroylglutamic acid was prepared by heating the free acid with magnesium oxide in the minimum amount of water. The solution was filtered to free it of excess magnesium oxide. On cooling, magnesium pteroylglutamate crystallized as needles. After three recrystallizations the magnesium salt was collected, air-dried, and then dried in a high vacuum at 110° for 4 hours.

$C_{19}H_{16}O_6N_7Mg_{1.5}\cdot H_2O$			
Calculated.	C 46.29,	H 3.66,	N 19.90, Mg 7.41
Found.	" 46.51, 46.44,	" 4.20, 3.70,	" 19.92, 19.78, " 7.38, 7.29

When the compound was reduced under the conditions described above, it was found to contain 0.09 per cent free amine as *p*-aminobenzoic acid and 28.25 per cent total amine. The difference between these two values multiplied by the factor, $(C_{19}H_{16}O_6N_7Mg_{1.5}\cdot H_2O)$/(molecular weight of *p*-aminobenzoic acid), gives a figure of 101.2 per cent. As the accuracy of the Bratton and Marshall determination is ±2 per cent, the figures indicate that the reductive cleavage is essentially complete.

An independent method of assessing the purity of the compound was based on a comparison of the extinction coefficients of the magnesium pteroylglutamate with the value for pteroylglutamic acid. The $E^{1\%}_{1cm.}$ value at 365 mμ for magnesium pteroylglutamate was 184. When this is corrected for the magnesium and water content, the $E^{1\%}_{1cm.}$ value is 205.5. The value for a highly purified preparation of the free acid is 206. This is further evidence for the essentially complete reduction of pteroylglutamic acid into its pteridine and aromatic amine components.

Time of Reduction—Samples were reduced for the lengths of time noted in Table I. Maximum amine liberation occurred in less than 10 minutes. Further reduction, especially with certain samples of zinc dust, led to somewhat lower values. This apparently arises from partial destruction of the aromatic amine that is formed on reductive cleavage (see below).

Stability of p-Aminobenzoic Acid and p-Aminobenzoylglutamic Acid to Reduction—*p*-Aminobenzoic acid and *p*-aminobenzoylglutamic acid were reduced under conditions similar to those used for the pteroyl derivatives. Definite destruction of the aromatic amines occurred (Table II). The inclusion of gelatin in the reducing solution protects the amines from inactivation. The exact nature of this protective action is unknown.

Stability of Diazo Compound to Light[2]—A series of experiments was carried out in which *p*-aminobenzoylglutamic acid was diazotized in red flasks which were non-actinic, in ordinary glassware in laboratory light (slight

TABLE I

Effect of Time and Acidity on Reduction of Air-Dried Pteroylglutamic Acid by Zinc

Time	Acid concentration	*p*-Aminobenzoic acid	
		1st determination	2nd determination
min.		*per cent*	*per cent*
1	0.5 N HCl	28.9	28.9
2	0.5 " "	28.8	28.9
5	0.5 " "	29.0	29.0
10	0.5 " "	29.5	29.5
20	0.5 " "	28.3	28.3
10	0.05 " "	25.3	27.4
10	0.1 " "	27.9	27.6
10	0.2 " "	28.0	27.6
10	0.5 " "	28.6	28.2
10	1.0 " "	27.9	28.0
10	0.1 " CH_3COOH	27.5	27.4
10	0.2 " "	28.0	27.4
10	0.5 " "	28.0	26.8
10	1.0 " "	28.6	27.0

TABLE II

Effect of Gelatin on Stability of p-Aminobenzoic Acid and p-Aminobenzoylglutamic Acid to Reduction by Zinc

Compound	Time of reduction	Before reduction	After reduction	
			No gelatin	With gelatin
	min.	*γ per ml.*	*γ per ml.*	*γ per ml.*
p-Aminobenzoic acid	10	100	79.0	100
	60	100	45.6	98
p-Aminobenzoylglutamic acid	10	103	92.3	103
	60	103	81.5	102

sunlight), and in ordinary glassware in direct sunlight. The results are presented in Table III. It is quite apparent that the *p*-aminobenzoylglutamic acid diazo compound is unstable to direct sunlight. In ordinary

[2] We are indebted to Dr. W. Seaman and Mr. J. T. Woods of the Calco Chemical Division, American Cyanamid Company, Bound Brook, New Jersey, for the information and data pertaining to the instability of the *p*-aminobenzoylglutamic acid diazo compound to light and the fact that zinc amalgam will serve as a satisfactory reducing agent.

laboratory light (no direct sunlight) the diazotization product appears to be stable. However, if there is a possibility of direct sunlight, the diazotization and coupling should be carried out in non-actinic glassware.

Stability of Pteroylglutamic Acid to Light—Since it has been shown that pteroylglutamic acid is decomposed by light to yield *p*-aminobenzoylglutamic acid (2), the analytical procedures for this compound should be carried out in the absence of direct or indirect sunlight.

Correlation with Biological Activity—In certain synthetic products the chemical method gives higher values than does microbiological assay. This is interpreted to mean that the isomeric 7-pteridyl compound is also reduced to yield an aromatic amine. The extent of reduction of the isomeric compound has not been studied on a pure product. However, a comparison of the chemical values with the values obtained on microbiological assay serves to indicate the proportion of active isomer present.

TABLE III

Stability of p-Aminobenzoylglutamic Acid Diazo Compound to Light

Conditions of lighting	*p*-Aminobenzoylglutamic acid
	per cent
Red flask	99.6
Laboratory light	98.9
Direct sunlight	56.9

When only the active isomer is formed, there is complete correlation between the chemical and biological activity.

Interfering Compounds—Any compound that will give rise to an aromatic amine on reduction, which will develop a color in the Bratton and Marshall determination, will interfere. The distribution of such compounds in natural products is unknown.

Interfering compounds in the crude products arising from synthesis are the isomeric compound mentioned above and oxidized amines. The oxidized amines are removed by preliminary purification of the active compound.

Utility of Method—The method is satisfactory with concentrates derived from natural sources when the content of the active compound is 5 per cent or greater. The method is entirely satisfactory for determining the potency of crude products derived from various synthetic reactions when only the naturally occurring isomer is present.

When the method is used for the various derivatives or analogues, the desired factor is obtained from the following ratio, (molecular weight of analogue)/(molecular weight of *p*-aminobenzoic acid).

DISCUSSION

On the basis that the reduction proceeds essentially to completion, the accuracy of the method is determined by the accuracy of the Bratton and Marshall procedure, which is ±2 per cent.

As the sensitivity of the colorimetric procedure is not great, the chemical method is of no value in determining the pteroylglutamic acid content of natural materials unless the active compound is present at a concentration sufficient to give a readable color.

Zinc dust containing from 1 to 3 per cent copper has been used as the reducing agent. The reduction is somewhat more vigorous but offers no particular advantages over zinc dust alone. Zinc amalgam (containing from 0.1 to 4.3 per cent zinc) is a satisfactory reducing agent.[2] In this modification the reduction is carried out for 30 minutes on a shaking machine. The values obtained with zinc amalgam are similar to those obtained with zinc dust when gelatin is present but somewhat higher than the values obtained with zinc dust alone.

Only approximate values could be obtained for pteroic acid by the chemical method because of the extreme insolubility of this compound in acid solution. This is a specific rather than a general property of this group of compounds.

SUMMARY

A chemical method for the determination of pteroylglutamic acid and related compounds is outlined. The method is based on the fact that these compounds are cleaved by reduction in an acid solution to yield a pteridine and an aromatic amine. The amount of aromatic amine formed during the reduction is determined by the method of Bratton and Marshall and is used as a measure of the pteroyl derivative present.

It is a pleasure to express our appreciation to Mr. L. Brancone and coworkers for the microanalyses.

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