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Note

Determination of the γ-aminobutyric acid agonist 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-3-ol in urine by high-performance liquid chromatography

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Decreased γ-aminobutyric acid (GABA) activity seems to be involved in certain neuroleptic and psychiatric disorders [1—4]. Therefore the development of specific GABA agonists with pharmacokinetic and toxicological properties acceptable for clinical use is of great interest.

Muscimol is a potent GABA agonist [5, 6], but too toxic for use in humans. Among a number of muscimol analogues synthesized, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-3-ol (THIP) (I, Fig. 1) has proved a potent and specific GABA agonist [6—8]. In pharmacological studies THIP is active after systemic administration in a variety of animal models [9].

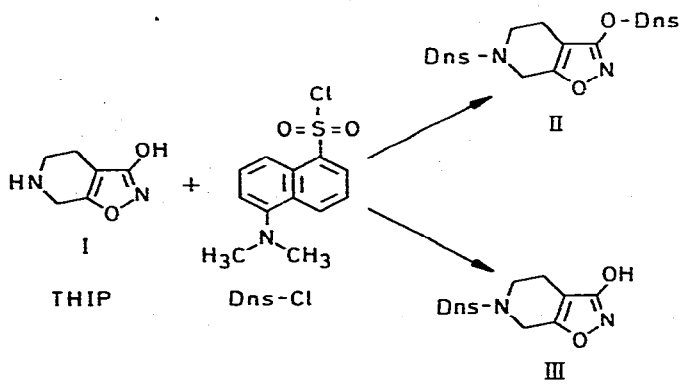

Fig. 1. Reaction of 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridine-3-ol (THIP) with Dns-Cl.

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Preliminary studies on the absorption, distribution, metabolism, and excretion of THIP in animal and man using ^{14}C-labelled THIP have been performed [10].

THIP is an amino acid analogue having pK_a values of 4.44 (enol group) and 8.48 (amine group) [11].

In this paper we present a high-performance liquid chromatographic (HPLC) method for the determination of THIP in urine. The excretion of unchanged THIP in the urine of man and rat has been determined by this method.

EXPERIMENTAL

Chemicals

THIP was a gift from Lundbeck & Co. A/S (Copenhagen, Denmark). 5-Dimethylamino-1-naphthalenesulfonyl chloride (Dns-Cl) was obtained from E. Merck (Darmstadt, G.F.R.). Other chemicals and solvents used were of analytical grade quality.

Apparatus

A liquid chromatograph consisting of a Waters Model 6000A pump, a Rheodyne Model 7120 loop injection valve (20 μl) and a Waters Model 440 ultraviolet absorbance detector (254 nm) was used. A 150 × 4.6 mm I.D. stainless-steel column packed with LiChrosorb RP-18, 5 μm, was used. The column was operated at room temperature with acetonitrile—water (75:25) as the mobile phase at a flow-rate of 2 ml/min.

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on glass plates (5 × 10 cm) pre-coated with a 250-μm layer of silica gel from Merck.

Butanol—acetic acid—water (4:1:1) was used as the eluent for analysis of THIP; 0.2% ninhydrin in ethanol was used as visualization reagent.

Analysis of dansylated THIP was performed using chloroform—methanol—acetic acid (100:4:1) as developing solvent.

Sample preparation

Dansylation of THIP in urine was made by mixing 10 μl of rat urine (or 100 μl of human urine), 25 μl of 0.1% adrenaline in water (internal standard), 300 μl of 0.05 *M* sodium borate buffer (pH 8.5) and 600 μl of 0.1% Dns-Cl in acetone in a glass tube. The reaction was carried out at 40°C for 40 min, and 20 μl of the reaction mixture were taken for HPLC.

Human study

Each of three healthy volunteers received a single oral dose of 15 mg of THIP dissolved in water. Urine was collected over a period of 48 h. The samples were stored at —20°C until analysis.

Rat study

Three rats together in a metabolic cage were given 200 mg/kg THIP orally in water. Urine and faeces were collected over the periods 0—8, 8—24 and 24—48

h after administration. The urine and faeces were stored at −20°C until analysis.

RESULTS AND DISCUSSION

THIP is a very polar compound due to its amphoteric character. The compound is soluble in water, whereas its solubility in organic solvents such as methanol, acetone and ethyl acetate is very low. Extraction of THIP into an organic solvent is therefore not feasible. Extraction of THIP as an ion-pair with different cations and anions was tried without any success. An HPLC method involving pre-column derivatization with Dns-Cl to enhance detectability has therefore been developed. As TLC analysis indicated unchanged THIP in the urine but not in faeces, the method has been developed for urine.

TLC of the reaction mixture after dansylation of THIP showed that two dansyl derivatives of THIP were formed. The derivative obtained in highest yield could be prepared by a slight modification of the procedure described by Frei et al. [12] for preparation of the tri-dansyl derivative of adrenaline. ^{1}H-NMR studies verified the derivative as a di-dansyl derivative of THIP (II, Fig. 1). The other dansyl derivative of THIP could be obtained by hydrolysis of the di-dansyl derivative with methanolic potassium hydroxide. This derivative was shown to be a mono-dansyl derivative of THIP by ^{1}H-NMR (III, Fig. 1). It is known that O-dansyl derivatives but not N-dansyl derivatives can be hydrolysed with methanolic potassium hydroxide [13]. The obtained mono derivative of THIP is therefore assumed to be the N-dansyl derivative.

The reaction of THIP and adrenaline with Dns-Cl was studied with regard to the influence of pH, contents of water in the acetone—water reaction mixture, temperature and time of reaction and excess of Dns-Cl.

The reaction of THIP and adrenaline with Dns-Cl as a function of pH is shown in Fig. 2. The amount of the di-dansyl derivative of THIP decreases with increasing pH due to hydrolysis of the di-dansyl derivative to the mono-dansyl derivative of THIP as shown by TLC. The amount of the tri-dansyl derivative of

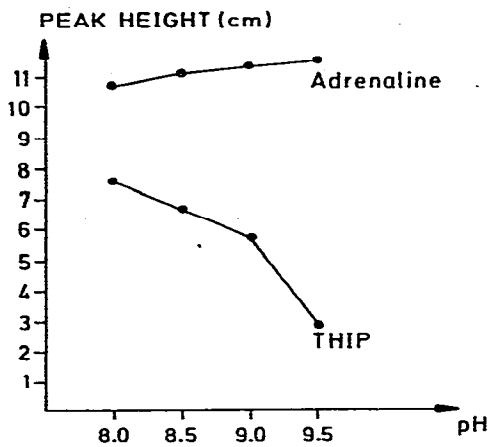

Fig. 2. Reaction of THIP and adrenaline with Dns-Cl expressed by peak height as a function of pH of the reaction mixture.

adrenaline increases with increasing pH. As a compromise, a pH value of 8.5 was chosen. Dansylation of THIP and adrenaline was reproducible at 40°C, and a 40-min reaction time at this temperature ensured complete reaction. The influence of the amount of water and the concentration of Dns-Cl in the reaction mixture were shown to be of minor importance for the dansylation of THIP and adrenaline.

Adrenaline was chosen as internal standard because it forms a tri-dansyl derivative with Dns-Cl. Compounds forming di-dansyl derivatives could not be used as internal standard, as the derivatives had the same HPLC retention times as the di-dansyl derivative of THIP. Preparation of a fresh adrenaline solution every day was necessary to prevent stability problems.

Due to higher contents of interfering substances in rat urine than in human urine it was not possible to obtain reproducible results by using more than 10 μl of rat urine, whereas 100 μl of human urine could be used without problems. A chromatogram of a rat urine sample containing THIP is shown in Fig. 3.

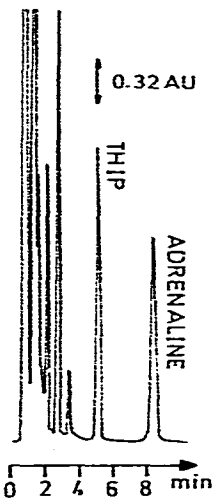

Fig. 3. High-performance liquid chromatogram after dansylation of rat urine spiked with THIP (1 mg/ml). Internal standard: adrenaline. Column: LiChrosorb RP-18, 5 μm (150 × 4.65 mm I.D.). Mobile phase: acetonitrile—water (75:25); flow-rate, 2 ml/min; UV detection (254 nm).

A UV detector (254 nm) was used as the fluorescence detector available (Perkin-Elmer Model LC 1000) did not show better selectivity or sensitivity. Detection limits were found to be 5 μg/ml of rat urine and 500 ng/ml of human urine. However, use of another fluorescence detector may give a better signal-to-noise ratio and thus a lower limit of detection [14].

The reproducibility of the method was 1%, 3% and 5% relative standard deviation (n = 6) analysing samples of urine containing THIP in concentrations of 1 mg/ml, 50 μg/ml and 5 μg/ml, respectively.

Excretion

The quantitative HPLC procedure was used to determine the excretion of unchanged THIP in urine from three human volunteers (Fig. 4) receiving an

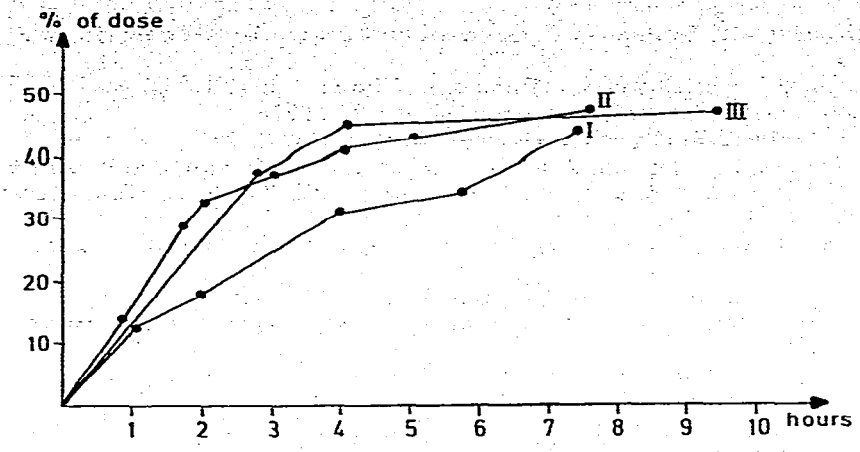

Fig. 4. Cumulative excretion of THIP in human urine following a single oral dose of 15 mg of THIP to three volunteers.

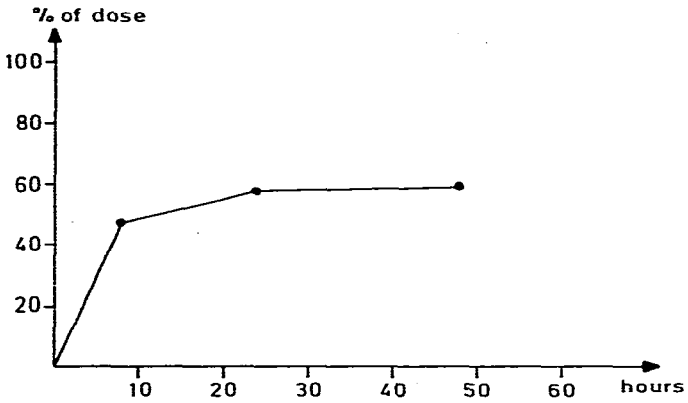

Fig. 5. Cumulative excretion of THIP in rat urine following administration of 200 mg/kg THIP to rats.

oral dose of 15 mg of THIP each, and rats (Fig. 5) given 200 mg/kg THIP orally. The volunteers excreted THIP in urine in amounts corresponding to 44%, 47% and 47% of the dose given, whereas the rats excreted amounts of THIP corresponding to 50% of the dose given. These results agree with the results obtained using ^{14}C-labelled THIP [11].

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REFERENCES

1 K.G. Lloyd, H. Möhler, P.H. Heitz and G. Bartholine, J. Neurochem., 25 (1975) 789.
2 P.L. McGeer and E.G. McGeer, J. Neurochem., 26 (1976) 65.

3 J.T. Coyle, R. Schwarty, J.P. Bennet and P. Compochiaro, Progr. Neuro-Psychopharmacol., 1 (1977) 13.
4 S.J. Enna, L.Z. Stern, G.J. Wastek and H.I. Yamamura, Life Sci., 20 (1977) 205.
5 S.R. Naik, A. Guidotti and E. Costa, Neuropharmacology, 15 (1976) 479.
6 J. Scheel-Krüger, J. Arnt and G. Magelund, Neurosci. Lett., 4 (1977) 351.
7 J. Scheel-Krüger, J. Arnt, C. Bræstrup, A.V. Christensen and G. Magelund, in P. Krogsgaard-Larsen, J. Scheel-Krüger and H. Kofod (Editors), GABA-Neurotransmitters, Academic Press, New York, 1979.
8 P. Krogsgaard-Larsen, G.A.R. Johnston, D. Lodge and D.R. Curtis, Nature (London), 268 (1977) 53.
9 A.V. Christensen, J. Arnt and J. Scheel-Krüger, Life Sci., 4 (1979) 1395.
10 B. Schultz, T. Aaes-Jørgensen, K.P. Bøgesø and A. Jørgensen, Acta Pharmacol., Toxicol., 49 (1981) 116.
11 P. Krogsgaard-Larsen, Acta Chem. Scand. B, 31 (1977) 384.
12 R.W. Frei, M. Thomsen and I. Frei, J. Liquid Chromatogr., 1 (1978) 443.
13 N. Seiler and L. Demisch, in K. Blau and G.S. King (Editors), Handbook of Derivatives for Chromatography, Heyden, London, 1978.
14 S. Honoré Hansen, J. Chromatogr., 226 (1981) 504.