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Separation and isolation of trace impurities in L-tryptophan by high-performance liquid chromatography*

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ABSTRACT

A high-performance liquid chromatographic (HPLC) profiling method was developed to separate trace impurities in L-tryptophan products associated with the eosinophilia-myalgia syndrome (EMS) epidemic. The test portion was dissolved in water, and the solution was filtered and chromatographed on a silica-based C_{18} reversed-phase HPLC column by using linear gradient elution with water and acetonitrile-water (80:20); both solvents contained 0.1% trifluoroacetic acid for ion-pairing. The method was used to profile 200 test samples from six manufacturers of L-tryptophan. The method was modified to include the use of a C_{18} disposable cartridge to retain the 1,1′-ethylidenebis(L-tryptophan) (peak E, peak 97 or EBT), the impurity most strongly associated with EMS, and to remove the L-tryptophan before HPLC separation and quantitation. Recoveries of EBT added to test portions (2 μg/g and above) averaged 80%.

INTRODUCTION

In the fall of 1989, physicians in New Mexico, USA, noted that several patients had developed a peculiar illness marked by eosinophilia and severe myalgia [1]. Epidemiologic investigations by these physicians and researchers at the Mayo Clinic and the Centers for Disease Control (CDC) [2–6] associated this illness with the consumption of L-tryptophan (L-Trp). The CDC case definition of the syndrome has been (1) an eosinophil count $> 1 \cdot 10^9/l$, (2) generalized debilitating myalgia and (3) no evidence of infection or neoplasm that would explain the eosinophilia or the myalgia [7]. To date, more than 1600 cases of eosinophilia-myalgia syndrome (EMS) have been reported, with 38 deaths [8]. Warnings and recalls of L-Trp by the Food and Drug Administration (FDA) in November 1989 [9] promptly ended the epidemic. Research was immediately initiated to determine the cause of EMS. Since L-Trp had been used previously without any reported toxicity, this research focused on the search for a contaminant or contaminants.

L-Trp was produced in Japan by six manufacturers. The bulk was made into capsules, powder or tablets by many distributors in the USA. It was used to treat insomnia, premenstrual syndrome, obesity and drug withdrawal.

In early February 1990, we developed a high-performance liquid chromatographic (HPLC) procedure to screen L-Trp products for minor impurities. Studies by the CDC and other researchers in Oregon and Minnesota found a strong association between EMS and L-Trp produced by Showa Denko K.K. between October 1988 and June 1989 [10]. A method for isolating and quantifying one of the impurities, 1,1′-ethylidenebis(L-tryptophan) (EBT, peak E or peak 97), was developed.

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EXPERIMENTAL

HPLC profiling of L-tryptophan

The laboratory sample was ground, 0.5 g of the powder was placed into a 15-ml polypropylene centrifuge tube and 5 ml of water were added. The mixture was vortexed for 2 min, centrifuged and filtered, and 50-100 μl of the filtrate were injected into the HPLC system. The system consisted of a Waters U6K injector, a 15-cm Delta-Pak C_{18}, 300-A (pore size), 3-μm (particle size) column, two pumps, a UV detector set at 220 and 280 nm and a Digital Professional 350 computer equipped with Waters software. The linear gradient elution used consisted of solvent A: 0.1% trifluoroacetic acid (TFA) in water, and solvent B: 0.1% TFA in acetonitrile-water (80:20). The mobile phase started with 100% solvent A for 2 min followed by a linear gradient to 80% solvent B at 37 min.

Isolation of EBT

A saturated solution of L-Trp (10 ml) was passed through a Waters C_{18} Sep-Pak cartridge that had been conditioned with 6 ml of methanol and 6 ml of water. The cartridge was washed with 30 ml of water, followed by 6 ml of acetonitrile-water (6:94) to eliminate most of the L-Trp. The contaminants were then eluted with 6 ml of acetonitrile-water (70:30). The solvent was evaporated by using a Speed Vac (Savant) vacuum evaporator, the residue was dissolved in 2 ml of water, the solution was filtered and 500 μl of the filtrate were chromatographed on a 30-cm NovaPak C_{18}, 3-μm (particle size) column by using a linear gradient from 20% solvent B to 60% solvent B over 23 min. The HPLC system and solvents A and B were the same as previously described.

Quantitation of EBT

EBT standard solution was prepared in 0.1 *M* triethylammonium acetate (pH 9.2) buffer. The L-Trp manufactured by Tanabe Seiyaku Co. contained no EBT and, therefore, was used as the control in the recovery studies. Various amounts of EBT were added to a 0.5-g test portion (from ground L-Trp tablets or contents of L-Trp capsules) in a 250-ml Erlenmeyer flask. The test portion was extracted with 100 ml of 0.1% TFA solution by shaking the mixture for 3 min, the extract was filtered and 50 ml of the filtrate was passed through a C_{18} Sep-Pak cartridge that had been conditioned with methanol and water. A vacuum manifold was used to adjust the flow-rate to about 10 ml/min. The cartridge was washed with 30 ml of water and 6 ml of acetonitrile-water (6:94). After most of the solvent had been eliminated by applying vacuum for an additional 2 min, a polyethylene 15-ml centrifuge tube was placed under each cartridge. A small amount of L-Trp and the impurities were then eluted with 6 ml of methanol-acetonitrile (1: 1). The eluate was concentrated in a Speed Vac vacuum evaporator to about 0.2 ml. The final extract was diluted to 2 ml with water, centrifuged at high speed and injected onto a 30-cm Delta Pak C_{18}, 5-μm (particle size) column. Solvents A and B were the same as previously described. The UV detector was set at 280 nm. A combination of gradient, isocratic and step gradient elution was used, starting with a linear gradient from 20% solvent B to 30% solvent B in 10 min, followed by isocratic elution for 15 min and then immediate step gradient elution to 80% solvent B for 10 min. The retention time of EBT was about 19.5 min.

RESULTS AND DISCUSSION

The HPLC fingerprint profiles (200 samples from different manufacturers) are relatively consistent from lot to lot of the individual manufacturers. HPLC profiling can be used to determine the source of an unknown lot. The variation in chromatographic patterns is probably related to differences in the manufacturing processes. The chromatograms from patient-related materials showed many more small peaks. More than 200 test samples of L-Trp were analyzed. Most of the patient-related materials were produced by Showa Denko K.K. Figs. 1 and 2 show typical chromatograms of L-Trp from two different manufacturers. Fig. 2 is that of a Showa Denko K.K. product.

Using similar HPLC profiling procedures. researchers at the CDC and at the Mayo Clinic have attempted to correlate the impurities found by HPLC with the incidence of EMS. They concluded that EBT (Fig. 2) may be associated with the illness [2,11]. Our effort has been centered on the isolation of EBT and some of the later-eluting components.

In the EBT isolation procedure, the disposable

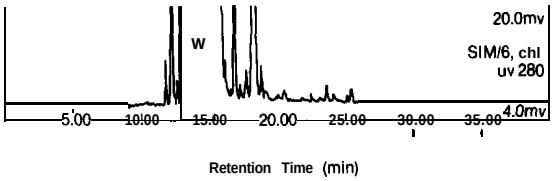

Fig. 1. Chromatogram of L-tryptophan (w) from manufacturer 1. Chromatographic conditions: 15-cm C_{18} column; linear gradient from 0 to 80% B over 35 min; detection at 280 nm. Mobile phase components: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile-water (80:20).

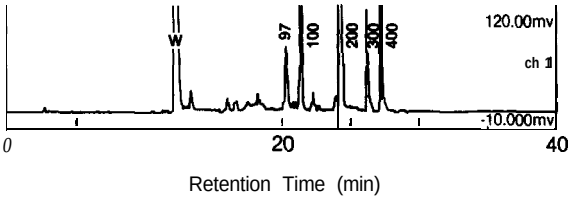

Fig. 3. Chromatogram of concentrated minor components in L-tryptophan (w). Chromatographic conditions: 30 cm C_{18} column; linear gradient from 20 to 60% B over 23 min; detection at 280 nm. Mobile phase components as in Fig. 1.

C_{18} cartridge was used to separate the bulk of the L-Trp from the contaminants. Components representing five of the HPLC peaks shown in Fig. 3 were collected and then analyzed separately by using various mass spectrometric (MS) techniques. In preliminary results obtained in collaboration with the University of Virginia, we found all five components to have molecular masses of less than 500, using a Fourier transform MS procedure. The exact molecular mass (M_r) of EBT (one of the five components, M_r 766) was determined in collaboration with PE Sciex, Canada. The MS data suggested several structures. High-resolution secondary ion MS performed at the FDA with a VG instrument gave the exact elemental composition, limiting the structure to an t-Trp dimer joined by an ethylidene bridge. However, it was not certain whether the two L-Trp molecules were joined with a bridge at the amino nitrogens or the indole nitrogens. By using a procedure developed by Showa Denko K.K., the component corresponding to peak E was synthesized and the structure was determined to be 1,1′-ethylidenebis(L-tryptophan), now known as EBT (Fig. 4) [12]. Researchers at the Mayo Clinic independently determined the structure of this compound, which was isolated from the contaminated L-Trp [13]. EBT is an aminal, an L-Trp dimer joined by an ethylidene linkage at the indole nitrogens.

With the availability of EBT as a reference standard (purity >95%), it became possible to develop a quantitative method for EBT in L-Trp tablets or capsules. (MS showed that the M_r of 766 and the fragmentation pattern for the standard and the isolated EBT were the same.) The solubility and stability of EBT were determined. Although EBT is readily soluble in either acidic or basic solution, it is more stable in basic solution. EBT in 0.1% TFA solution kept at 5°C decomposed completely within 1 week (no HPLC peak at the retention time of the peak produced by the original solution before storage). A solution of 10 mg EBT/100 ml of 0.1 M triethylammonium acetate (pH 9.2) buffer kept at 5°C for 1 month gave an HPLC peak that was the same height as the peak produced by the original solution before storage for the same injection volume. L-Trp is more soluble in 0.1% TFA solution

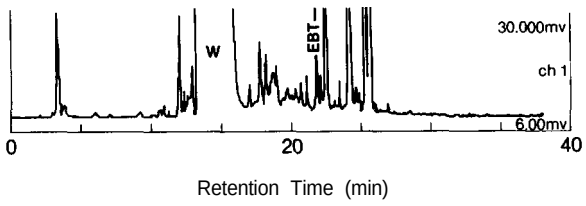

Fig. 2. Chromatogram of L-tryptophan (w) from manufacturer 2. Chromatographic conditions and mobile phase components as in Fig. 1.

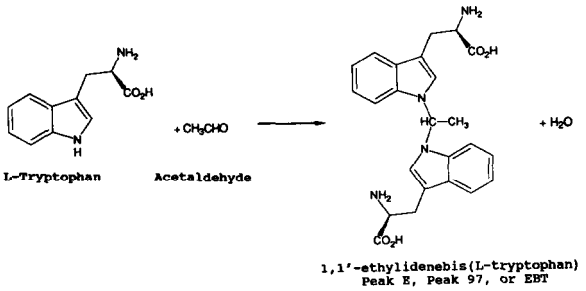

Fig. 4. Synthesis of 1,1′-ethylidenebis(L-tryptophan)

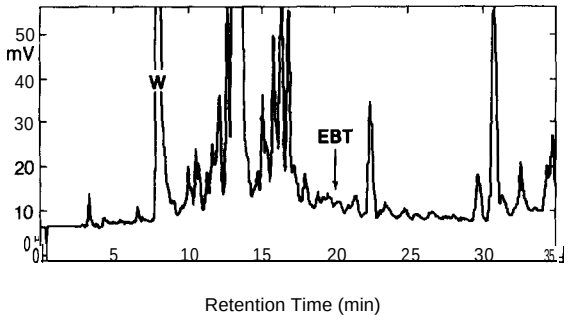

Fig. 5. Chromatogram of non-patient-related material. Chromatographic conditions: 30-cm C_{18} column; linear gradient from 20 to 3% B over 10 min, isocratic elution for 15 min, step-gradient elution to 80% B, hold for 8 min. Mobile phase components as in Fig. 1. W = L-Trp.

than in water (pK_a values for L-Trp are 2.43 and 9.44); therefore, spiked test portions were dissolved in TFA solution. It is not advisable to dissolve the compound in basic solution because most silica gel bonded-phase C_{18} cartridge packings will be destroyed at a $pH > 7$. Recoveries of EBT added at levels of 0.6, 1, 2 and 4 μg/g of L-Trp were 117, 113. 84 and 82%, respectively. The high recoveries for the lower levels were due to slight background interferences.

This method was used to analyze some of the patient-related and non-patient-related materials. All of the patient-related materials were found to contain EBT at $> 70\,\mu$g/g of tablet, whereas the non-patient-related materials either did not contain EBT or contained significantly less EBT, as shown by the absence of a peak at the approximate retention time of EBT (Figs. 5 and 6).

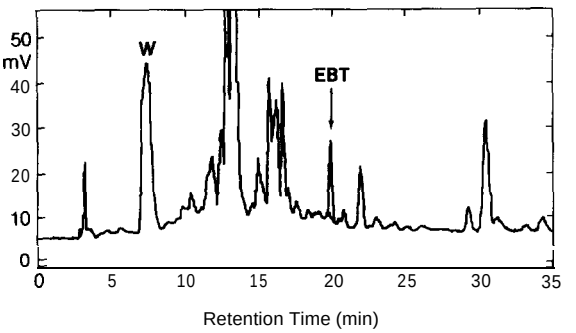

Fig. 6. Chromatogram of patient-related material. Chromatographic conditions and mobile phase components as in Fig. 5.

CONCLUSIONS

All L-Trp preparations contain various minor impurities. These vary with the manufacturing processes. One of the impurities, EBT (peak E or peak 97), from Showa Denko K.K. products was isolated. The structure was elucidated and a quantitative method of analysis was developed. Although preliminary testing in an animal model indicates that EBT may have some role in EMS, other factors may be involved [14].

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REFERENCES

1 P. A. Hertzman, W. L. Blevins, J. Mayer, B. Greenfield, M. Ting and G. J. Gleich, N. *Engl. J. Med.*, 322 (1990) 869.
2 E. A. Belongia, C. W. Hedberg, G. J. Gleich, K. E. White, K. L. MacDonald and M. T. Osterholn, N. *Engl. J. Med.*. 323 (1990) 357.
3 Centers for Disease Control, *Morbid. Mortal. Weekly Rep.. 38 (1989) 785.*
4 Centers for Disease Control, *Morbid. Mortal. Weekly Rep., 38 (1989) 842.*
5 Centers for Disease Control, *Morbid. Mortal. Weekly Rep.. 39 (1990) 14.*
6 Centers for Disease Control, *Morbid. Mortal. Weekly Rep.. 39 (1990) 89.*
7 E. M. Kilbourne, L. A. Swygert, R. M. Philen, R. K. Sun, S. B. Auerbach, L. Miller, D. E. Nelson and H. Falk. *Ann. Intern. Med.*, 112 (1990) 85.
8 R. Philen, Centers for Disease Control, personal communication, 1991.
9 Food Chrm. News, Nov. 27 (1989) 49.
10 L. Slutsker, F. C. Hoesly, L. Miller, L. P. Williams, J. C. Watson and D. W. Fleming, *J. Am. Med. Assoc., 264* (I 990) 213.
11 Centers for Disease Control. *Morbid. Mortal. Weekly Rep.. 39 (1990) 589.*
12 M. J. Smith, E. P. Mazzola, T. J. Farrell, J. A. Sphon, S. W. Page, D. Ashley, S. R. Sirimanne, R. H. Hill. Jr. and L. L. Needham. *Tetrahedron Lett., 32 (1991) 991.*
13 A. N. Mayeno, F. Lin, C. S. Foote, D. A. Loegering, M. M. Ames, C. W. Hedberg and G. J. Gleich, *Science,* 250 (1990) 1707.
14 L. J. Crofford, J. I. Rader, M. C. Dalakas, R. H. Hill, S. W. Page, L. L. Needham, L. S. Brady. M. P. Heyes, R. L. Wilder, P. W. Gold, I. Illa, C. Smith and E. M. Sternberg, *J. C/in. Invest..* 86 (1990) 1757.