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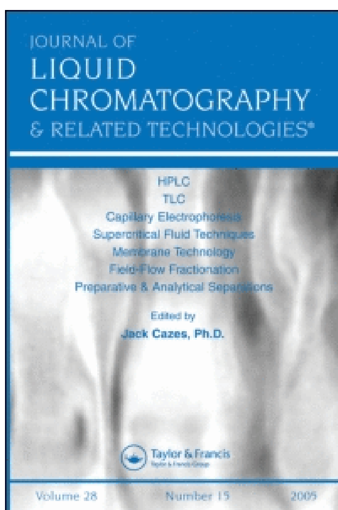

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SOLID PHASE EXTRACTION AND HPLC ANALYSIS OF TRYPTAMIDE IN PLASMA

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ABSTRACT

A method for extraction and quantification of tryptamide in plasma is described in this report. The method employs Amberlite XAD-2 column extraction followed by HPLC with ultraviolet detection. The procedure is simple, rapid and reproducible. It has been applied to the measurement of tryptamide in plasma of rats dosed orally with this antiinflammatory and analgesic compound.

INTRODUCTION

Tryptamide (N-nicotinoyl-tryptamine) is a compound synthetized by Misztal and Grabowska (1). Current studies have shown that tryptamide reveals strong antiinflammatory and analgesic properties in rats and mice (2,3). Taking also into account that its toxicity and ulcerogenic activity is very mild (4), tryptamide has a chance to be

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a valuable and safe drug, especially in therapy of rheumatism.

In order to perform pharmacokinetic studies a simple and sensitive method for the estimation of tryptamide in biological fluids is needed. The first published method (5) requires a time-consuming liquid-liquid extraction followed by insufficiently specific and sensitive spectrophotometric determination.

The present paper describes an assay of tryptamide in rat plasma based on the solid-phase extraction using a non-ionic adsorbent Amberlite XAD-2 column, and a high performace liquid chromatography with a UV detection. Th procedure is simple and reliable. It can be applied to analyse tryptamide in plasma of rats dosed orally with this compound.

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MATERIALS AND METHODS

Materials and Reagents

All solvents were HPLC grade. Acetonitrile, methanol and methylene chloride were from Merck (Darmstadt, FRG). Bidistilled deionized water was used to prepare aqueous solutions. Chlordiazepoxide (internal standard) and tryptamide were donated by Cracow Pharmaceutical Works Polfa (Poland). Amberlite XAD-2 was purchased from Rohm and Haas Co (Philadelphia, USA).

The tryptamide stock standard solutions (1.0 mg/ml) was prepared in methanol. The working standard solution

of tryptamide (10 µg/ml) was prepared in water. The solutions of internal standard (1.0 mg/ml and 10 µg/ml) were prepared in the same manner as tryptamide solutions. The water solutions of the compounds were stored in brown bottles at 4°C and made up fresh weekly. The methanol solutions were stored at -4°C made up fresh every two weeks.

Amberlite XAD-2 resin, 20-50 mesh, was cleaned by extraction in a Soxhlet extractor altering between methanol, acetonitrile, diethyl ether and finally with methanol. The dried material was moderately ground in a mortar, suspended in methanol and decanted. The suspension of the purified non-sized resin was used to prepare extraction columns.

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High Pressure Liquid Chromatography

Separations were carried out on a Techma-Robot type 302 HPLC instrument (Warsaw, Poland). The chromatograph was equipped with a stainless-steel column (250 x 4 mm I.D.) packed with octadecil silica (Lichrosorb RP-18; 10 µm particle size; Merck, FRG), which was manufactured by ZOCH (Lublin, Poland). A fixed wavelength (254 nm) UV detector (ICHF-PAN, Warsaw, Poland) was used.

The mobile phase consisted of acetonitrile and water (40:60, v/v). The flow-rate was 1.0 ml/min. The column was kept at room temperature.

<u>Solid-Phase Extraction</u>

A plug of a silanized glass wool was introduced into small pasteur pipettes (0.6 cm I.D.). On top of the plug, the pipettes were filled with the cleaned Amberlite XAD-2 resin to a height of about 5 cm. The columns were conditioned prior to use with 4 ml of methanol followed by 4 ml of distilled deionized water at a flow-rate about 0.5 ml/min. The flow-rate was regulated by changing the tightness of the plugs. The liquids were able to pass through the columns under their own gravity.

Rat plasma was used fresh, or stored frozen. 0.5 ml of plasma was spiked with a known amount of tryptamide, and 0.5 ml of internal standard working solution. Then the mixture was diluted with a physiological salt solution up to 2.5 ml, and the whole volume was pipetted gradually onto the top of Amberlite XAD-2 column. The column was rinsed twice with 2 ml of water, with the second rinse drawn down through by vacuum. Tryptamide and internal standard were eluted with two successive 2-ml volumes of methanol. The solvent was evaporated at 50°C under N_2, and the residue was reconstituted in 0.2 ml of methanol. The reconstituted samples were injected, using 5 µl of aliquots, directly into the chromatograph.

Calibration curves were generated over the concentration range 1-40 µg/ml by admixing control plasma

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with known concentration of tryptamide and internal standard. Peak heights were determined for each standard and sample from the chromatograph. A least-squares regression analysis was performed by plotting peak height ratios (tryptamide:internal standard) of the standards on the ordinate vs. the concentration (μg/ml) of the calibration standards on the abcissa. The concentrations of tryptamide in the samples were determined from the calibration curves.

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Animal Experiments

Male Wistar rats weighing 200-250 g were used. The animals were maintained on a standard laboratory diet , and fasted for 16h prior to experiment. The rats were given per os 4 ml of suspension of tryptamide (25 mg/kg) in 2% solution of methylcellulose. At various intervals (5-300 min) rats were decapitated, and blood samples were collected in heparinized test tubes. Blood plasma was obtained by centrifugation.

RESULTS

Typical chromatograms of a rat control plasma extracts are shown in Fig. 1. The retention times for tryptamide and the internal standard are 5.0 and 9.1 min, respectively. No peaks are present on the chromatogram of the drug-free pooled plasma which could disturb the determination of tryptamide.

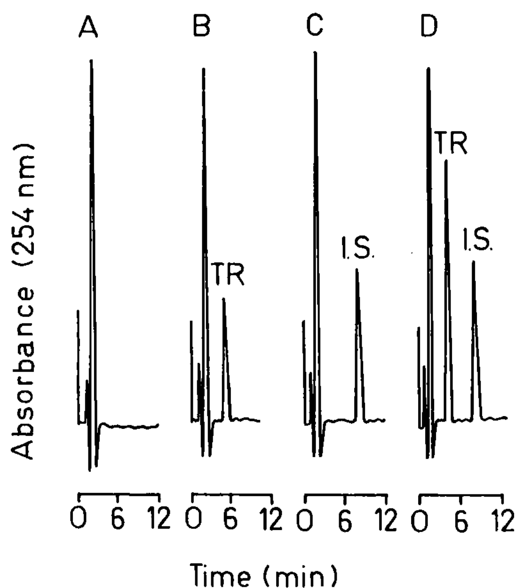

Figure 1. Typical chromatograms of extracted blank rat plasma (A), extracted rat plasma spiked with: 5 µg/ml of tryptamide (B), 5 µg/ml of internal standard (C), 10 µg/ml of tryptamide and 5 µg/ml of internal standard (D). Retention times: tryptamide (TR) - 5.0 min, internal standard (IS) - 9.1 min.

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Next experiments aimed at establishing an elution selectivity and effectiveness of various organic solvents in relation to tryptamide and internal standard. The pooled plasma samples spiked with the said compounds (10 µg of each) were parallely passed through three similar Amberlite XAD-2 columns, and eluted with the following solvents: methanol, acetonitrile and diethyl ether. Fractions of 1 ml of the eluates were collected and treated as described in the Methods. The results of

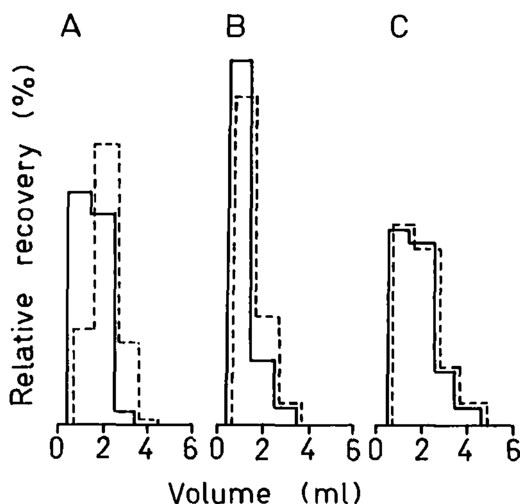

Figure 2. Elution profiles of tryptamide (—) and internal standard (---) from Amberlite XAD-2 columns. Samples of each compound (10 µg/ml) were parallely applied on three analytical columns, and eluted with methanol (A), acetonitrile (B) and diethyl ether (C). Each fraction (1 ml) was evaporated, dissolved in 200 µl of methanol, and 5 µl aliquot was injected into HPLC apparatus.

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the experiments are presented at Fig. 2. It is visible that with each solvent used most of the compounds applied on the columns are eluted within first two 1-ml fractions. Similar pictures were also obtained with acetone, ethyl acetate and methylene chloride (data not shown). We chose arbitrarily methanol as a mobile phase, and used to collect 4 ml of the eluate for the recovery precautions.

Six point calibration curves of tryptamide subjected to multiple determinations on alternating days using

TABLE 1.

Linearity and Precision of Calibration Curves

Curve	Slope	Y Intercept	Correlation Coefficient
1	10.1272	-0.0758	0.9995
2	10.4179	0.1069	0.9947
3	11.4580	0.0375	0.9998
4	10.8040	0.1176	0.9965
$\bar{x}$	10.7017	0.0470	0.9976
SD	0.5753	0.0360	
CV(%)	5.380		

TABLE 2.

Accuracy and Precision of the Tryptamide Assay

Concentration added (µg/ml)	Concentration found (µg/ml) (mean ± SD)	Assay Precision (CV%)	n
Within run			
4	3.65 (0.23)	6.3	4
12	11.47 (0.58)	5.1	4
36	35.36 (0.76)	2.1	4
Within day			
4	3.60 (0.31)	8.61	3
36	35.61 (1.27)	3.6	3

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contol rat plasma are linear (correlation coefficient 0.9947-0.9995) over the working range 1-40 µg/ml (Table 1). The mean regression equation is y = 10.7017 + 0.0470, where x and y represent the peak height ratio tryptamide/ internal standard and tryptamide concentration (µg/ml), respectively. The limit of detection of this method is 0.3 µg/ml. Levels below this limit can be measured, if desired, by increasing the injection volume, and/or diminution the volume of the reconstituted sample.

Accuracy and precision of the assay were tested by using rat plasma spiked with 4 to 36 µg/ml of tryptamide. The data presented in Table 2 show that the obtained concentrations varied from 91.29% to 98.22%, and from 90.0% to 98.91% in relation to within run and within day, respectively.

Amberlite XAD-2 resin exhibits a considerable capacity for tryptamide, since as much as 150 µg of this compound in spiked plasma aliquot was fully retained by the column. There is also possibility to process different volumes of plasma (within 0.1-2.0 ml tested) without appreciate effect on accuracy of the assay. Noteworthy is that pH of the sample to be analyzed did not significantly affect the adsorption of tryptamide at least in the range tested, that is.between 3.7 and 10.5.

The application of the assay is demonstrated (Fig. 3) with the plasma tryptamide concentration-time profile

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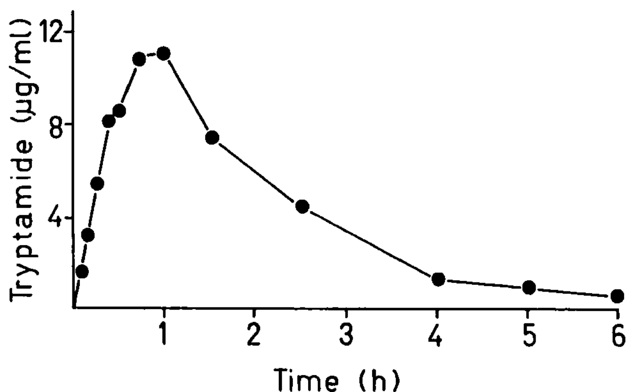

Figure 3. Plasma level of tryptamide following a single oral dose of 25 mg/ml (n = 4).

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after single dose of 25 mg/kg of tryptamide. A mean peak concentration of tryptamide (11 μg/ml) was observed after 60 min following administration. The area under the concentration vs. time curve was approximately 24.18 (μg/ml).h.

DISCUSSION

Over the past decade solid-phase extraction (SPE) has emerged as a powerful tool for isolation and purification of different compounds including drugs and their metabolites. Trace quantities of these compounds can be extracted efficiently from complex sample matrices like, for instance, plasma, urine, or tissue. Three groups of sorbents have mainly gained the acceptance for the SPE technique: non-ionic macroporous resins, bonded silicas,

and recently ISRP supports (6,7). Among the first group of sorbents, styrene-divinylbenzene polimer Amberlite XAD-2 has been especially widely used since early seventies, at first for the qualitative (8), then for the quantitative (9) extraction of drugs from biological material.

In this work, the minicolumns made of ordinary pasteur pipettes and loosely packed with Amberlite XAD-2 have been successfully used for the extraction of tryptamide from rat plasma in order to quantify it by HPLC. The extracts are very clean, so there are no problems with the determination of tryptamide. The extraction procedure is simple, fast and cheap; the columns can work almost infinitely. It can be recommended for modestly equipped laboratories which do not possess an automated SPE system, like that distributed by Du Pont (10).

The recovery, precision and accuracy of the assay are good enough to use it for the pharmacokinetic studies with tryptamide in animals, and possible in humans. It is believed that the proposed assay has many advantages over previously described one (5).

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