Synthesis of ^{35}S-Labelled Djenkolic Acid

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Abstrak

Keracunan jengkol yang kadang-kadang terjadi setelah memakan buah jengkol (Pithecellobium lobatum Benth.) telah dilaporkan oleh negara-negara ASEAN, seperti Indonesia, Malaysia dan Thailand. Di negara-negara ini pohon jengkol dapat tumbuh dan buahnya dimakan oleh sebagian penduduknya. Asam jengkolat, ialah sejenis asam amino berunsur belerang dan terdapat di dalam buah jengkol dalam bentuk asam amino bebas. Bila dimakan, sebagian dari asam amino ini terurai oleh bakteri usus dan menghasilkan bau jengkol yang khas pada ekskreta sedangkan asam jengkolat yang diserap oleh usus diekskresi oleh ginjal dan menjadi penyebab gejala-gejala keracunan jengkol. Belum banyak yang diketahui tentang cara-cara penguraian asam amino yang langka ini di dalam tubuh manusia. Pemberian asam jengkolat yang radio-aktip kepada binatang percobaan dan sukarelawan akan sangat membantu memecahkan persoalan ini. Dengan tujuan ini telah disintesis ^{35}S-asam jengkolat. Dilaporkan dua cara untuk memperoleh ^{35}S-asam jengkolat dengan aktivitas spesifik yang cukup tinggi untuk percobaan-percobaan.

Abstract

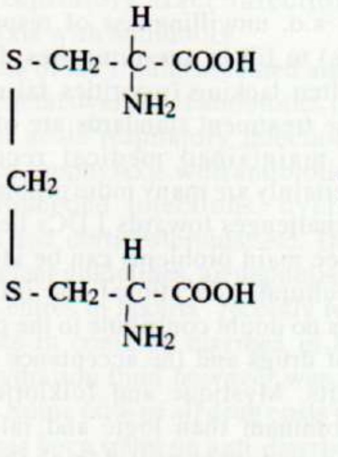
Cases of djenkol poisoning, which sometimes occur after consumption of the djengkol bean (Pithecellobium lobatum, Benth.) have been reported in ASEAN member countries, such as Indonesia, Malaysia and Thailand, where this bean is relished by certain layers of the population. Djenkolic acid, a sulfur containing amino acid present as the free amino acid is concomitantly ingested during the consumption of the beans. Some of this amino acid is broken down by the intestinal bacteriae producing the well-known offensive djenkol odour in the excretae, while the absorbed djenkolic acid is excreted by the kidney and is responsible for the symptoms of djenkol poisoning. However not much is known of other possible metabolic reactions of this unusual amino acid in the human body. Administering radio-active djenkolic acid to experimental animals and human volunteers would greatly help to solve this problem. With this in mind ^{35}S-labelled djenkolic acid was synthesized. Two methods for obtaining ^{35}S-djenkolic acid with appropriate specific activities are hereby reported.

Keywords: *^{35}S-labelled djenkolic acid, L-(^{35}S) cysteine hydrochloride, cysteine thioacetal of formaldehyde, S-carboxymethylcysteine.*

INTRODUCTION

Djenkolic acid is a sulfur-containing amino acid found in the djenkol bean *(Pithecellobium lobatum, Benth.)*. It was identified by Van Veen and Hyman in 1933 as the toxic substance of the bean, responsible for the symptoms of djenkol poisoning, which sometimes occur after the consumption of the beans.[1]

The chemical structure of this unusual amino acid was subsequently confirmed by Du Vigneaud and Patterson in 1937 by its chemical synthesis from cysteine and methylenechloride.[2] Djenkolic acid can be considered as the cysteine thioacetal of formaldehyde.



L - djenkolic acid

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Unlike most of the other sulfur-containing amino acids, djenkolic acid is never found as a protein constituent but is present in the bean as a free amino acid.[3] The metabolic fate of djenkolic acid in the human body has thus far not been elucidated.

Van Veen reported an increased amount of urinary sulfur after consumption of the beans. It was found that djenkolic acid could not replace cystine in the diet of the growing rat and attributed this to the fact that djenkolic acid is not absorbed by the intestine of the rat.

Greenberg et al.,[4] succeeded in isolating and purifying an enzyme from rat liver, capable of decomposing djenkolic acid into thio-methylcysteine, pyruvic acid and ammonia. The newly formed thiol is unstable and breaks down spontaneously. Cysteine and hydrogen sulfide have been identified among the breakdown products.

In our earlier work we found that after the consumption of djenkol beans, besides djenkolic acid, a new sulfur-containing compound could be identified in human plasma by paper- chromatography. The same results were obtained when pure djenkolic acid was administered to monkeys and human volunteers.[5]

Because of the possible metabolism of this sulfur-containing amino acid and also to study the biochemical conversion of djenkolic acid in the mammalian body directly, we have to use labelled djenkolic acid.

This paper describes two methods for the chemical synthesis of ^{35}S-djenkolic acid.

MATERIAL

L-cysteine monohydrochloride monohydrate G.R. was purchased from Nakarai Chemicals Ltd. L-(^{35}S) cysteine hydrochloride with a specific activity of 2425 uCi/ml was obtained from Radiochemical Centre Amersham.

8 % Formaldehyde solution was freshly prepared by diluting 1 vol. of formalin G.R. (Nakarai Chemicals Ltd.) with 4 vols. of 6 N HCl.

2-Mercaptoethanol was obtained from Wako Pure Chemicals Industries Ltd.

Fresh iodoacetate solution was prepared by dissolving 930 mg of iodoacetate (Merck) in 600 µl of 6 N NaOH.

Authentic djenkolic acid was prepared according to the method of Armstrong and Du Vigneaud, while S-carboxymethylcysteine was synthesized according to the method of Ratner and Clark.[6] Both compounds were subsequently used as reference compounds in all the experiments.

METHOD

The method for the synthesis of djenkolic acid described by Armstrong and Du Vigneaud[6] was followed, but the amounts of reactants were scaled down to milligrams without significantly altering the reactions conditions. Our earlier experiments had shown that a satisfactory separation of djenkolic acid from unreacted cysteine/cystine cannot be achieved by either paper chromatography or high-voltage electrophoresis.

Therefore the following 2 methods of obtaining pure radio-active djenkolic acid are hereby reported.

a. "open method". In this method the djenkolic acid formed is converted into its monohydrochloride, which is relatively more insoluble in HCl than the corresponding HCl salts of unreacted cysteine/cystine.

b. "closed method". Here the cystine present in the reaction mixture is reduced to cysteine by reacting it with 2-mercaptoethanol followed by S-carboxymethylation with iodoacetate. Under the conditions specified djenkolic acid does not react with 2- mercaptoethanol or iodoacetate.

PROCEDURE

In a reacti-vial (Pierce) with a maximum volume of 1 ml were mixed:

11.1 mg (0.063 millimoles) of L-cysteine monohydrochloride monohydrate

72 µl of L-(^{35}S) cysteine hydrochloride solution

72 µl conc. HCl

13 µl of 8% formaldehyde solution

The reaction mixture was stirred by a magnetic stirrer and left overnight in a heating block at 70°C. The following day the mixture was carefully neutralized by the addition of 6 N NaOH until pH 7.

A white precipitate, consisting mostly of djenkolic acid was observed during this neutralization procedure.

Open method

After cooling in ice, the mixture was centrifuged and the supernatant carefully removed. To the precipitate were added small quantities of a 1 % HCl solution, under heating the vial in a boiling water-bath, until the precipitate has just dissolved.

Approximately 200 µl of the HCl solution were needed. The solution was cooled in ice to precipitate the djenkolic acid monohydrochloride salt. The supernatant was removed and the precipitate was again disolved in minimal amounts of 1 % HCl. This procedure of recrystallization was repeated two more times.

The resulting precipitate was dried under vacuum over NaOH and P_2O_5 overnight. The dried material thus obtained weighed 6.4 mg and had a total activity of 8.23 x 10^7 cpm. measured by a Packard Tricarb Liquid Scintillation Spectrometer Model 3320 with a counting efficiency of 80 %.

Previous experiments carried out using "cold" cysteine by this method yielded pure djenkolic acid monohydrochloride with crystal form and melting point (250-300°C, dec.) in accordance with the characteristics described by Armstrong and Du Vigneaud.[6]

Closed method

In this method the pH of the reaction mixture was carefully adjusted to 8 - 9.

An amount of 0.28 millimoles (20 μl) 2-mercaptoethanol was added and the mixture was stirred for 4 hours at room temperature. An equimolar amount of iodoacetate was then added and the mixture stirred for another 30 minutes.[7]

At the end of this period 20 ul of 12 N HCl were added to dissolve the precipitate. The solution was applied to Whatman 3 mm filterpaper and run for about 40 hours in a descending system with butanol : acetic acid : water = 12 : 3 : 5 as chromatographic solvent.

Authentic djenkolic acid and S-carboxymethylcysteine were run alongside serving as markers. In this chromatographic system djenkolic acid and S-carboxymethylcysteine separated nicely.

The position of radio-active djenkolic acid was located by both scanning the resulting chromatogram on a Packard chromatogram scanner Model 7201 and by spraying the authentic samples with the polychromatic ninhydrin reagent.[8] With this reagent djenkolic acid appears as a blue-grey spot, while S-carboxymethylcysteine is stained bright green.

The corresponding fraction containing the radioactive djenkolic acid was cut out and eluted with 0.1 N HCl.

The resulting solution was dried under reduced pressure over NaOH and P_2O_5.

The dried material yielded a total activity of 7,91 x 10^7 cpm with a weight of 7.5 mg.

RESULTS AND DISCUSSION

The original method for the synthesis of djenkolic acid in quantities of several grams as described by Armstrong and Du Vigneaud in 1947 is straightforward with a yield of 68 %. However, when the amounts of the reacting materials were scaled down to several milligrams, the problem of purification of the resulting product arises.

As satisfactory separation of unreacted cysteine/cystine from djenkolic acid was difficult to accomplish with those small amounts, two methods of obtaining pure ^{35}S-djenkolic acid are hereby reported.

While the "open method" is shorter and relatively easier, the possibility of radio-active contamination is greater and the yield is low. On the other hand, the "closed method" reduces the first mentioned drawback, but requires more effort and time.

It should be stressed that in the latter method the pH of the reaction mixture during reduction and carboxymethylation has to be carefully maintained between 8 and 9 as above or below these values the yields will be lower due to undesirable side reaction.[9]

We obtained 23 % of the theoritical amount with a specific activity of 5542 uCi/mg by the "open method", while the "closed method" resulted in a 40.8% yield with a specific activity of 4804 uCi/mg. We considered these specific activities appropriate for metabolic tracing studies. To our knowledge no work on the synthesis of ^{35}S-labelled djenkolid acid has thus far been reported.

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REFERENCES

1. Van Veen AG, Hyman AJ. Het giftige bestanddeel van de djengkolboon, Geneesk Tydschr Nederl Indie, 1933; 73 : 991-1003.
2. du Vigneaud V, Patterson WI. The synthesis of Djenkolic Acid. J Biol Chem, 1937; 114 : 533-8.
3. Oen LH, Kusumahastuti T, Tadjal W. Form and Distribution of Djenkolid Acid in the Djenkol Bean. Indon J Nutrition, 1972; 4: 2-5.
4. Greenberg DM, Mastalerz P, Nagabushanam A. Mechanism of Djenkolic Acid Decomposition by Cystathionase, Biochem Biophys Acta, 1964; 81 : 148-64.
5. Oen LH, Setiadi E. An Unknown Sulfur-containing Compound in the Plasma of Men and Rhesus Monkeys after Administration of pure Djenkolic Acid (To be published).
6. Armstrong MD, du Vigneaud V. A New Synthesis of Djenkolic Acid, J Biol Chem 1947; 168: 373-7.
7. Hirs CHW. Reduction and S-carboxymethylation of Proteins in Methods of Enzymology, Vol XI, CHW Hirs (ed) Acad Press, New York, 1967: 199-203.
8. Moffat ED, Lytle RI. Polychromatic technique for the identification of amino acids on paper chromatograms. Anal Chem 1959; 31:926.
9. Gurd FRN. Carboxymethylation in Methods of Enzymology, vol XI, CHW Hirs (ed). Acad Press, New York, 1967 : 532-41.