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QUANTITATIVE DETERMINATION OF THE γ-AMINOBUTYRIC ACID AGONIST, 4,5,6,7-TETRAHYDROISOXAZOLO[5,4-*c*]PYRIDIN-3-OL, IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method was developed to determine pharmacologically relevant concentrations of the γ-aminobutyric acid agonistic compound THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol) in serum. THIP is extracted from serum by a cation-exchange column, derivatized by dansyl chloride, and further separated and quantitated by reversed-phase high-performance liquid chromatography using measurement of ultraviolet absorption at the optimal wavelength (265 nm). The lower detection limit of the method is 0.07 μmol l^{-1} (10 ng ml^{-1}) when 2 ml of serum are used for extraction. This sensitivity is sufficient for pharmacokinetic studies in man following administration of a presumably therapeutic dose of THIP. Blood samples collected from 28 patients treated with one or more of 26 drugs relevant for co-administration with THIP did not contain substances which would affect the reliable quantitation of THIP, if present.

INTRODUCTION

The compound 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) (Fig. 1) is a heterocyclic compound related to γ-aminobutyric acid (GABA). It has been shown to be a GABA-receptor agonist [1, 2], and the assessment of the possible therapeutic value of the substance is at present in progress.

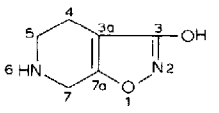

Fig. 1. Structure of THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol).

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A reversed-phase ion-pair high-performance liquid chromatographic (HPLC) method for determination of THIP in aqueous solution has been reported [3]. It provides a sensitive detection of THIP, especially when an electrochemical detector is used. However, the application of this method for quantitation of THIP in extracts from biological material, in particular serum, has not been successful.

The procedure described in the present paper is related to a method for the determination of THIP in urine [4]. The inclusion of an extraction step for concentration of THIP from the sample prior to HPLC analysis has decreased the lower detection limit several hundred times. This makes the quantitative determination of pharmacologically relevant serum concentrations of THIP possible, and the method has been used in human and animal studies of THIP pharmacokinetics. The extraction procedure used is inspired from a method for GABA purification [5] which in turn is a modification of a separation method for biogenic amines [6].

EXPERIMENTAL

Instrumentation

HPLC was carried out using a Pye Unicam LC-XPD pump (Cambridge, Great Britain) delivering a constant flow of 2.00 ml min^{-1}, a Rheodyne 7125

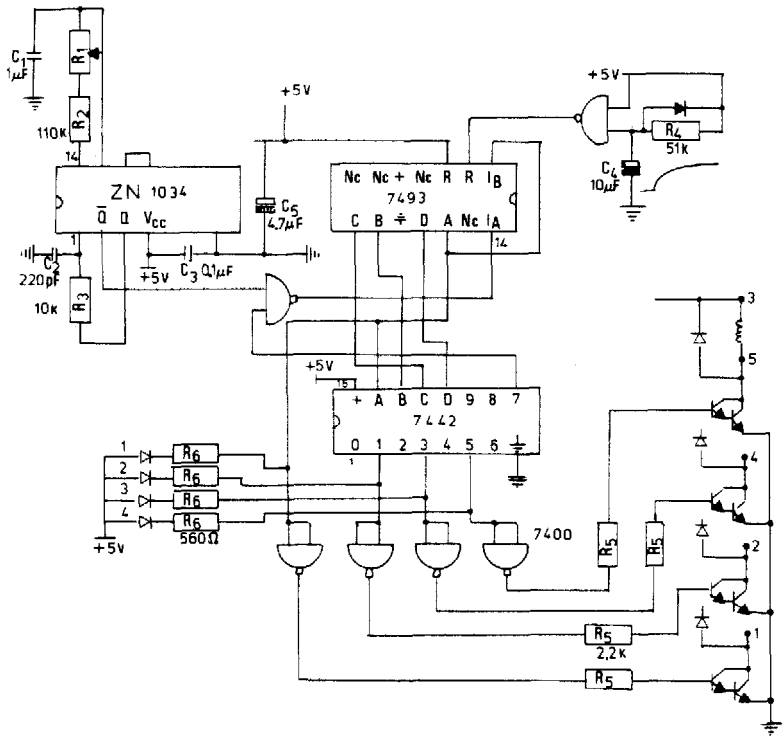

Fig. 2. Diagram for device for unattended rinsing of extraction columns. R_1 is varied (approx. 200 Ω) for time (volume) adjustment. The four electromagnetic valves are driven from the Darlington couplings in the right side of the diagram. A conventional power supply (5 V for the timer electronics, 20 V for the electromagnetic valves) is not shown.

injection valve (Berkeley, CA, U.S.A.) equipped with a 80-μl loop, and a Uvikon 725 variable-wavelength spectrophotometric detector (Kontron, Zürich, Switzerland) operated at a wavelength of 265 nm. The detector signal was recorded and processed by a Kipp & Zonen Model BD 9 recorder (Delft, The Netherlands) and a Hewlett-Packard Model 3390 A reporting integrator (Avondale, PA, U.S.A.). The column consisted of a 40 mm long precolumn and a 120 mm main column, both of them packed with Spherisorb S5-ODS (5 μm particle size) by a slurry technique using the Pye Unicam pump. Both column tubes were made by Knauer (Oberursel, G.F.R.), internal diameter 4.6 mm, connected end to end by a socket. Ultraviolet (UV) absorbance spectra of the components eluted from the HPLC column were recorded by a Uvikon LC 720 detector. Some experiments on alternative fluorescence monitoring were carried out using a Kontron SFM-23 LC fluorescence detector.

A device has been constructed for unattended rinsing of cation-exchange columns before use. Four electromagnetic valves (Gemü type 102, EPDM for water and PTFE for hydrochloric acid, sodium hydroxide, and buffer solution, made by Gebr. Müller, Ingelfingen, G.F.R.) were controlled by a timer including a counting circuit (diagram in Fig. 2) for delivery of the flushing solutions to a sixteen-pronged manifold as given under Extraction.

Materials

Chemicals used were AG 50W-X4 (200—400 mesh) cation exchanger (Bio-Rad Labs., Richmond, CA, U.S.A.), ammonium bicarbonate (certified grade, Fisher, Fair Lawn, NJ, U.S.A.), Dns chloride (5-dimethylamino-1-naphthalene sulfonyl chloride) (Sigma, St. Louis, MO, U.S.A.), ethanol 96% or (for protein precipitation) absolute (DDSF, Copenhagen, Denmark), muscimol base (purum grade, Fluka, Buchs, Switzerland), Spherisorb S 5-ODS (Phase Sep, Queensferry, Great Britain), tetrahydrofuran (HPLC grade, Rathburn, Walkerburn, Great Britain), THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol, monohydrate; synthesized by our company), water redistilled in an all-glass apparatus (used for all solutions), and acetone (dried by anhydrous sodium sulphate), citric acid 1-hydrate, disodium ethylene diamine tetraacetate (EDTA), hydrochloric acid (fuming), methanol, sodium hydroxide, disodium tetraborate 10-hydrate, sodium dihydrogen phosphate 1-hydrate and disodium hydrogen phosphate 2-hydrate (all pro analysi grade, E. Merck, Darmstadt, G.F.R.). Minisorb® 70 × 11 mm (3 ml) tubes with stoppers, and plain polyethylene tubes 100 × 16 mm (11 ml) (Nunc, Roskilde, Denmark). Vacutainer® tubes (siliconized, without anticoagulant; B-D, Ireland). Serological glass pipettes, disposable, 5.5 mm I.D., total length 150 mm (Bilbate, Daventry, Great Britain).

Buffer solutions

Buffer solutions were prepared from the amounts given below by dissolving in the appropriate volume of water required for 1000 ml of solution: 0.1 *M* sodium phosphate buffer, pH 6.5, containing 0.1% EDTA: 8.83 g of sodium dihydrogen phosphate 1-hydrate, 6.40 g of disodium hydrogen phosphate 2-hydrate, 1.00 g of EDTA. Citrate buffer pH 3.0: 8.47 g of citric acid 1-hydrate, 80.6 ml of 1 *N* sodium hydroxide, 30.0 ml of hydrochloric acid.

0.025 *M* sodium citrate buffer pH 4.5: 5.25 g of citric acid 1-hydrate, 40.0 ml of 1 *N* sodium hydroxide, adjusted to pH 4.50 ± 0.05 if necessary. 0.05 *M* sodium citrate buffer pH 5.35: 10.50 g of citric acid 1-hydrate, 110 ml of 1 *N* sodium hydroxide, adjusted to pH 5.35 ± 0.05 if necessary. 0.05 *M* sodium borate buffer pH 8.5: 9.53 g of disodium tetraborate 10-hydrate were dissolved in 430 ml of water, pH adjusted to 8.50 ± 0.02 by 2 *N* hydrochloric acid, and finally made up to 500 ml with water.

Final version of the procedure

Extraction. A 75-g portion of cation-exchange resin was rinsed in a Büchner funnel with 300 ml of 0.1 *M* sodium phosphate buffer, pH 6.5, containing 0.1% EDTA, followed by 150 ml of water, 300 ml of 60% aqueous methanol, 300 ml of 2 *N* hydrochloric acid, 300 ml of 2.4 *N* ethanolic (60%) hydrochloric acid, and 150 ml of water in the order stated. The washed resin was packed from a slurry into serological glass pipettes fitted with a plug of glass wool at the shoulders of the pipette. The height of the resin bed was 75 mm.

The newly packed columns (and columns used once or twice before) were mounted in the column rinsing apparatus and treated with a sequence of 2 *N* sodium hydroxide containing 1% EDTA—water—2 *N* hydrochloric acid—water—0.1 *M* sodium phosphate buffer, pH 6.5, containing 0.1% EDTA—water, 20 ml of each. During the extraction of a sample, the column (washed within three days) was connected through a three-way stopcock to a vertically mounted 20-ml glass syringe. The plunger was loaded with a 165 g weight.

Before application onto the column, a 2.00-ml sample of serum was pipetted into a Minisorb® tube, the internal standard, (2.1 μg of muscimol) was added, dispensed as 60 μl of a 35 μg ml^{-1} solution, and proteins were precipitated by addition of 1.0 ml of ethanol. Sixteen samples were prepared at the same time. The tubes were stoppered and the contents mixed by turning. Then the samples were centrifuged (18,000 *g* at 5°C for 20 min). The supernatant from each sample was decanted into an 11-ml polypropylene tube, 6.0 ml of citrate buffer pH 3.0 were added, and finally the pH of the mixture was adjusted to 3.0 by the addition of approximately 70 μl of 2 *N* hydrochloric acid. This mixture was centrifuged (18,000 *g* at 5°C for 20 min).

The clear supernatant was injected by means of a polypropylene syringe through the side-arm of the three-way stopcock into the glass syringe and thereupon passed through the cation-exchange column. When no solution remained in the glass syringe, the following solvents were passed through in the same manner: water (8.0 ml), 0.025 *M* sodium citrate buffer pH 4.5 (8.0 ml), 0.05 *M* sodium citrate buffer pH 5.35 (4.0 ml), water (8.0 ml), 0.05 *M* ammonium bicarbonate (0.9 ml) (freshly prepared). Then the weights were removed, the column tips rinsed with water, and each column was eluted with 2.0 ml of 0.05 *M* ammonium bicarbonate. The eluate was collected in a 4-ml glass tube, which had been rinsed and deactivated by ethanol in an ultrasonic bath. The tube was closed by a perforated stopper, and the eluate was deaerated by standing 5 min in an ultrasonic bath. Then the samples were frozen at −18°C and freeze-dried at 0.02—0.05 Torr for about 16 h. If less than 4.0 ml serum were

available for the duplicate determination, the extraction procedure was carried out using an aliquot of serum of at least 650 μl without any modification of the procedure.

Derivatization. The freeze-dried residue from each sample was dissolved in 45 μl of sodium borate buffer pH 8.5 and 90 μl of a 7 mg ml^{-1} solution of dansyl chloride in dry acetone were added. The tube was stoppered and placed in a 40°C water bath for 40 min, centrifuged (1500 *g* at 5°C for 10 min), and stored in ice water until analysis.

High-performance liquid chromatography. Eighty microlitres of the reaction mixture, corresponding to almost the entire liquid phase of the prepared sample, were injected into the HPLC system. The mobile phase used in the HPLC system was a tetrahydrofuran—water (50.3:49.7) mixture, deaerated by helium purging. The flow rate was 2.00 ml min^{-1}. Sample concentrations of THIP were calculated from the chromatograms using a known relation between THIP serum concentration and the THIP-Dns/muscimol-Dns peak height ratio. This relation was established by linear regression analysis of data obtained by extraction and HPLC analysis of standard samples. These were made by adding known amounts of THIP to human blank serum to produce concentrations in the range 0—4 μmol l^{-1} (0—561 ng of non-aqueous THIP per ml).

In the characterization of the performance of the method the coefficient of variation (C.V.) and the limit of sensitivity were estimated from analysis of six 2-ml aliquots of each of six standard serum samples representing the above range.

Experiments on analytical interferences

Serum was prepared from blood samples collected from in total 28 patients of both sexes undergoing antineoplastic, antiinflammatory or antihypertensive treatment, and the serum samples were analysed by the method described above, using detection by UV-absorption measurement. The only deviation from the established procedure was the omission of internal standard addition. The drugs administered were the following: acetylsalicylic acid, atenolol, bendroflumethiazide, bleomycin, CCNU, cholestyramine, cisplatin, cyclophosphamide, dactinomycin, diclofenac, digoxin, doxorubicin, 5-fluorouracil, ibuprofen, indomethacin, melphalan, methotrexate, misonidasol, mitopodozide, naproxen, paracetamol, penicillamine, prednisone, tolmetin, vinblastine, and vincristene. Also studied was the compatibility of the analytical method with the use of Vacutainers®.

RESULTS

Chromatography

The chromatogram of a serum sample obtained from a human volunteer 2 h after peroral administration of 10 mg of THIP (representing the lower part of the presumed therapeutic dose range) is reproduced in Fig. 3. Detection was made by measurement of UV absorbance and fluorescence (in series). Also shown is the chromatogram of a sample of human blank serum, without muscimol added, detected by UV-absorption measurement at 265 nm. The arrows indicate the position of peaks referring to THIP and muscimol, if present.

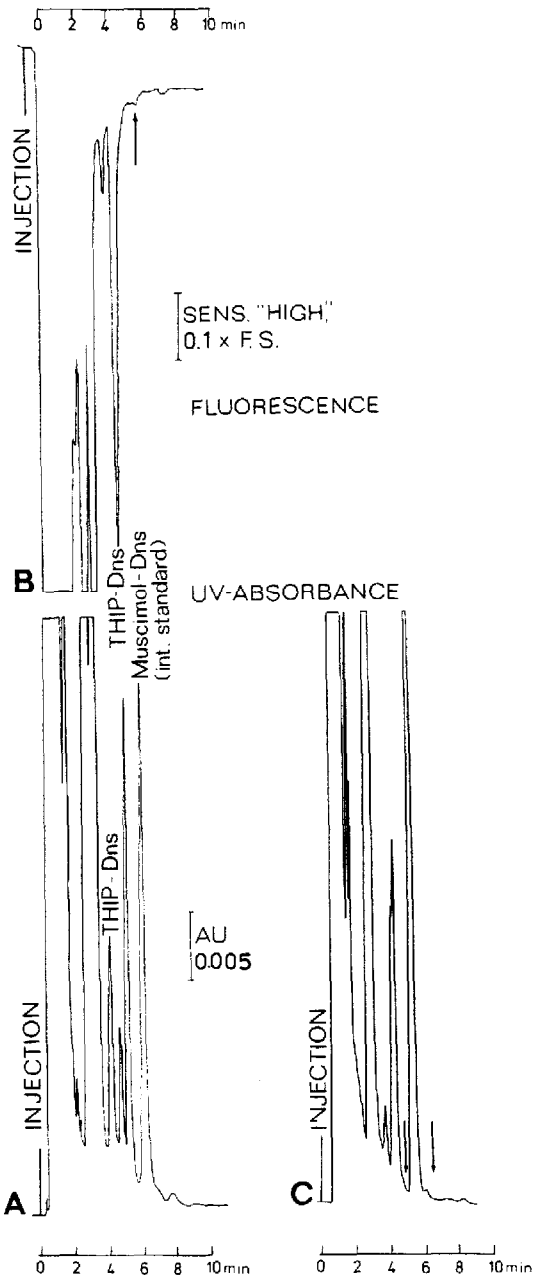

Fig. 3. Chromatograms of (A) a serum sample obtained from a human volunteer 2 h after peroral administration of 10 mg of THIP, detection by measurement of UV absorbance at 265 nm; (B) the same sample, monitored by fluorescence measurement, excitation wavelength 346 nm, emission wavelength 514 nm, the arrow indicates the position of muscimol-Dns; (C) human blank serum (without muscimol added), UV detection, arrows indicate the positions where THIP-Dns and muscimol-Dns would elute.

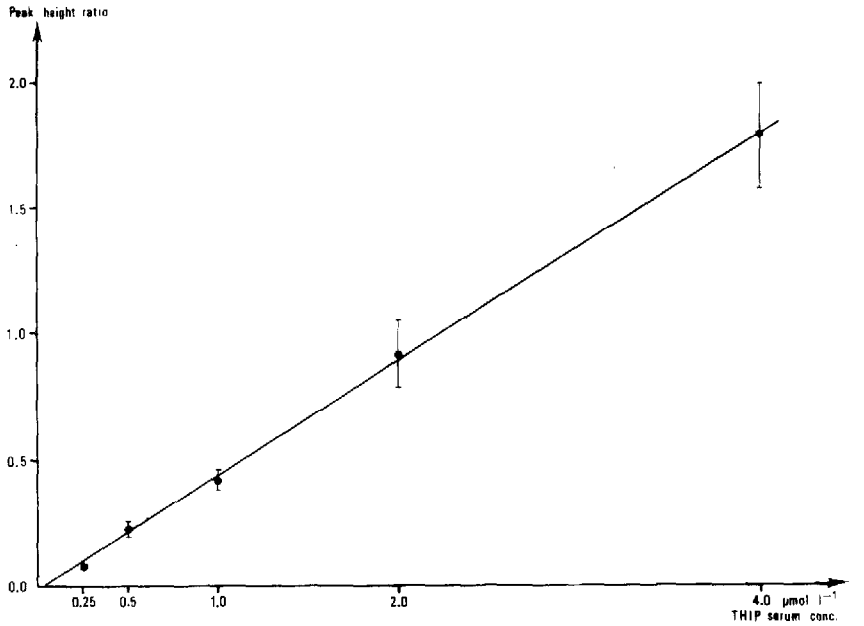

Fig. 4. Calibration curve constructed from peak height ratios. Mean of six determinations at each concentration. Error bars indicate the standard deviation.

Calibration curves, precision and linearity

Fig. 4 shows graphically the relation between THIP serum concentration and the peak height ratio. Each point represents the mean of six determinations, performed on the same day. The correlation coefficient in linear regression analysis was 0.9857.

A variance ratio test revealed the experimental points to belong to a straight line at the 0.90 probability level. The following values of C.V. were calculated in the five groups of THIP-spiked samples (the numbers in parentheses are the corresponding nominal THIP concentrations): 8.5 (0.25 μM); 13.7 (0.50 μM); 9.8 (1.0 μM); 14.3 (2.0 μM); 11.9 (4.0 μM). No correlation between C.V. and concentration was apparent.

Limit of sensitivity

From the data referring to the calibration curve shown (Fig. 4) it can be estimated that the lower limit of sensitivity is about 0.07 μmol l^{-1} (10 ng ml^{-1}). The intercept of the calibration curve with the abscissa axis was at 0.04 μmol l^{-1}, but the concentration must be 0.07 μmol l^{-1} (0.067 μmol l^{-1}) to separate the calculated peak height ratio from zero by twice the standard deviation (estimated from the C.V. of the 0.25 μM group).

A series of experiments on determination of THIP in serum samples spiked with relatively low THIP concentrations, 0.02 μmol l^{-1}, 0.03 μmol l^{-1}, or 0.04 μmol l^{-1}, revealed 0.03 μmol l^{-1} to be the lowest concentration able to produce an integrator signal exceeding the blank sample baseline at the position of THIP-Dns in the chromatogram.

Analytical interferences

In the analysis of the serum samples collected from patients receiving other drugs, no peaks occurred in the chromatograms, with one exception, at the positions where THIP or the internal standard, muscimol, would elute (in the form of Dns derivatives) if present in the serum sample. The single sample which contained an interfering substance, eluting at the position of THIP-Dns, had been obtained from a patient treated with indomethacin and mitopodozide. The size of the peak would correspond to a THIP serum concentration of about 0.15 μM. The interfering substance is not likely to be mitopodozide or a metabolite of this drug since it did not appear in any of the other samples from the several patients treated with this drug. Nor does indomethacin obviously account for the interfering peak, since the peak was absent in the chromatograms of the sample collected from the other indomethacin-treated patient.

Determination of the THIP concentration in six aliquots of THIP-spiked serum which had been contained in Vacutainer blood collection tubes revealed the recovery of THIP from these tubes to amount on an average 97.4% of the recovery of THIP from plain glass tubes. Recoveries were assessed from the areas under the peaks corresponding to THIP. Serum blank samples from Vacutainers did not contain interfering substances. In conclusion, Vacutainers (without anticoagulant) can be used for blood sample collection without affecting the determination of THIP quantitatively or qualitatively.

Application to pharmacokinetic studies

The proposed method for quantitative determination of THIP in serum has been applied for pharmacokinetic studies in man, dog, baboon, and rat. A

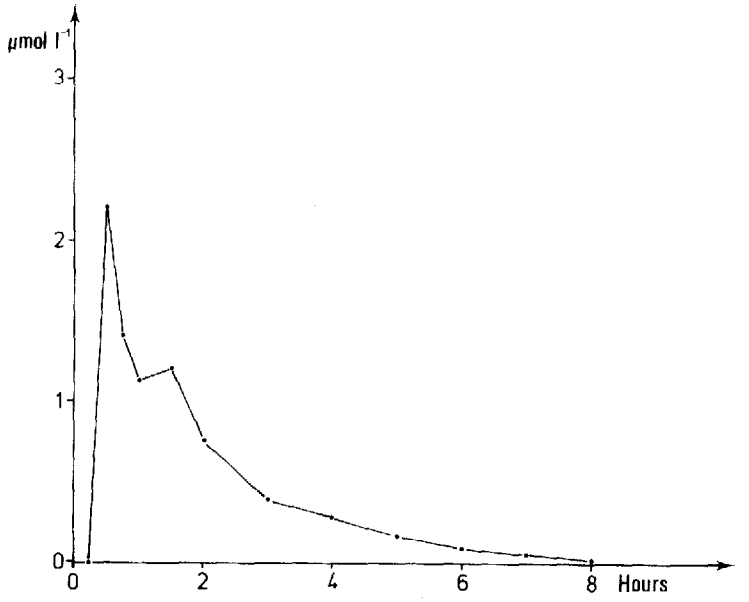

Fig. 5. Time course of THIP serum concentration after administration of a capsule containing 15 mg of THIP to a healthy, human volunteer.

representative observation on the time course of serum THIP is shown in Fig. 5. The curve refers to a healthy, human volunteer who received an oral dose of 15 mg of THIP.

DISCUSSION

The procedure for THIP serum concentration determination may seem troublesome and time-consuming, but experience has shown a capacity of duplicate determination of twelve samples per working day to be a realistic long-term schedule for one technician.

During the development of the present method, the use of alternative procedures and materials was of course considered:

Extraction

The efficient extraction of THIP from biological samples implied considerable trouble. Irrespective of the pH of the solvent, THIP is too polar to be extractable by solvents less polar than water, so extraction of THIP by means of an anion or cation exchanger, or as an ion-pair, was attempted. For ion-pair extraction the counter-ions trifluoroacetic acid or perfluorooctanosulfonic acid were used; chromatography did not reveal THIP to be extracted in this way.

Extraction of THIP by means of an ion-exchange material was attempted using strong as well as weak anion or cation exchangers. The influence of application buffer pH and resin bed height on column binding efficiency, and the suitability of various buffer solutions for selective elution of substances other than THIP, was studied using ^{14}C-labelled THIP, which was quantitated by liquid scintillation counting. This method was also used for evaluation of serum sample pretreatment (deproteinization), choice of solution for effective elution of THIP, and choice of eluate fraction to be collected for HPLC. The recovery of THIP in the extraction procedure was found to be 101.1 ± 2.3% when determined by [^{14}C] THIP.

Evaporation of water and ammonium bicarbonate from the extracts is necessary before derivatization and HPLC analysis. Freeze-drying was preferred to the use of a rotary evaporator because the former procedure resulted in a more complete ammonium bicarbonate sublimation, was less time-consuming, and allowed the use of smaller test tubes for eluate collection and evaporation.

Derivatization

The concentration of Dns chloride in the reagent used has been observed to influence the ratio of derivatives from THIP and muscimol detected in the HPLC analysis. A concentration of 7 mg ml^{-1} was found most suitable since in the range 5—10 mg ml^{-1} the ratio was rather constant, whereas the use of a great excess of dansyl chloride produced potentially interfering substances.

High-performance liquid chromatography

Nucleosil 5 C_{18} was compared with Spherisorb S5-ODS for use as column packing material. When tested by a benzene—naphthalene—anthracene mixture, the Nucleosil column was the more efficient one, but the opposite was the case when tested with authentic samples in the HPLC system described in the present paper.

The optimum wavelength setting for the UV-absorbance detector was found by means of a Uvikon LC 720 detector, which is able to record a UV-absorbance spectrum of the components anywhere in the chromatogram when eluent flow has been temporarily stopped. The UV absorbance of the Dns derivatives from THIP and muscimol is maximum at 254 nm in the medium used as mobile phase, but a wavelength of 265 nm was found to offer a more favourable proportion between the absorbance of the THIP and muscimol derivatives on the one hand and the absorbance of components eluting close to THIP-Dns on the other hand. However, monitoring at 254 nm will be satisfactory if only a detector equipped with this wavelength is available.

Detection by fluorescence monitoring was attempted using the excitation/emission wavelength combinations 254/514 nm or 346/514 nm (selected on the basis of absorption and fluorescence spectra). As is seen from Fig. 3, the component eluting just after THIP-Dns was not detected in fluorescence measurement. However, probably owing to the noise of the fluorescence detector, application of this technique was not found to improve the sensitivity of the method. In addition, muscimol-Dns was not detected by the fluorescence detector, neither did any of 30 other substances plausible for use as internal standard possess the desired combination of extractability, ability to form a fluorescent Dns derivative, favourable retention characteristics in the HPLC system, and stability in solution.

Interference from metabolites

THIP is known to undergo conjugation with glucuronic acid in mouse, rat, and man [7]. Conjugation at the enolic oxygen atom as well as at ring nitrogen in position 6 is imaginable; an O-monoglucuronide may extract in a similar way as unconjugated THIP in the cation-exchange extraction procedure. However, the conjugate will not form the same Dns derivative as free THIP does.

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