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Note

Determination of the γ-aminobutyric acid agonist 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol by high-performance liquid chromatography using UV or electrochemical detection

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4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) is a heterocyclic analogue of γ-aminobutyric acid (GABA). It has been observed to be a GABA receptor agonist and may be a drug of clinical value[1,2].

A high-performance liquid chromatographic (HPLC) method for the determination of THIP has been developed in an attempt to establish a procedure for the determination of THIP in biological materials, or in pharmaceutical preparations. The pK_a values of THIP are 4.4 and 8.5[1], and its zwitterionic structure makes it very hydrophilic. Several column packing materials and mobile phase compositions were tried, and the use of an octadecylsilica material and a buffered mobile phase containing perfluorooctanesulphonic acid was found to offer the most stable system as regards constancy of the retention times of THIP and other sample components. As the UV absorption of THIP is maximal at a wavelength of 217 nm and relatively low at wavelengths exceeding 230 nm, electrochemical detection was considered a favourable alternative to measurement of UV absorption for monitoring THIP in the eluate from the column. A slightly modified version of the coulometric detector designed by Lankelma and Poppe[3] was used.

EXPERIMENTAL

Materials

In the final version of the method the following chemicals were used: lithium perchlorate, sodium acetate (both pro analysi grade, Merck, Darmstadt, G.F.R.), perfluorooctanesulphonic acid (Prosynth grade, Riedel-de Haën, Seeltze-Hannover, G.F.R.), tetrahydrofuran (HPLC grade, Rathburn, Walkerburn, Great Britain) and water redistilled in all-glass apparatus; these chemicals were used for the preparation of the mobile phase, consisting of a 90:10 mixture of 0.02 *M* sodium acetate solution (pH 4.5) and tetrahydrofuran, with added perfluorooctanesulphonic acid (5 m*M*) and lithium perchlorate (50 m*M*). The mixture was degassed by occasional purging with helium. The stationary phase was Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.). THIP monohydrate (Lu 2-030) had been synthesized by this company.

During the development of the chromatographic method, the following ion-pairing reagents and column packing materials had also been tried: dioctyl sulpho-

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succinate, sodium salt (Sigma, St. Louis, MO, U.S.A.), perfluorohexanesulphonic acid (Riedel-de Haën), PIC B-7 (Waters Assoc., Milford, MA, U.S.A.), trifluoroacetic acid (Merck-Schuchardt) and LiChrosorb-NH_2 (5 or 10 μm), LiChrosorb RP-2 (5 or 10 μm), Nucleosil 10 CN, Spherisorb S5 CN and Spherisorb S5 ODS.

Equipment

HPLC was carried out with an LC-XPD pump (Pye Unicam, Cambridge, Great Britain), operated at a constant flow-rate, a Rheodyne 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 50-μl loop, a Uvikon 725 variable-wavelength UV absorbance detector (Kontron, Zürich, Switzerland), in most experiments operated at 217 nm, and, placed in series downstream from the UV absorbance detector, an electrochemical detector constructed by our company's engineer, largely according to published instructions[3]. The inlet and outlet tube mountings had been reinforced and made more resistant towards dissolution of the glue by passing the tubes through acrylic 9 mm O.D. bushes cemented to the outside of the auxiliary electrode plate. The holes in this glassy carbon electrode plate were drilled 3 mm wide, permitting a ring-formed liner made from PTFE to be pressed around the end of each metal tube, flush with the polished inner surface of the plate. This liner protected the glue against components of the mobile phases. A spacer of thickness 110 μm separated the glassy carbon electrodes. The working electrode had been conditioned in hot paraffin wax as advised[3]. The wiring of the potentiostat had been modified so as to extend its zeroing capability.

The detector signals were recorded and processed by a Model BD 9 two-channel recorder (Kipp & Zonen, Delft, The Netherlands) and two Model 3390A reporting integrators (Hewlett-Packard, Avondale, PA, U.S.A.). The column materials were packed into Knauer tubes (Knauer, Oberursel, G.F.R.) of I.D. 4.6 mm and length 120 mm by a slurry technique, using the Pye Unicam pump and a reservoir of the Kirkland type[4].

Voltammetry of THIP

To determine a suitable potential for operation of the electrochemical detector, a hydrodynamic voltammogram for THIP was constructed. A 50-μl aliquot of a 100 μM aqueous solution of THIP was injected into the HPLC system at a series of detector electrode potentials. The potential was initially +0.70 V, and was increased in steps of 0.02 V until +1.02 V was applied. The mobile phase flow-rate was 2.0 ml min^{-1}. Two injections were made at each potential, and the resulting mean heights and mean areas of the chromatographic peak from THIP were plotted against applied potential.

Linearity and precision

Aqueous solutions of THIP were prepared at concentrations of 1, 2.5, 5, 10, 50, 100, 200 and 500 μM and 1, 1.5, 2, 3, 4 and 5 mM.

In one series of experiments, 50-μl aliquots of the solutions in the range 1–500 μM were injected into the HPLC system, making six cycles in all. A potential of +0.98 V was applied to the working electrode of the electrochemical detector. In another series of experiments, 10-μl aliquots of all of the solutions were injected, in six cycles, using a detector potential of +0.95 V. At both potential settings the electrode

had been allowed to equilibrate overnight while the voltage was applied and the mobile phase was recirculated. The UV absorbance detector was operated at 217 nm. The mobile phase flow-rate was 2.0 ml min^{-1}.

RESULTS AND DISCUSSION

Chromatographic system

Perfluorooctanesulphonic acid was preferred for use as counter ion for the ion-pairing with THIP because it was able to produce an ion pair that was relatively strongly retained on the column, and the retention of which was reasonably stable towards the influence of changes in the ambient temperature and the qualitative composition of the sample injected. In contrast, systems using a citrate-buffered mobile phase containing 0.15% trifluoroacetic acid and a stationary phase carrying amino or cyano groups were very susceptible to such changes, although an arbitrarily large retention time and a good peak shape could be obtained by adjustment of the concentration of trifluoroacetic acid.

When eluted with a mobile phase containing perfluorooctanesulphonic acid, perfluorohexanesulphonic acid or dioctyl sulphosuccinate, a C_{18} silica retained the ion pair more strongly than did a C_2 silica of the same particle size, as would be expected. Nucleosil 5 C_{18} seemed to offer a better shape of the peak from THIP than did Spherisorb S5 ODS.

Using the final choice of column and mobile phase composition, THIP was eluted from the column in 3.2 min. A chromatogram is shown in Fig. 1. The sample injected was an aqueous solution containing THIP, which had been formed by hydrolysis of a derivative from THIP. The left-hand trace refers to measurement of UV absorption at 217 nm and the right-hand trace is the signal from the electrochemical detector, operated at an electrode potential of +0.98 V.

The application of electrochemical detection involves restrictions in the choice of the composition of the mobile phase. The pH must allow oxidation (or reduction) of the solute of interest. A large proportion of organic solvent in the mobile phase will increase the impedance of the mobile phase (*iR*) and with it the uncompensated resistance between the reference electrode and the working electrode, and this may make an increase in the applied potential necessary in order to avoid an unacceptable limitation of the linear working range[5], although the cell design advised by Lankelma and Poppe[3] reduces the influence of *iR* on the potential of the working electrode. In my experience, lithium perchlorate is useful for simultaneous adjustment of the

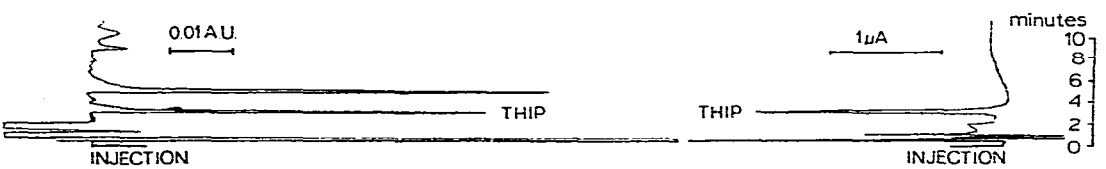

Fig. 1. Chromatogram of a sample containing THIP formed by hydrolysis of a derivative from THIP. Mobile phase: 0.02 *M* sodium acetate (pH 4.5)–tetrahydrofuran (90:10) with added perfluorooctanesulphonic acid (5 mmol l^{-1}) and lithium perchlorate (50 mmol l^{-1}); flow-rate, 2.0 ml min^{-1}. Column: 120 × 4.6 mm I.D., packed with Nucleosil 5 C_{18}. Detectors (placed in series): UV absorption, wavelength 217 nm, 0.1 a.u.f.s. (left-hand trace), and electrochemical detector, electrode potential +0.98 V (right-hand trace).

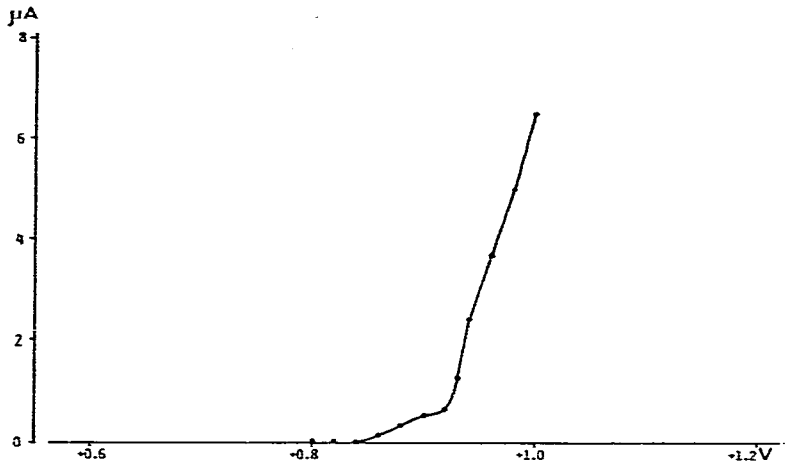

Fig. 2. Peak current (μA) from the electrochemical detector as a function of applied working electrode potential when 5-nmol samples of THIP were injected into the HPLC system.

retention time, suppression of peak tailing and increasing the electrical conductivity of the mobile phase. The use of it as a supporting electrolyte has been reported recently[6].

Choice of electrode potential

The voltammogram obtained from mean peak heights is shown in Fig. 2. No difference was noted when mean peak areas were plotted instead. When a working electrode potential of +0.84 V or less was applied, no measurable oxidation of THIP occurred. Application of a potential exceeding +1.01 V caused the operational amplifiers of the potentiostat to work in the saturated manner, *i.e.*, the amplifier outputs were maximal and thus not able to grade the signal.

An electrode potential of +0.98 V was selected for further experiments in which the detection of low THIP concentrations was desired, whereas a potential of +0.95 V was used for experiments on the linear response range because the lower potential allowed larger amounts of THIP to be detected without amplifier overload.

Calibration graphs: linearity and precision

Fig. 3 shows double logarithmic plots of the response from THIP (mean values) *versus* the amount injected, obtained by the electrochemical detector at electrode potentials of +0.98 and +0.95 V (below), and by the UV absorbance detector (above). The bars indicate standard deviations, but are diminutive at most points when UV absorption was measured. The coefficient of variation (C.V.) ranged from 16.5 to 34.2 in electrochemical detection and from 2.2 to 191.1 in UV detection, but in the latter instance the C.V. did not exceed 12.8 for sample amounts ranging from 0.5 to 50 nmol, whereas high C.V. values were found even with the larger sample sizes when electrochemical detection was used.

The electrochemical response curve obtained from peak areas had a shape similar to that in Fig. 3, but in this instance the C.V. was even larger (20.8–62.2). A reason for the "flatness" of the lower part of the electrochemical detection curves may be addition of a relatively large signal from noise to the detector signal with these

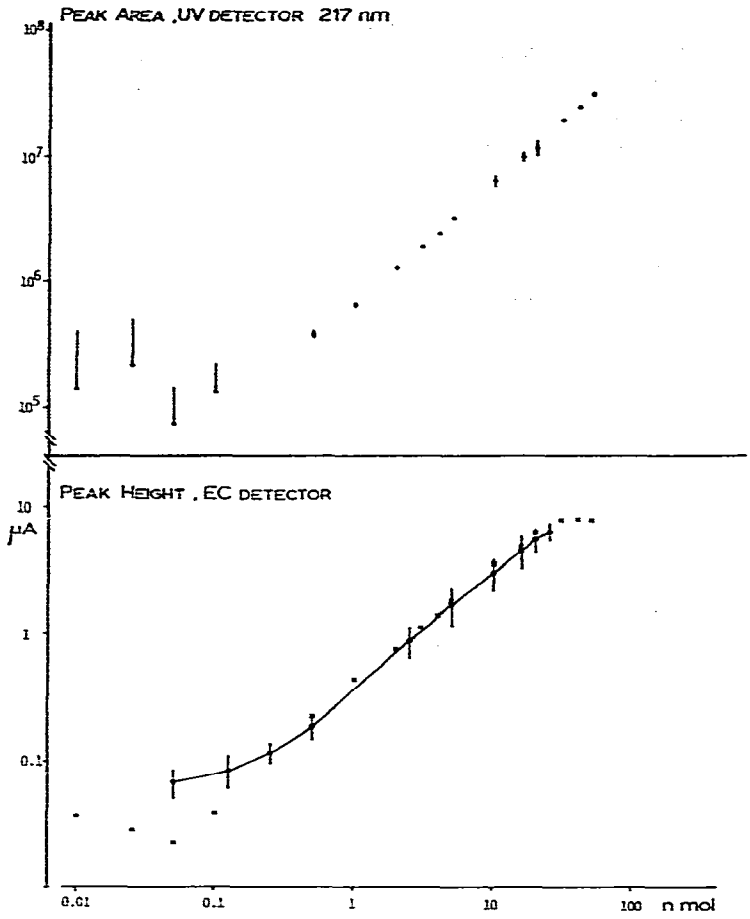

Fig. 3. Detector responses to 0.01–50 nmol of THIP injected into the HPLC system. Above: UV absorbance at 217 nm (peak areas, integrator units). Below: electrochemical (EC) detector operated at +0.98 V (●) or +0.95 V (×) (peak heights, μA). Bars indicate standard deviations (n = 6).

small amounts, and similarly uncertain responses were recorded from the UV detector in this range. The sigmoidal shape of the upper part of the electrochemical detection curves is likely to reflect saturation of processes in the electrochemical detector cell, or amplifier overload.

The electrochemical detector is likely to work in the voltammetric mode at the mobile phase flow-rate and the spacer thickness used[3], and in this instance peak heights should be used for quantitation[7]. In accordance with this, the C.V. was smaller when peak heights were used.

CONCLUSION

The procedure described here is a reliable means for quantitation of the GABA agonist THIP in dilute aqueous solutions. When an electrochemical detector with a large electrode surface area was used, amounts of 50 pmol per injection could be

detected with a C.V. of 25.6 ($n = 6$). Detection by a UV absorbance detector operated at 217 nm is accurate for samples of 500 pmol or more per injection. An advantage of the use of the electrochemical detector is the specificity that can be obtained.

The method has been used successfully in studies on the *in vitro* hydrolytic formation of THIP from THIP derivatives, of which only one was detectable (oxidizable) at the potential used.

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