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Short Communication

Sensitive determination of 1,1′-ethylidenebis(L-tryptophan) in L-tryptophan by gradient HPLC with fluorimetric detection

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Introduction

A dimeric impurity of L-tryptophan, 1,1′-ethylidenebis(L-tryptophan), has been associated with eosinophilia–myalgia syndrome (EMS) which has resulted in death and injury of a number of patients who have consumed L-tryptophan as a dietary food supplement or in medicinal products. The source of the impurity has been traced to a change in the fermentation process used by one manufacturer for the biosynthesis of the tryptophan precursors [1].

As the concentration of 1,1′-ethylidenebis(L-tryptophan) in batches associated with EMS is typically less than 0.01% w/w [2], a sensitive and specific method must be employed for its quantitation. Two liquid chromatographic methods based upon gradient elution development and single-wavelength spectrophotometric detection of 1,1′-ethylidenebis(L-tryptophan) in tryptophan have been published [1, 3]. This work was undertaken to investigate whether fluorescence detection offers any advantage in sensitivity and limits of detection and quantitation.

Experimental

Instrumentation

Two HPLC systems were used. System 1 consisted of a Philips PU 4100 liquid chromatograph with a Gilson Model 231 sample injector fitted with a 50-μl Rheodyne loop and a Perkin–Elmer LS 30 luminescence spectrometer detector, and a Shimadzu C-R3A Chromatopac integrator. System 2 comprised a Shimadzu LC-9A pump, Spark Holland autosampler and LDC Analytical Spectromonitor 3100 ultraviolet detector with an Anachem oven and Shimadzu C-R3A integrator. System 2 was used only to compare the performance of the fluorimetric and UV detectors.

Reagents

Acetonitrile was Rathburn HPLC-grade (Walkerburn, Scotland, UK) (minimum 80% transmittance at 220 nm) or Rathburn 'far UV'-grade (minimum 95% transmittance at 220 nm). Water was de-ionized. Trifluoroacetic acid (TFA) was Fisons (Loughborough, UK) SLR grade. A sample of 1,1′-ethylidenebis(L-tryptophan) reference material [4] was kindly supplied by Dr Samuel W. Page of the Food and Drug Administration (Washington DC, USA).

Preparation of standard

A stock solution of 1,1′-ethylidenebis(L-tryptophan) in water (1.05 mg ml^{-1}) was prepared. Working standard solutions were prepared by making appropriate dilutions of the stock solution. The stock solution was stored at 4–8°C and was found to be stable over long periods (at least 6 months).

Preparation of the sample

Approximately 100 mg of L-tryptophan powder was accurately weighed into a 20-ml

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volumetric flask. About 15 ml of water was added and the flask was shaken for 5 min and sonicated briefly until dissolution was complete. The sample was diluted to volume with water.

Chromatographic conditions

A 20 × 0.3 cm i.d. Nucleosil 5-μm ODS column maintained at 35°C was used at a flow rate of 0.4 ml min^{-1} giving a back-pressure of about 1200 psi at the start of the gradient. The analytical detector wavelengths were set at 280 nm (excitation) and 342 nm (emission) for fluorimetric detection, and at 220 nm for ultraviolet detection. HPLC-grade acetonitrile was used throughout except for the comparison of detection limits, when 'far UV'-grade was used.

Mobile phase composition. Mobile phase A was 0.1% TFA in water. Mobile phase B was 0.1% TFA in acetonitrile–water (80:20, v/v). The following gradient was used: 0–2 min (100% mobile phase A); 2–28 min (linear gradient to 50% A); 28–35 min (linear gradient to 100% B); 35–43 min (composition held); composition returned to initial conditions in 10 min.

Results and Discussion

As the principal objective of the study was to compare the performance of the fluorescence and spectrophotometric detectors in terms of sensitivity and limits of detection and quantitation, it was considered that the gradient programme already in use at this laboratory for screening samples of L-tryptophan should be used for the chromatographic development. This eluent was the same as that used by Bolongia *et al.* [1], but differed slightly in the gradient programme. While the gradient defined above was adequate for the majority of samples, it was noticed that with some samples of L-tryptophan, which were subsequently shown not to contain the impurity, other impurity peaks eluted at or near the retention time of the analyte impurity peak. For these samples, it was necessary to increase the time of the second segment of the gradient to 30 or 35 min to achieve sufficient resolution in order to confirm the absence of the impurity.

Validation

Linearity. The detector response (peak area) was proportional to the concentration of the impurity over the range tested (between 0.05 and 3 μg ml^{-1}, equivalent to 0.001–0.6% w/w of the L-tryptophan concentration). The regression was: $y = 998.8x - 1.75$ with a correlation coefficient of 0.9999 ($n = 9$), where x is in μg ml^{-1}.

Precision. The repeatability of the analytical system was determined by using two samples of L-tryptophan found to contain 0.011 and 0.0013% w/w of impurity. Six consecutive replicate injections of each sample gave a relative standard deviation (RSD) of 0.6 and 1.5%, respectively.

Recovery. A solution containing L-tryptophan with no detectable 1,1′-ethylidenebis(L-tryptophan) was spiked with an aliquot of the impurity solution at two concentrations of 0.11 and 0.42 μg ml^{-1} (equivalent to 0.0022 and 0.0084% w/w, respectively). Recoveries obtained by comparing peak areas with standard solutions at these two concentrations were 109 and 100%, respectively.

Detection and quantitation limits. The limit of detection (LOD) for both the fluorimetric and ultraviolet detectors was determined by chromatographing a series of six 1,1′-ethylidenebis(L-tryptophan) standard solutions at concentrations between about 0.05 and 0.3 μg ml^{-1}, and a solvent blank injection, using HPLC system 2. The LOD was calculated from the formula [5]: LOD (μg ml^{-1}) = $3 \cdot s_d/S$, where s_d is the standard deviation of the baseline noise and S is the sensitivity of the method (i.e. the slope of the calibration curve, mm μg^{-1} ml). The standard deviation of the baseline noise was taken as one-fifth of the peak-to-peak noise recorded around the retention time of the 1,1′-ethylidenebis(L-tryptophan) peak in the solvent blank [6]. The chromatographic conditions were identical for each of the detection methods, and 'far UV'-grade acetonitrile was used in the eluant. The LOD for the fluorimetric measurements was 0.015 μg ml^{-1} (0.75 ng injected on-column) and for the ultraviolet measurements 0.041 μg ml^{-1} (2.0 ng injected on-column). These are equivalent to impurity levels of 0.00030 and 0.00082% w/w, respectively.

The corresponding limit of quantitation (LOQ) determined by using the formula $10 \times s_d/S$, was 0.001 and 0.0027% w/w for the

fluorimetric and spectrophotometric methods, respectively. The repeatability, as the RSD of five replicate area measurements obtained with the fluorimetric detector at a concentration of impurity close to the calculated LOQ of 0.047 μg ml^{-1} (equivalent to 0.00093% w/w), was 6.9%. Precision of this order at the LOQ was considered to be acceptable and confirms the value for the LOQ predicted by statistical treatment of baseline noise. The efficiency of the column for the impurity peak at the time the measurements were made was approximately 1.1×10^6 plates m^{-1}.

The LOD and LOQ values obtained with the fluorimetric detector were about 40% of those of the ultraviolet detector. As the sensitivities of the two detectors were similar, the higher limits observed with the ultraviolet detector were attributed to the higher baseline noise due to the baseline drift that occurred with increasing concentration of acetonitrile as the gradient progressed. 'Far UV'-grade acetonitrile was used to minimize this effect as far as possible. An advantage of fluorimetric detection is that the less expensive HPLC-grade acetonitrile can be used without increasing the noise level.

Application

The method described was used to screen 30 samples of L-tryptophan starting material from several manufacturers for the presence of 1,1′-ethylidenebis(L-tryptophan). Screening was performed by chromatographing a sample solution, adding one drop of a 1,1′-ethylidenebis(L-tryptophan) solution (about 2 μg ml^{-1}) to the HPLC vial, and chromatographing the spiked solution to confirm the retention time of 1,1′-ethylidenebis(L-tryptophan). 1,1′-Ethylidenebis(L-tryptophan) was detected in six samples, all from the same manufacturer. These were then assayed by the method described, using System 1 with fluorimetric detection. Concentrations up to 0.0122% w/w were found, although two of these were below the LOQ. Figure 1 shows a chromatogram of one of these samples with a concentration of 1,1′-ethylidenebis(L-tryptophan) corresponding to 0.0013% w/w.

The fluorimetric LC method developed is

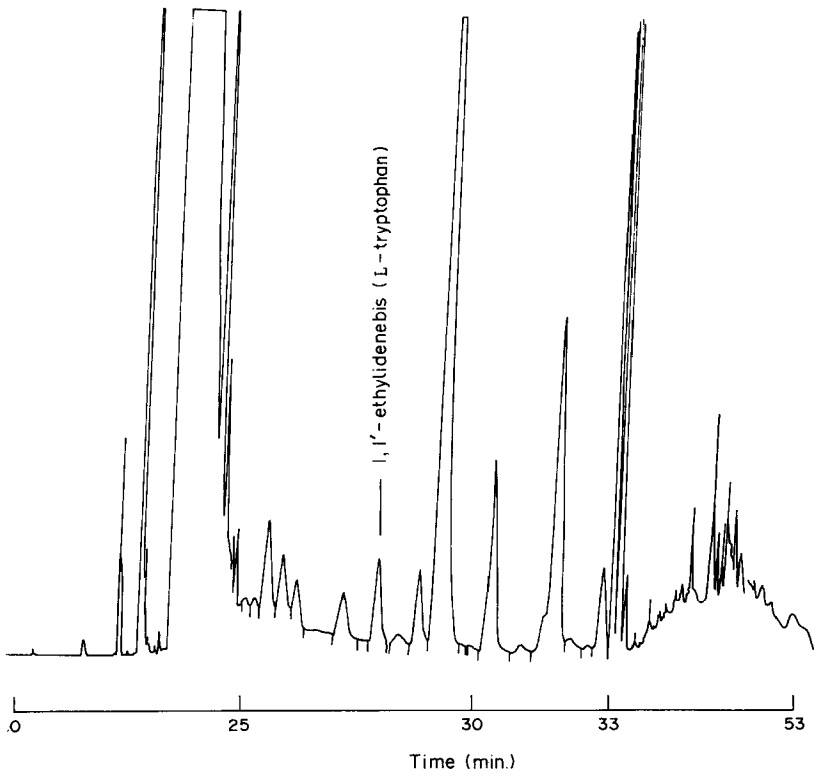

Figure 1
HPLC chromatogram with fluorimetric detection of a sample of L-tryptophan containing 1,1′-ethylidenebis(L-tryptophan) at a concentration of 0.0013% w/w. (Chart speed: 0–25 min and 35–53 min — 2 mm min^{-1}; 25–33 min — 10 mm min^{-1}.)

straightforward and more sensitive than that based on UV detection. The method should be suitable for the routine screening of 1,1′ ethylidenebis(L-tryptophan) in samples of L-tryptophan.

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