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Spectrophotofluorometric Analysis of 2-[[3-(Trifluoromethyl) phenyl]amino]-3-pyridine Carboxylic Acid in Urine

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Abstract□ A sensitive spectrophotofluorometric method was developed for the analysis of 2[[3-(trifluoromethyl) phenyl]amino]-3-pyridine carboxylic acid (I) in urine. The method is based on the fluoroscence behavior of the I-aluminum complex in absolute ethanol. This fluorophore has activation and emission wavelengths of 355 and 450 nm, respectively. Optimum conditions for the reaction were investigated. The fluorescence was linear in the range of 0.25 3.0 ug of I/ml. Replicate studies of spiked urine samples, each containing 2.5 ug of I/ml showed good precision with a relative standard deviation of 0.019. Overall recovery percent from five spiked urine samples was 99.4 ± 1.32 %.

Keywords□ Spectrophotofluorometric analysis —2[[3-(trifluoro methyl) phenyl] amino]-3-pyridino carboxylic acid—Antiinflammatory agent—metal chelate fluorescence.

2[[3-(Trifluoromethyl)phenyl]amino]-3-pyridine carboxylic acid(I) was described by Hoffmann and Faure in 1966[1]. It is a pyridic isotere of the flufenamic acid and its antiinflammatory and analgesic activity has been clearly shown in animal and man by many investigators[2,3,4]. This product is now crurently used in human therapy. For pharmacokinetic studies on (I) and its dosage forms, a simple, yet sensitive analytical procedure for (I) in urine is needed. Previous determination of (I) in body fluids were based on UV assay which had been used with dog plasma samples and also liquid scintillation counting was used to study ^{14}C-I metabolism. In order to confer greater specificity on the method, a gas chromatographic method was developed. This involved chromatographing (I) as its trimethylsilyl ester. Recently, Cowen and Salmon reported the assay method of (I) in plasma and urine by high performance liquid chromatography using reversed-phase columns. However, development of assay methods for drugs in body fluids is largely governed by speed and ease of use along with high sensitivity. Thus in many cases an assay involving a suitably simple extraction followed by fluorescence measurement often represents the ideal.

This paper reports the sensitive spectrophotofluorometric analysis of (I) in urine based on its complex formation with aluminum chloride in absolute ethanol.

EXPERIMENTAL

Materials and Reagents

Five hundred milligrams of aluminum chloride, anhydrous (Wako pure chemical.) were dissolved in 100 ml of spectrograde anhydrous ethanol (Merck) and stored at room temperature. A pH 4.0 buffer solution was prepared by adding 0.05M sodium tartrate to 0.1N tartric acid using a pH meter. Powdered samples of (I) (Squibb & Sons Co.) were used in the preparation of standard solutions.
All other chemicals utilized were the highest grade of the commercially available materials.

Apparatus

Fluorescence measurements were made with Shimazu RF-510 Spectrofluorophotometer equipped with 150W xenon arc lamp energy source and R452 photomultiplier.Fluorescence spectra were recorded on Varian model 1976 laboratory strip chart recorder. Fluoresce nce intensity is presented in arbitrary units and spectra have not been corrected.

Spectrofluorophotometer was calibrated daily using a solution of 0.1 per cent quinine bisulfate in 0.1N H_2SO_4. UV absorption spectra were made with an Pye Unicam SP8-100 spectrophotometer with 1-cm quartz cells.

Mettler microbalance H54AR, Fisher Accumet 230A pH/ion meter and Vortex Genie mixer were also employed.

Preparation of Standard Curve

Five milligrams of (I) were weighed accurately and dissolved in 100.0 ml of absolute ethanol. Then a series of standard solutions containing 0.1–2.5 μg of I/ml was prepared and 1.0 ml of the each standard solution was pipeted into a 10-ml glass-stoppered volumetric flask.

To this flask, 0.1 ml of aluminum chloride reagent was added and diluted with absolute ethanol to the mark. The fluorescence intensity was measured using excitation and emission wavelengths of 355 and 450nm against a blank, respectively.

Assay of (I) in urine

One ml of human ruine containing (I) was placed in a 15-ml stoppered volumetric flask. Two ml of tartrate buffer (pH 4.0), 8ml of ethyl ether, and 0.5 g of sodium chloride were added to the flask.

The contents were mixed mechanically using Vortex Genie mixer for one min and were centrifuged for 10 min at 3000 rpm. Six ml of the organic layer was transferred into a clean 15-ml stoppered volumetric flask and complete evaporation of the ethyl ether was done on a warm water bath at 35°C. To the residue, 0.1ml of 0.5% aluminum chloride solution and five ml of anhydrous ethanol was added and the fluorescence was measured using excitation and emission wavelengths of 355 and 450 nm, respectively.

Urine samples spiked with known concentrations of (I) were assayed under the same experimental conditions for calibration purposes.

A control urine sample was utilized to assess background fluorescence arising from biological matrix.

RESULTS AND DISCUSION

The investigation to the fluorescence of

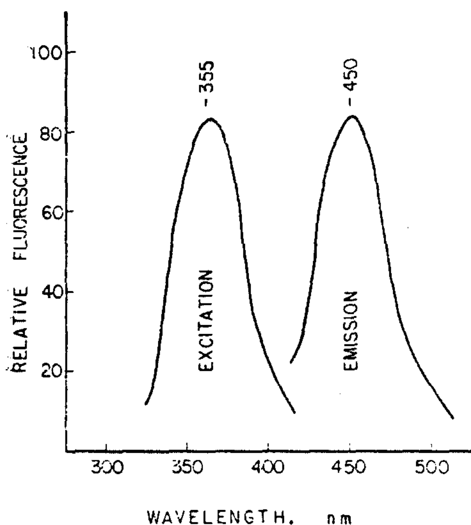

Fig. 1: Activation and emission spectra of I-aluminum chloride complex in absolute ethanol.

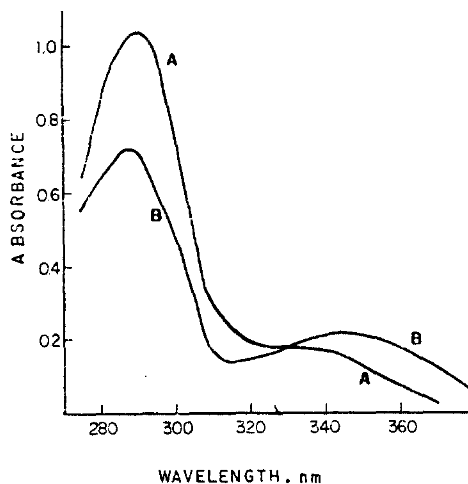

Fig. 2: Absorption spectra of I (curve A) and Ialuminum chloride complex (curve B) in absolute ethanol.

(I) alone showed that this compound demons trates native fluorescence in ethanolic solution. The fluorophore had activation and emission maxima of 355 and 440 nm, respectively. But its sensitivity was too week to be analytically useful. In the presence of aluminum chloride, on the other hand, (I) showed a intensive blue fluorescence in ethanol with a maximum activation wavelength corresponding to the native fluorescence. But the maximum wavelength of fluoroscence was shifted to the long-wavelength, 450 nm. (Fig. 1)

And its fluorescence was more sensitive about 100-times than that of native fluorescence. Fig. 2 shows the absorption spectral shift obtained when (I) was treated with condition of the assay procedure described.

From the changes in electronic absorption, excitation or emission spectra produced by the addition of aluminum ion as shown on Fig.1 and Fig. 2, it is esteemed that the fluorogens of the reaction are aluminum chelate of (I)

Then consequently, investigations are being continued on the precise structure and other analytical properties of this metal chelate in this laboratory.

The concentration optimum of the aluminum chloride reagent was determined by adding varying volumes of 0.5% ethanolic aluminum chloride anhydrous to a series of 10-ml volumetric flasks, each containing 1.0 ml of 0.5 μg of I/ml, and measuring the fluorescence against a blank after diluting each solution to volume with ethanol. Each blank contained the same amount of reagent as in the solution studied. The maximum was obtained when the concentration of the reagent was above 5×10^{-4}% in the final solution. The reaction time and temperature were determined by following

the fluorescence developement at room temperature, 37°, and 50°.

No difference in fluorescence intensity was obtained. Therefore, room temperature was chosen for the convenience without heating process. Moreover, the fluorescence of the reaction product was stable for at leats 3 hrs and showed very little, if any, decay after 24 hrs. Under the proposed experimental conditions, for the preparation of standard curve, a linear fluorescence response was obtained from 0.25 to 3.0 μg of I/ml. The application of the procedure to the determination of I in biological fluids required investigation into extractability of the drug using different conditions.

Different authors[5] had reported the usage of various pH solution for the fextraction of (I) from biological fluid with ethyl ether. Among the choices were adjusting the pH of urine containing spiked quanitites of (I) to pH 4.0 with tartrate buffer and adding the sodium chloride for the salting out agent. When the proposed method was applied to the analysis of (I) in human urine, another linear fluorescence response was made over the concentration range from 0.5 to 10 μg of I/ml due to the fluorescence derived from blank urine.

But this does not diminish the usefulness of the fluorometric assay of (I) since the detection limit of this method remains about 40-times more sensitive than that of UV for the urine samples yet.

The reproducibility of the analysis of (I) in urine was determined by analyzing nine replicate spiked urine samples. At the concentration level of 2.5 μg of I/ml, the coefficinet of variation was 0.019 (Table I).

Table I : Reproducibility of fluorophore development of (I) in spiked urine samples at 2.5 μg/ml.

Sample	Relative Fluorescence
1	24.8
2	24.1
3	25.1
4	24.6
5	24.5
6	25.1
7	23.9
8	24.2
9	23.9
Average	24.5
Coefficient of Variation	0.019

Table II : Recovery of (I) from spiked urine samples.

Amount Weighed, μg	Analyzed at Concentration Level, μg/ml	Amount Found, μg	Recovery, %
176	0.88	167	95.0
270	1.35	266	98.5
560	2.80	566	101.1
1150	5.75	1144	99.5
1260	6.30	1295	102.8
	Overa! 11 Recovery		99.4
	Standard Error of the Mean		1.32

Recovery studies of (I) from urine samples were also performed. The overall recovery of five spiked urine samples was 99.4% with a standard error of the mean of 1.32% (Table II).

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