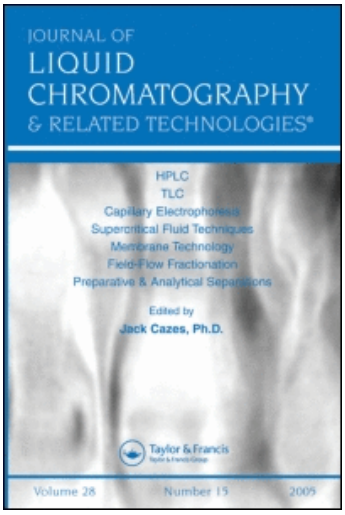

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Halina Szumilo[a]; Tadeusz Dzido[a]
[a] Department of Inorganic, Analytical Chemistry Medical Academy, Lublin, Poland

To cite this Article Szumilo, Halina and Dzido, Tadeusz(1992) 'Demonstration of Tryptamide and its Metabolites with Solid Phase Extraction, TLC, and HPLC in Rats', Journal of Liquid Chromatography & Related Technologies, 15: 2, 337 — 349
To link to this Article: DOI: 10.1080/10826079208017174
URL: http://dx.doi.org/10.1080/10826079208017174

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(2), 337-349 (1992)

DEMONSTRATION OF TRYPTAMIDE AND ITS METABOLITES WITH SOLID PHASE EXTRACTION, TLC, AND HPLC IN RATS

HALINA SZUMILO AND TADEUSZ DZIDO
Department of Inorganic and Analytical Chemistry
Medical Academy, Staszica 6
Lublin 20 081, Poland

ABSTRACT

Tryptamide is a compound of potent anti-inflammatory and analgesic activity. It is now under pharmacokinetic and metabolic studies in animals. We describe here a sorbent extraction method for TLC and HPLC analysis of tryptamide and its four partially identified conjugated and non-conjugated metabolites in urine, plasma and tissue of rats. The extraction was carried out by passing the samples through pre-cleaned Amberlite XAD-2 resin columns, washing them with saline and eluting with methanol. The conjugated metabolites were cleaved using beta-glucuronidase and sulfatase. The HPLC separation was carried out with Lichrosorb RP-18 and isocratic elution in three solvent systems: I, acetonitrile-water (40:60); II, acetonitrile-water (24:76); III, acetonitile-NH_4HPO_4 (pH 2.7)(16:84). Detection was accomplished with a UV detector at 254 nm.

INTRODUCTION

In contemporary therapy non-steroid anti-inflammatory drugs and non-narcotic analgesics have gained popularity and wide acceptance. Among of them are these having indole ring in the molecule, such as indomethacin (and its

structural analogs sulindac and clomethacin) and indoprofen (1). Main drawbacks of these drugs are their toxicity and ulcerogenic activity.

In several reports which appeared in 1987 (2-5) a detailed pharmacological characterization of tryptamide (N-3-pyridoyl-tryptamine), a compound long ago synthetized by Misztal and Grabowska (6), was described. It was demonstrated that tryptamide (TR) exhibits potent anti-inflammatory and analgesic properties in rats and mice, and that its toxicity is low (4,5). Pharmacokinetic studies carried out by us (7,8) and others (9) showed effective absorption of TR from gastrointestinal tract and its considerable fast elimination in rats and rabbits.

Preliminary metabolic study of TR was performed in rats (10). Several metabolites were found to occure in urine following po or ip administration. Spectral analysis indicated that they probably are hydroxylated derivatives, having hydroxyl groups at either indole (A,D), or pyridine (B,C) rings of basic TR structure (Fig. 1).

Here, we describe TLC and HPLC analysis of TR metabolites mostly isolated from urine, and from oher materal of rats as well.

MATERIALS AND METHODS

Materials and Reagents

Tryptamide (TR) base was donated by Pharmaceutical Works Polfa (Krakow, Poland). For animal experiments, TR

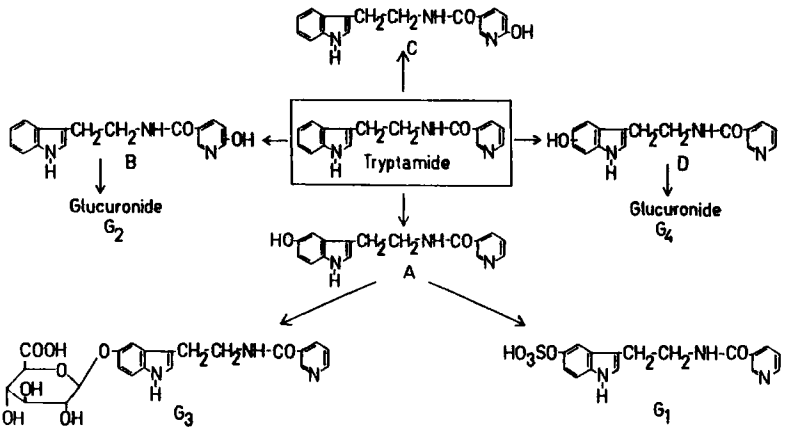

Figure 1. Preliminary scheme of metabolic pathways of tryptamide in rats.

was dissolved in a mixture of propylene glycol-ethanol-saline (4:1:5), or suspended in 1% methylcellulose solution. Beta-glucuronidase also containing sulfatase activity was a Sigma (USA) product. Lichrosorb RP-18 (10 um particle size) and silica-gel 60 F254 plates were from Merck (Germany). Amberlite XAD-2 was purchased from Rohm and Haas Co.(USA). The resin was cleaned as previously described (7).

Male Wistar rats, waighing 200-250 g, were used throughout the experiments. The animals were maintained on a standard granulated diet and water ad lib. They were kept individually in metabolic cages. After administration of 5 ml-portions of TR preparation (po - by

stomach-tube), 24-h urine samples were colleted in bottles containing azide and toluene as preservatives.

Isolation of urine metabolites

Three groups of rats were used: 1, control (non-treated); 2, treated with TR via po route; 3, treated with TR via ip route. Each animal received 100 mg/kg of TR, and the period of urine collection was 24 h. The portions of filtered urine (20 ml) were passed through Amberlite XAD-2 columns (1 x 12 cm). The columns were washed with water, the metabolites were eluted with methanol (30 ml) and the eluates were concentrated to about 2 ml at 50°C under N_2 (crude extract). These were maintained at -18°C until processed, or subjected to separation and isolation of metabolites using TLC. Following TLC of the 100 ul crude extract samples, the bands of separated metabolites were scraped off and eluted with methanol.

To split the conjugated metabolites, aliquots (100 ul) of the crude extracts were evaporated to dryness, the residues were suspended in 1 ml of 0.5 M acetate buffer, pH 4.7 and treated with 500 ug (200 units) of beta-glucuronidase. The tubes were stoppered and the mixtures were incubated at 37°C for 16 h in the toluene atmosphere. Afterwards, the precipitates (if present) were discarded by centrifugation and the supernatants were passed through pre-cleaned Amberlite XAD-2 columns (7). The eluates were subjected to TLC or HPLC analysis. Samples non-treated with the enzyme were run parallely for comparison.

Extraction of TR metabolites from blood, liver and microsomal incubation mixture

Samples of liver were homogenized in 1.15% KCl-25 mM phosphate buffer, pH 7.2. The homogenates were centrifuged for 20 min at 10 000 x g. Aliquots (1 ml) of the material (tissue extract, blood plasma) were treated with beta-glucuronidase and passed through Amberlite XAD-2 columns (7).

Microsomal fraction was prepared from livers of rats previously treated with phenobarbital at a dose of 80 mg/kg for three consecutive days. A standard ultracentrifugation technique was applied for this purpose. Metabolism in vitro was performed using incubation mixture which contained in 5 ml: phosphate buffer, pH 7.4, 300 umol; NADP, 1.5 umol; glucose-6-phosphate, 25 umol; nicotinamide, 50 µmol; glucose-6-phosphate dehydrogenase, 0.5 unit and the microsomal fraction, about 15 mg protein. The reaction was started by adding 50 µl of 0.1 M TR in methanol. After the incubation at 37°C in a reciprocating shaker (usually 15 min), the samples were cooled in an ice-bath and the metabolites as well as the remaining TR were extracted and cleaned using the Amberlite XAD-2 technique (7).

Chromatographic analysis

TLC of TR metabolites was performed against available indole and pyridine derivatives. Two solvent systems were elaborated: I, chlroform-methanol-acetic acid (70:20:5) and II, chloroform-methanol-acetic acid (80:10:2.5). Spots were detected by sprying the plates with an Ehrlich

reagent containing 250 mg of p-dimethylaminobenzaldehyde in 100 ml of a mixture of ethanol and conc. HCl (1:1). The spots were coloured blue or red-blue. The chromatograms could be preserved several months when covered with glass plates and stored in plastic bags in the darkness.

Most of HPLC experiments were carried out using an apparatus and Lichrosorb RP-18 prepacked column (4 x 250 mm) as previously described (7). Some of them were performed with a Hewlett-Packard Model 1050 liquid chromatograph (Palo Alto, USA) containing a 20 µl sample injector, Lichrosorb RP-18 column (4 x 100 mm) and a spectrophotometric detector. Three solvent systems were applied: I, acetonitrile-water (40:60); II, acetonitrile-water (24:76) and III, acetonitrile-$NH_4H_2PO_4$(pH 2.7)(16:84).

Tentative balance determinations of TR metabolites excreted in urine

The animals received 25 mg/kg of TR via femoral vein at local anasthesia and urine was collected after 10 h, 1, 2 and 3 days. Afterwards, 1 ml-samples of non-treated and beta-glucuronidase-treated urine were processed using Amberlite XAD-2 columns as described before. HPLC of the isolated metabolites was performed in the solvent system II. A percentage of the particular metabolite was estimated by means of an area standardization method and a following equation: $X_i = S_i/\Sigma S_i \times 100$, where X_i denotes the percentage of the particular metabolite, S_i- area of its peak in HPLC, and ΣS_i- the sum of areas of all metabolites.

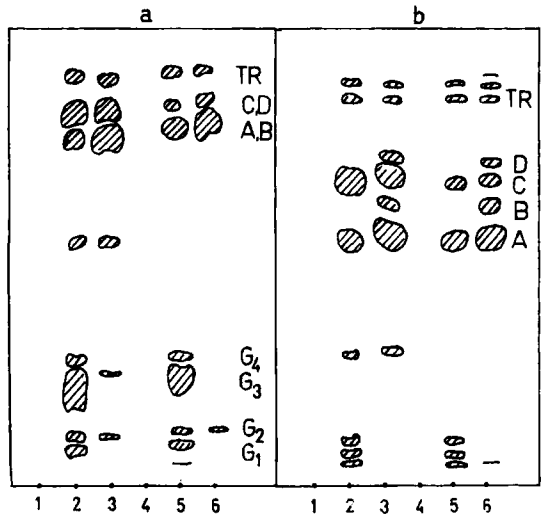

Figure 2. TLC separation of urinary tryptamide (TR) metabolites before and following beta-glucuronidase treatment. a. TLC in solvent system I (chloroform-methanol-acetic acid, 70:20:5). b. TLC in solvent system II (chloroform-methanol-acetic acid, 80:10:2.5). 1, control urine; 2, TR ip urine; 3, TR ip urine enzyme-treated; 4., control urine enzyme treated; 5, TR po urine; 6, TR po urine enzyme-treated. G_1, G_2, G_3, G_4 - conjugated metabolites; A,B,C,D - non-conjugated metabolites.

RESULTS AND DISCUSSION

Fig. 2 shows a TLC pattern of urine metabolites in two solvent systems: I (a) and II (b). The chromatograms reveal a group of slowly migrated metabolites (G_1-G_4), which are probably conjugated metabolites, and a group of fast migrated, presumably non-conjugated metabolites (A,B,C,D). One can see that the solvent I is more suitable for the separation of the former, and the solvent II for the separation of the latter. A and C seem to be major

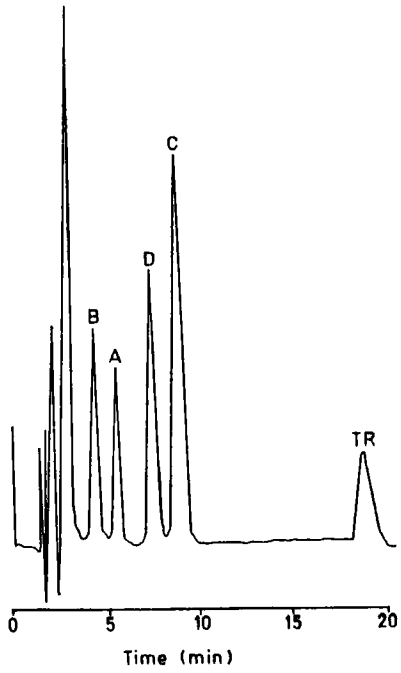

Figure 3. HPLC pattern of purified urinary non-conjugated tryptamide (TR) metabolites (A,B,C,D) in solvent system II (acetonitrile-water, 24:76).

metabolites which are present both in conjugated and free forms. B and D are rather minor metabolites, their occurrence in free form is hardly visible. None of them was present in control urine samples. The profiles of po and ip urine metabolites look much the same, which indicates that the biotransformation of TR in gastrointestinal tract of rats is insignificant.

The postulated conjugated metabolites (G_1-G_4) were individually eluted from the chromatogram and treated

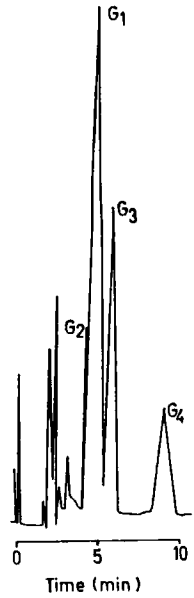

Figure 4. HPLC pattern of purified urinary conjugated tryptamide (TR) metabolites (G_1,G_2,G_3,G_4) in solvent system III (acetonitrile-$NH_4H_2PO_4$, pH 2.7, 16:84).

with beta-glucuronidase, followed by rechromatography. It appeared that G_1 and G_3 were converted into A, G_2 into B and G_4 into D (data not shown). Since the enzyme preparation also contains sulfatase activity, it is reasoable to suggest that the isolated metabolites (especially A) can simultaneously occure in urine at different proportions as glucuronides and sulfates.

Fig. 3 shows the HPLC separation of non-conjugated metabolites in the solvent system II. The resulted retention times are: 4 min (B), 5 min (A), 7.4 min (D) and

TABLE 1

Preliminary Balance Determinations of Tryptamide (TR) Metabolites Excreted in Urine of Rats Following Intravenous Administration in a Dose 25 mg/kg

Time	Metabolites (%)[x]				
	A	B	C	D	Total
10 h	15.4	2.4	16.0	0.5	34.3
1st day	33.2	10.6	17.0	1.5	62.3
2nd day	0.9	1.3	0.3	0	2.5
3rd day	0.7	0.2	0.1	0	1.0
Total	50.2	14.3	33.4	2.0	
Free	16	37	93	27	
Conjugated	84	63	7	73	

x The values represent the percentage of the individual metabolite estimated by HPLC using area standardization method as stated in the Methods. Each value is an average of two determinations.

8.2 min (C); TR - 19 min. The separation of the major constituents of conjugated metabolites in the solvent system III is shown on Fig. 4. The following retention times were found: 4.8 min (G_2), 4.9 min (G_1), 5.8 min (G_3) and 9 min (G_4). Both of these solvents are not convenient for the analysis of TR, since retention times of this compound proved to be too long then. Previously described (7) less polar solvent I is more suitable for that.

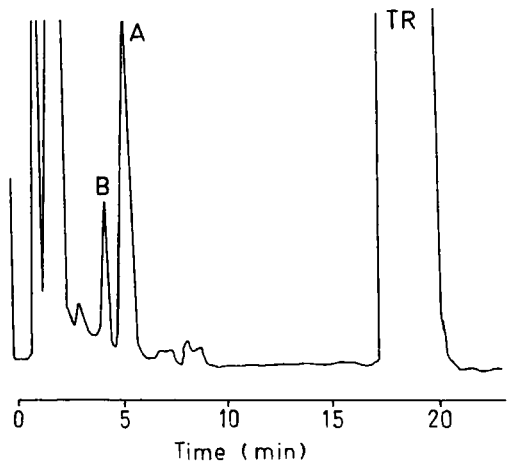

Figure 5. Formation of tryptamide (TR) metabolites A and B in vitro with rat liver microsomal incubation mixture. Incubation lasted 15 min. HPLC solvent system II was used (acetonitrile-water, 24:76).

Table 1 shows the relative proportions of TR metabolites in the course of the urine excretion. It can be seen that almost 97% of the total metaboites eliminated via urine were excreted during first 24 h. Taking into account the content, an order may be formed as: A>C>B>D. Metabolite A amounted to about 50%, and A plus C to about 84% of the total metabolites. Metabolites A, B and D are mostly present as the conjugates(63-84%), while C is rather excreted in free form. Only minute amount of TR was found intact (0.12-0.8% of the administrated dose), that gives an evidence of its efficient biotransformation in rats.

In blood, the metabolites were observed during first 6 h only, and C predominated. In liver A and B rose up during first hour then dropped fast, C reached a peak value at 2 h, D was not detected (data not shown). The metabolites A and B were produced in the microsomal incubation mixture in vitro (Fig. 5).

In conclusion, TR undergoes efficient metabolism in rats being transformed into at least four probably hydroxylated metabolites which occure both in free and conjugated forms (glucuronides and sulfates). These can be isolated from urine, blood and tissue by the sorbent extraction using Amberlite XAD-2 resin. The elaborated chromatographic systems enable their separation and analysis.

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