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Journal of Pharmaceutical and Biomedical Analysis
22 (2000) 711–715

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Short communication

# HPTLC determination of tryptophanase

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Received 26 November 1998; received in revised form 11 August 1999; accepted 26 August 1999

*Keywords:* Tryptophanase; Indole; HPTLC

## 1. Introduction

Tryptophan metabolites such as indole, skatole are possibly involved in the development of chronic diseases such as rheumatoid arthritis [1], colon carcinogenesis [2]. Tryptophan indole lyase (E.C 4.1.99.1) produced by the intestinal flora is responsible for deamination of L-tryptophan to indole [3]. Indian medicinal plants are claimed to have a preventive or curative effect on these chronic diseases [4]. A number of such medicinal plant extracts were therefore screened for the presence of inhibitors of the tryptophan indole lyase. The determination of its activity is usually based on estimation of the indole, which is enzymatically produced according to the following reaction (Fig. 1). While screening a number of plant extracts, it is essential to separate the indole from the interfering plant constituents for an accurate indole quantitation.

Various analytical methods such as colorimetry [5] using aldehydes in strong mineral acids or nitrosoindole derivative [6], spectroflurometry [7] to measure the indole released by the enzyme have been reported. A subsequent rapid and sensitive LC method [8] obviated the pre-separation of indole, but still needed sample clean up to remove the interfering constituents of plant extracts tested for inhibition, making the procedure unsuitable for the screening. This led to the development of a high performance thin layer chromatographic method for estimation of enzyme catalysed indole, in presence of any interfering plant constituents. HPTLC provides visualisation of the separated components of the sample. It provides on line identification of the analyte by in situ spectrum scanning and post chromatographic derivatisation along with $R_f$ comparison with the standard. It requires very little sample clean up since the layer is disposable. Several samples can be run simultaneously using a small quantity of mobile phase, thus lowering time and cost per analysis. The present work describes a simple yet sensitive, specific and reproducible HPTLC method for measuring the tryptophanase activity based on the indole formation.

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PII: S0731-7085(99)00306-4

## 2. Experimental

### 2.1. Reagents and chemicals

Analar grade solvents were purchased from Ranbaxy Chemical Ltd. Delhi (India). Tryptone, yeast extract, glucose required for the enzyme production were purchased from Hi Media (India). Indole and L-tryptophan were purchased from Sisco Research Laboratories, India.

### 2.2. Instrumentation

A Remi cyclomixer was used for mixing and vortexing of the samples. The samples were spotted on Camag HPTLC aluminium plates with silica gel 60 $F_{254}$ (E. Merck) with a layer thickness of 0.2 mm using a Camag Linomat Model IV. The samples were streaked in the form of narrow bands of 3 mm length at a constant rate of 15 s/μl using a nitrogen aspirator. The solvent system [9] consisted of benzene and acetone (9:0.1 v/v). The length of the chromatographic run was 7 cm and the time required for each run was approximately 10 min. The separation was visualised by irradiation of the plates with a 254-nm ultraviolet lamp. Densitometric analysis of the separated indole was carried out using the Camag TLC scanner II in the absorbance mode (276 nm). The scanning speed was kept at 1 mm/s. Integration of the chromatogram was performed using the Camag TLC scanner–integrator system LCI-100.

### 2.3. In situ identification of enzymatically liberated indole

The initial peak identification was done by comparison of the $R_f$ value. The identification was confirmed by co-chromatography of the standard indole and sample, and by recording the in situ spectrum of the two.

### 2.4. Sample preparation

#### 2.4.1. Standard curve of indole in toluene

Stock solutions of indole (50 and 5 μg/ml) were prepared in toluene. Appropriate quantities of these stock solutions were spotted to obtain indole in the range of 10–400 ng.

#### 2.4.2. Sample preparation for the enzyme assay

The *Escherichia coli* cells enriched in tryptophanase were produced in the laboratory using the reported procedure [3,5]. The cells were sonicated (Branson Sonifier-450). The supernatant obtained after centrifugation at 10 000 rpm (20 min, 5°C) was used as the enzyme source. Portions of this solution were first incubated at 37°C during 10 min with 0.2 ml of the pyridoxal phosphate solution (100 μg/ml) and then with 0.3 ml of a 0.01 M L-tryptophan solution for 60 min. The reaction was stopped by adding 0.3 ml of 2 M sodium hydroxide solution. The enzymatically liberated indole was extracted with 1 ml of toluene by mixing (4 min). The toluene layer, separated by centrifugation, was subjected to HPTLC analysis.

### 2.5. Validation of the assay

#### 2.5.1. System and method precision

System precision of the assay was tested at 50 and 100 ng level of indole and method precision study was carried out by using independent sample ($n = 6$) of indole in toluene at 50 and 100 ng. A sample of enzyme of 200 U per 0.3 ml was checked for the method precision. (1 U is the amount of enzyme required to produce 1 μmol of the product per minute at 37°C).

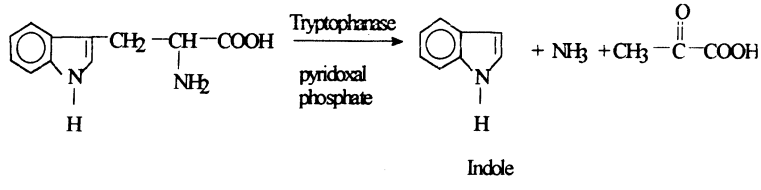


Fig. 1. Reaction catalysed by tryptophanase in presence of cofactor.

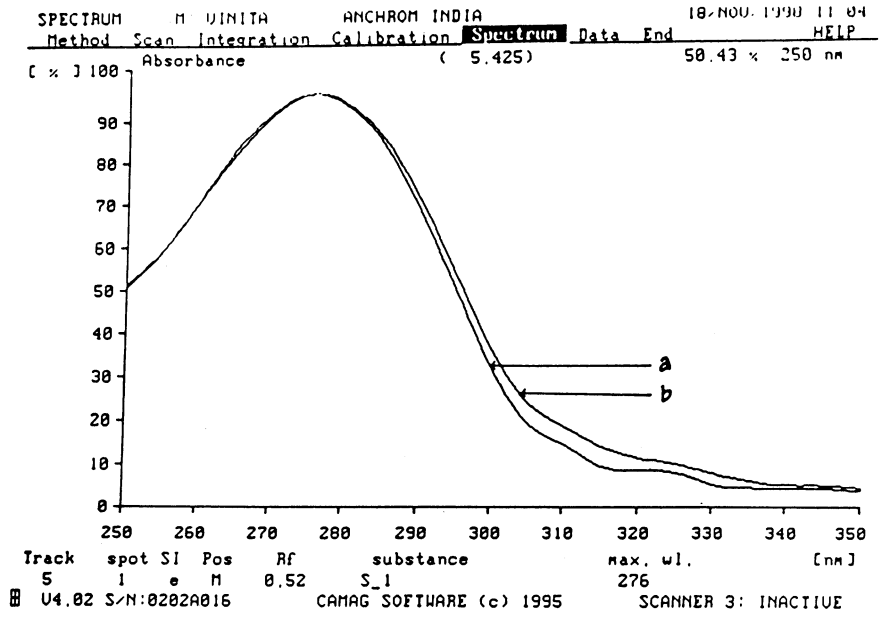


Fig. 2. In situ spectrum matching of enzyme catalysed indole. This confirms the identification of the enzyme-liberated indole.

*2.5.2. Limit of detection and limit of quantitation*

LOD was determined on the basis of signal to noise ratio. Mean of 15 noise peak areas and their absolute standard deviation values are determined. LOD is the amount of applied sample producing a peak area, which is equal to the sum of mean blank area and three times its S.D.

LOQ is the amount of applied sample producing a peak area, which is equal to the sum of mean blank area and ten times its S.D.

*2.5.3. Stability of indole in solution*

Solutions of same concentration were prepared in toluene and water and stored at room temperature for the durations of 30, 60, 120 and 240 min. These were then applied on the same chromatoplate. After development the chromatographic peaks were evaluated for the relative standard deviation between their peak areas.

*2.5.4. Extraction efficiency of the indole from the aqueous phase*

Appropriate aliquots of stock solution of indole in distilled water (10 μg/ml) were pipetted out in the glass stoppered centrifuge tubes. Enzyme solution (0.3 ml), 0.2 ml of pyridoxal phosphate (100 μg/ml), 0.3 ml of phosphate buffer (pH 7.8, 0.05 M) and 0.3 ml of 2 M sodium hydroxide were added. To each tube 1 ml of toluene was added and indole was extracted by vortexing during 4 min. The toluene layer was separated by centrifugation for 5 min at 3000 rpm. Twenty microlitres of these solutions was spotted to obtain concentrations of 50 and 100 ng, respectively.

*2.5.5. Linearity range*

The linearity of the standard curve was determined from 10 to 400 ng. The data was evaluated by the polynomial regression analysis.

*2.5.6. Ruggedness of method*

The, within day and between day variation of the assay at concentrations of 200 and 300 ng, was determined to check ruggedness of the method.

## 3. Results

*3.1. Optimisation of chromatography*

The HPTLC method developed was applied to

discover inhibitors of the tryptophanase enzyme from medicinal plants. As the method involved extraction of indole by toluene, plant constituents might be extracted resulting in interferences during the HPTLC process. The mobile phase was optimised in such a way that the indole could be quantified without any interferences (Fig. 3). The $R_f$ value of $0.53 \pm 0.01$ was obtained using benzene–acetone (9:0.1 v/v) as the eluent.

### 3.2. In situ identification of the indole

The indole produced by enzyme reaction of tryptophanase was identified with standard ($R_f = 0.53 \pm 0.01$). The increase in the area of the peak after co-chromatography confirmed the same. The in situ spectrum scanning of the reference and enzyme-liberated indole, revealed the spectral matching ($\lambda_{max} = 276$ nm) as depicted in Fig. 2. The spot of indole was further identified by its color reaction with the Ehrlich reagent [8].

### 3.3. Validation of the method

#### 3.3.1. Accuracy and precision studies

The relative standard deviation at 50 and 100 ng were found to be 1.33 and 0.98%, respectively, indicating high precision of the method. The accuracy of the method was examined at 50 and 100 ng levels of indole in toluene and at 200 U per 0.3 ml of the tryptophanase solution. The relative standard deviation was found to be 1.42, 1.07 and 5.33% respectively.

#### 3.3.2. Limit of detection and quantitation

The limits of detection and quantitation were 10 and 50 ng, respectively.

#### 3.3.3. Standard curve of indole

The polynomial regression data for the calibration plots ($n = 6$) showed linear relationship over a range of 50–300 ng. The mean values of correlation coefficient, slope and intercept were

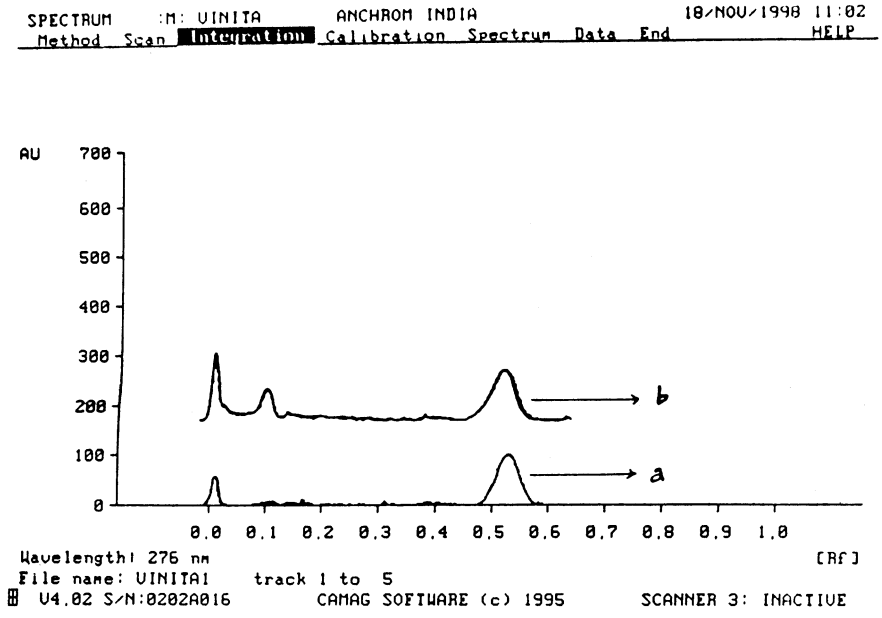


Fig. 3. Typical chromatogram of indole (a), indole in presence of the interfering substances (b), from plant phytoconstituents. The chromatogram shows well resolved peak of indole from that of the interference.

0.996 ± 0.0014 (S.D.), 1605.92 ± 24.33 (S.D.) and 78 411.2 ± 2038.025 (S.D.).

*3.3.4. Stability of indole in solution*

The stability of the analyte in the solution and during the analytical procedure ensures the validity of the method. As indole polymerises to form diindole, triindole and undergoes resinification under acidic conditions [10], the stability of indole in toluene and aqueous mediums at various time intervals, at room temperature were checked. A low R.S.D. of 0.23 and 0.42% at 100 ng level indicated stability of indole in toluene and water, respectively, over the assay period.

*3.3.5. Extraction efficiency*

This study was undertaken to determine the extraction efficiency of indole from the enzyme solution using the described method. The results indicated that the recovery of indole using the described extraction method was 98.2 (R.S.D., 2.45%) at 50 ng, 98.27 (R.S.D., 1.75%) at 100 ng.

*3.3.6. Ruggedness of the method*

The coefficient of variation for within day and between day analysis was 2.53 and 3.96% for 200 ng and 1.76 and 2.3% for 300 ng, respectively.

## 4. Discussion

Colorimetric methods for quantifying indole, based on its reaction with aldehydes in presence of strong mineral acid [1], or sodium nitrile [5] have been found to be linear over a narrow range with limit of quantitation 5 and 0.05 μmol/ml, respectively. Moreover, these methods cannot be applied for screening inhibitors from medicinal plants because of the interferences arising from the plant extracts. The spectroflurometric method reported [6] was not found to be suitable for this application, as many phytochemicals themselves are flurogenic thus giving poor specificity.

The present HPTLC method overcomes the problem of narrow linearity range. The chromatographic separation before quantitation permits determination of low levels of indole without any interferences. Although, the LC method [7] obviated the pre-separation of indole, it still needed sample clean up to remove the interfering plant constituents. HPTLC affords little sample clean up as the layers are disposable. The low limit of quantitation enables to measure around 80% inhibition of the enzyme exhibited by some medicinal plant extracts under investigation. Plate to plate variation and limit of quantitation (as compared to LC) can be limiting factors against the use of HPTLC as a method of analysis. However this limitation can be overcome by spotting known concentrations along with the sample in each chromatographic run. The minimum solvent consumption, off line technique, zero waiting time for instrument set up makes the method more suitable for the present application and thus can be a method of choice for routine analysis.

## Acknowledgements

The authors wish to thank UGC/DSA New Delhi, India for financial assistance to the Pharmaceutical Division. One of the authors, VPK is grateful to U.G.C., Ministry of Education, New Delhi, India for senior research fellowship.

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