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MICROBORE SINGLE-COLUMN ANALYSIS OF PHARMACOLOGICALLY ACTIVE ALKALOIDS FROM THE FLY AGARIC MUSHROOM *AMANITA MUSCARIA*

MICHAEL G. GORE* and P. M. JORDAN

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU (Great Britain)

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SUMMARY

Microbore single column conditions are described which allow the separation and quantification of the substituted isoxazole alkaloids muscimol and ibotenic acid, the pharmacologically active principles of *Amanita muscaria*. The procedure permits the evaluation of levels of the alkaloids at all stages of purification from fungal extracts and provides a convenient quantitative method for monitoring the formation of muscimol by the decarboxylation of ibotenic acid.

INTRODUCTION

The remarkable halucinogenic and fly killing properties of the fly agaric mushroom *Amanita muscaria* have been known and exploited for centuries. The active constituents of the fungus were found to be ibotenic acid [amino(3-hydroxy-5-isoxazolyl)acetic acid] and its decarboxylation product muscimol (3hydroxy-5-aminomethylisoxazole)[1–5] (Fig. 1). These two compounds have, in recent years, come into prominance by virtue of their potent pharmacological effects in the mammalian central nervous system[6]. Ibotenic acid, a powerful glutamate agonist[7], may be envisaged as a conformationally restricted glutamic acid. Muscimol has also neurotransmitter properties, being a powerful γ-aminobutyric acid agonist[7], by virtue of its structural resemblance to γ-aminobutyric acid. Both compounds have recently received much attention as probes for investigating the nature of glutamate and γ-aminobutyric acid binding to their respective receptors and have been suggested as possible models for the development of potential drugs in the treatment of specific nervous disorders such as Huntington's Chorea and epilepsy.

Since the chemical syntheses of both ibotenic acid and muscimol are difficult[8] it is often more convenient to isolate the compounds from the mushroom which is readily available.

A major problem arising during the purification of ibotenic acid and muscimol from the mushroom is one of determining the amounts of the alkaloids in crude extracts and of monitoring their levels during the isolation procedure. Large quan-

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tities of other amino acids and ninhydrin positive compounds are present in the mushroom which co-chromatograph with the alkaloids on conventional thin-layer chromatographic (TLC) systems. In addition, the levels of the alkaloids vary with age and subspecies of the mushroom and it is important to have a knowledge of their level prior to the purification. Furthermore, the preparation of muscimol in our laboratory by the decarboxylation of ibotenic acid required an accurate, rapid and reliable means of determining the levels of two compounds.

In this paper we report a microbore single column method for the quantitative determination of both ibotenic acid and muscimol and its application during the purification of ibotenic acid from the mushroom. The method has been used to locate the two alkaloids in the mushroom and for monitoring the chemical decarboxylation of ibotenic acid into muscimol.

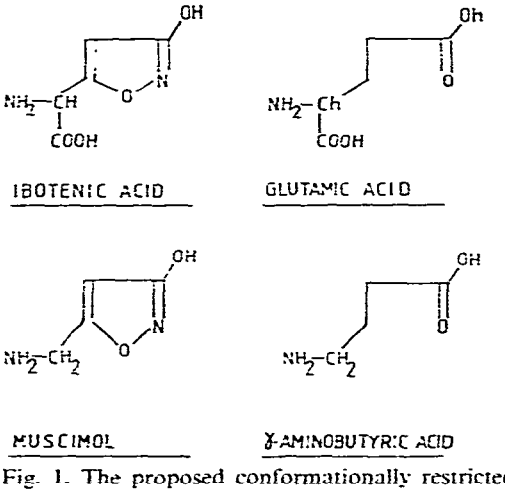

Fig. 1. The proposed conformationally restricted two-dimensional structures of glutamic acid and γ-aminobutyric acid and their structurally similar analogues, ibotenic acid and muscimol (respectively).

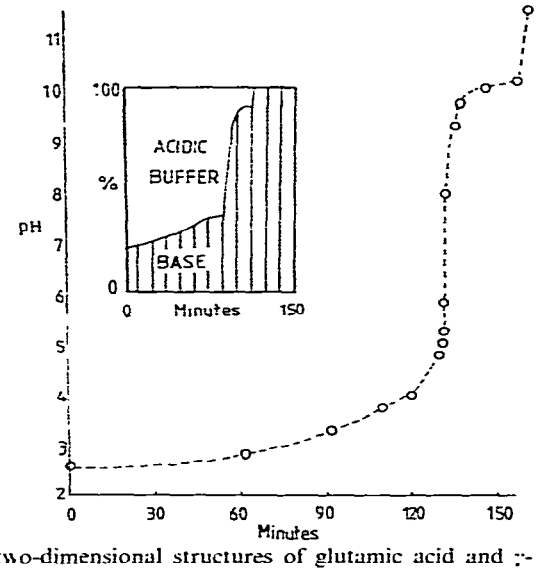

Fig. 2. A diagrammatic representation of the pH gradient employed obtained by mixing the acid and basic buffers (see Experimental) in the proportions indicated by the inset.

EXPERIMENTAL

Reagents

All reagents used are from BDH (Poole, Great Britain) and were of the Analar grade.

Sample preparation

Protein free extracts of *Amanita muscaria* were prepared by homogenising a given dry weight in 90% ethanol containing a known amount of the internal standard norleucine. After centrifugation at 30,000 *g* for 30 min the supernatant was taken, evaporated to dryness at 10°C and the dried extract redissolved in 20 m*M* HCl immediately before application to the column. Preliminary results suggested that the amounts of both ibotenic acid and muscimol measured were dependent on the acidity and temperature of this solution and the length of time between dissolving the extract

and applying the sample to the column. Further details of this phenomenon are given in the Results and discussion section.

Instrumentation

A Rank-Hilger J180 automated amino acid analyser was used to separate and detect the amino acids extracted from the *Amanita muscaria*. A single column system was employed (350 × 2.7 mm I.D.) and eluted amino acids were detected at 570 nm and 440 nm by reaction with ninhydrin in the presence of potassium cyanide as reducing agent. The buffer used to separate the various amino acids was generated "*in situ*" by mixing an acidic and a basic solution together in various ratios to give a pH gradient described in Fig. 2. The composition of the two buffers used for the elution process and for the salt free buffer used while loading the samples are given below.

Buffers

"*Salt-free*" *loading buffer (pH 2.1)*. 21.0 g citric acid, 100 ml methanol, 2.5 ml thiodiglycol (25%, w/w, in water), 3.5 ml of a 10% Brij 35 solution and distilled water to 1 l.

Acidic buffer (pH 1.9). 21.0 g citric acid, 11.7 g NaCl, 50 ml isopropanol, 2.5 ml thiodiglycol (25%, w/w, in water), 3.5 ml of a 10% Brij 35 solution all made up to 1 l with distilled water.

Basic buffer (pH 11.9). 19.0 g disodium tetraborate, 4.2 g NaOH, 10 ml of a 4% EDTA solution, 3.5 ml of a 10% solution of Brij 35 all made up to 1 l with distilled water.

Ninhydrin. 10 g ninhydrin, 216 g sodium acetate trihydrate, 400 ml α-methoxyethanol, 100 ml glacial acetic acid, 10 ml of 10% solution of Brij 35 were dissolved in distilled water and the volume made up to 1 l.

Reducing agent. 1 ml of a 1% solution of KCN was diluted into a stock solution of 1 l of distilled water containing 5 ml of 4 *M* NaOH and 3.5 ml of 10% Brij 35.

Colour development. The eluent from the chromatographic column (0.2 ml/min) is mixed with the ninhydrin solution (0.2 ml/min) and cyanide solution (0.025 ml/min). A nitrogen bubble is used to segment the stream (1 bubble every 2 sec) and the resultant mixture incubated at 90°C for 7 min prior to passing through the colorimeter where the absorbance at 570 nm and 440 nm was measured.

Quantitation of amino acids

Purified ibotenic acid (monohydrate, mol.wt. 176) and muscimol (free base, mol.wt. 125) were weighed out and their colour coefficients in the ninhydrin complex estimated relative to that of the norleucine–ninhydrin complex. These calculated "colour coefficients" were then used to quantitate these amino acids in elution profiles of extracts from *Amanita muscaria* as an estimate in purification or biosynthetic experiments.

RESULTS AND DISCUSSION

A reliable and quantitative separation of ibotenic acid and muscimol could be achieved using a microbore column and a citrate-buffer elution system as described in the legends to Figs. 3 and 4.

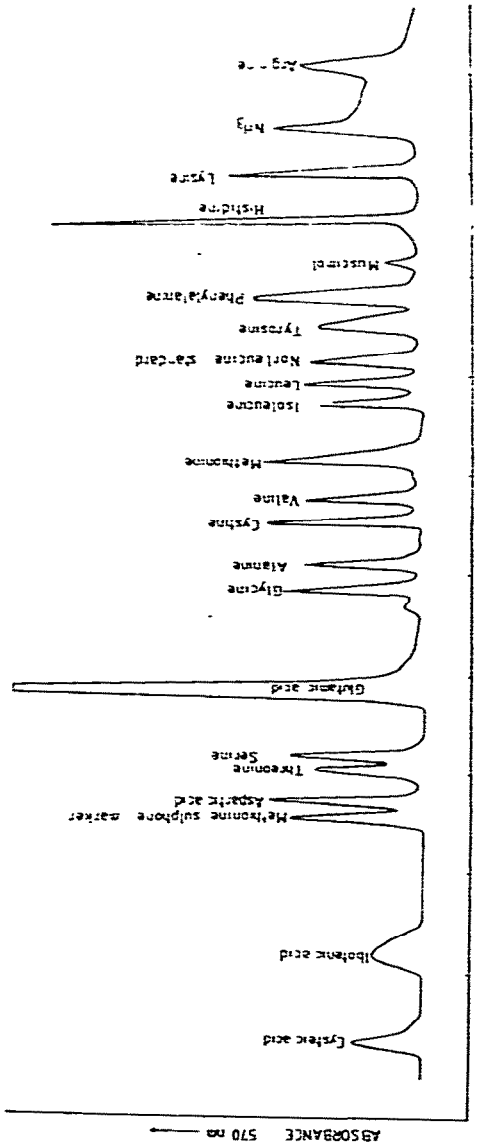

Fig. 3. An elution profile of a standard mixture of amino acids also containing ibotenic acid and muscimol. All amino acids were present at 10 nmol/ml in the sample with the exception of L-glutamate which was present at 50 nmol/ml.

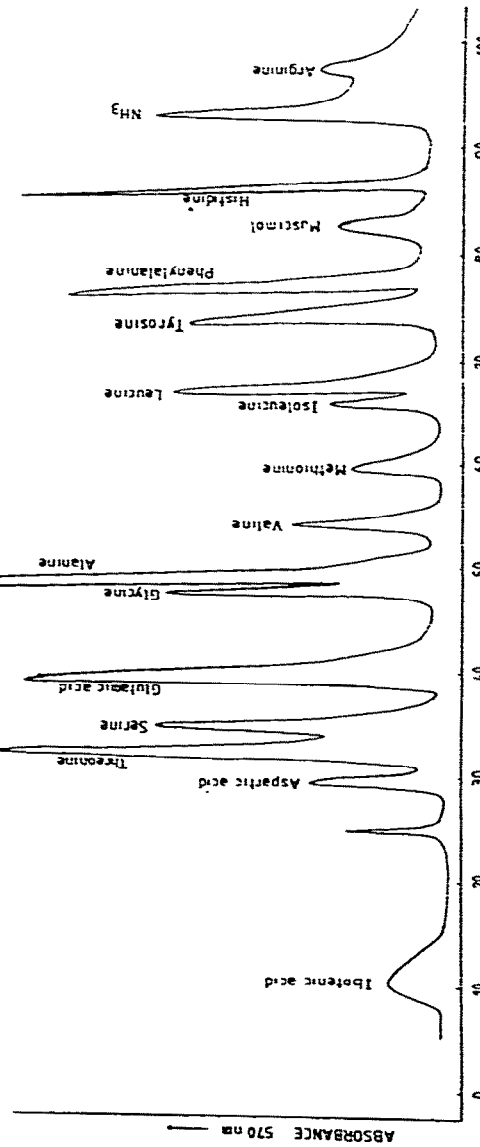

Fig. 4. An elution profile given by a protein free extract of *Amanita muscaria*. Dried fungus was deproteinised by 95% ethanol and the supernatant from a 30-min centrifugation at 20,000 *g* was evaporated to dryness and the amino acids redissolved in 20 m*M* HCl (500 μl).

The loading buffer system employed was at an initial pH of 1.8 to enhance the binding of the ibotenic acid. Under the conditions used, ibotenic acid had a retention time of 11 min and muscimol chromatographed near histidine with a retention time of 83 min, Fig. 3. The colour coefficients for both compounds determined under the above conditions were 0.42 and 0.48 for ibotenic acid and muscimol respectively relative to the standard norleucine (colour coefficient = 1.0). Although the ibotenic acid is retained by the resin, the affinity is relatively weak as exemplified by early elution from the column and the tailing of the peak, Fig. 4. Attempts to lower the pH to increase binding of ibotenic acid promotes the partial decarboxylation to muscimol. The pH chosen thus represents a compromise which allows satisfactory binding without decomposition. The method was used to determine the levels of ibotenic acid and muscimol in different parts of the mushroom, *Amanita muscaria* (Table I). The yellow region under the red cap cuticle of the mushroom has the highest concentration of ibotenic acid (548 nmol/g) with the white flesh below also containing substantial quantities (153 nmol/g). Knowledge of the distribution of the alkaloids was of great importance in the purification of the compounds since it allowed the portions of the mushroom richest in the alkaloids to be removed prior to homogenisation, thus greatly lowering the amount of starting material required.

The method was also of use for determining the levels of ibotenic acid and muscimol in the whole mushroom and for monitoring the purity of the isolated alkaloids with respect to amino acid impurities (Figs. 3 and 4).

Ibotenic acid is unstable in crude extracts or in acid due to enzymic and non-enzymic decarboxylation to muscimol. Fig. 5 shows the result of the decarboxylation process proceeding under mild conditions. Here 50 nmol of ibotenic acid were incubated for 0 min (Fig. 5a) or for 240 min (Fig. 5b) in 20 m*M* HCl at room temperature (21°C). The acid catalysed decarboxylation of ibotenic acid to muscimol is therefore a source of error in the estimation of either amino acid if for example an acidic protein precipitation procedure precedes the estimation. Under the conditions of Fig. 5 the yield of muscimol is almost quantitative with no indication of unwanted side products.

TABLE I

DISTRIBUTION OF IBOTENIC ACID AND MUSCIMOL IN MATURE MUSHROOM OF *AMANITA MUSCARIA*

Weighed amounts of tissue from various parts of the mushroom were homogenised in two volumes of water. The homogenate was stirred for 30 min and then centrifuged for 30 min at 20,000 *g*. An aliquot (100 μl) of the supernatant was deproteinized by the addition of 400 μl of ethanol and after removal of the precipitate the supernatant was freeze dried. The residue was redissolved in 20 m*M* HCl (500 μl) prior to microbore column analysis. Values quoted are nmol/g wet weight tissue and are calculated from the area of the trace as described.

Tissue sample	*Ibotenic acid (nmol/g wet weight)*	*Muscimol (nmol/g wet weight)*
Pigmented cuticle	None detectable	113.9
Yellow flesh	548.0	366.0
White flesh	153.0	308.7
Gill	73.0	365.0
Stalk —cross section	48.4	79.9

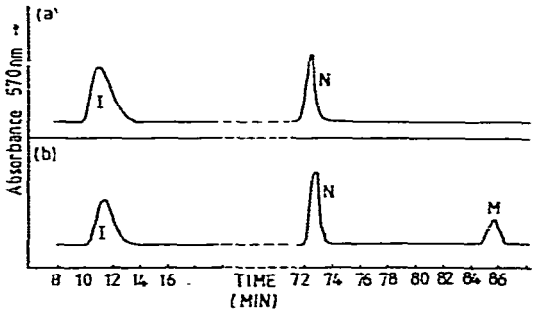

Fig. 5. Evidence of acid catalysed decarboxylation of ibotenic acid to muscimol. A solution of ibotenic acid 50 nmol ml and norleucine (20 nmol ml) in 20 m*M* HCl (21°C) was analysed immediately (a) and then after 240 min at 21°C (b). Trace b shows a peak co-chromatographing with muscimol. Peaks: I = ibotenic acid; N = norleucine; M = muscimol.

In summary we have developed a reproducible and quantitative method for the determination of the alkaloids ibotenic acid and muscimol which is sensitive enough to detect down to 30 ng of compound, and which allows the estimation of the alkaloids in crude extracts of *Amanita muscaria*. The method has the potential of further development for use in the estimation of the alkaloids in biological fluids such as blood or urine thus allowing the evaluation of kidney clearance or metabolism of the compounds under *in vivo* experimental conditions.

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