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Determination of histamine in trypsine by capillary zone electrophoresis – a possible alternative to the bioassay?

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A rapid capillary electrophoresis separation was developed in order to separate histamine from trypsine. A calibration curve with a determination limit of 1.0 μg/ml histamine in 1mg/ml trypsine was obtained. The method is suitable for the detection of small amounts of histamine contamination in trypsine with regard to the standards of the European Pharmacopoeia.

1. Introduction

Various substances of biological origin are still monitored in routine quality control for the presence of histamine as a possible impurity [1, 2]. Histamine is a mediator and a vasoactive compound with toxicological importance [3]. Contamination with this amine of biological origin can be caused by extraction of histamine-rich tissues, by inadequate purification, or by degradation of the amino acid histidine in the final product [4]. At present the manufacturing processes of chymotrypsine and trypsine have to be validated in such a way that the substances meet the requirements of the monographs. For aprotinine the test is specified without reservation in the monograph. The European Pharmacopoeia lays down a quantitative test for histamine impurity on a preparation of guinea pig small intestine. The test is based on the measurement of histamine induced muscle contraction [5]. The maximum quantity of histamine tolerated depends on the compound being examined and ranges between 1 μg/g and 5 μg/g. If the test fails in a certain manner the test for blood depressing substances (tested in cats) has to be used as described in the Pharmacopoeia. This experiment with non-specific modifications is listed as a purity check in further monographs e.g. kanamycine or streptomycine.

Conventional analytical procedures can be applicable as an alternative to animal testing for histamine. HPLC, and CE with UV- or fluorescence detection, ELISA or electrochemical biosensors are possible methods. They are used for histamine determination in the quality control of food, e.g. fish, cheese and wine, within the ppm-/ppb-range [6–14]. These analytical procedures are more sensitive, more robust, and have a similar or lower detection limit in comparison with the bioassay. Therefore retaining the bioassay is inconsistent with the state of science.

The aim of this paper is to present a method to determine small amounts of histamine in trypsine. It could be a potential alternative to the bioassay.

2. Investigations and results

In order to separate and to quantify histamine in trypsine a capillary electrophoresis method was developed having regard to the properties of the substances. Histamine is a biogenous amine which is positively charged in acidic conditions (imidazolium-cation) [2]. Trypsine is a basic protein built of 223 amino acids with histidine in the catalytic centre, and can produce histamine by enzymatic decarboxylation or bacteriological degradation [3]. A citric acid buffer system with a low pH-value was chosen to achieve a fast separation and at the same time give the opportunity of direct UV-detection. A similar system has been used successfully for the determination of histamine in food [10]. Advantages of the capillary electrophoresis technique are rapid separation time, easy sample preparation in most cases and high efficiency. The calibration range of histamine was set such as to include the limiting value of the monograph. Calibration was by injecting trypsine matrix spiked with different amounts of histamine. The temperature in all experiments was 30 °C, the field strength was varied. Histamine was detected after a migraton time of 4.8 min when the separation was performed at 285 Vcm^{-1}. Trypsine was detected after a migrating time of 9.1 min. After the field strength was raised to 428 Vcm^{-1}, the migration time of histamine was decreased to 3.0 min and tryp-

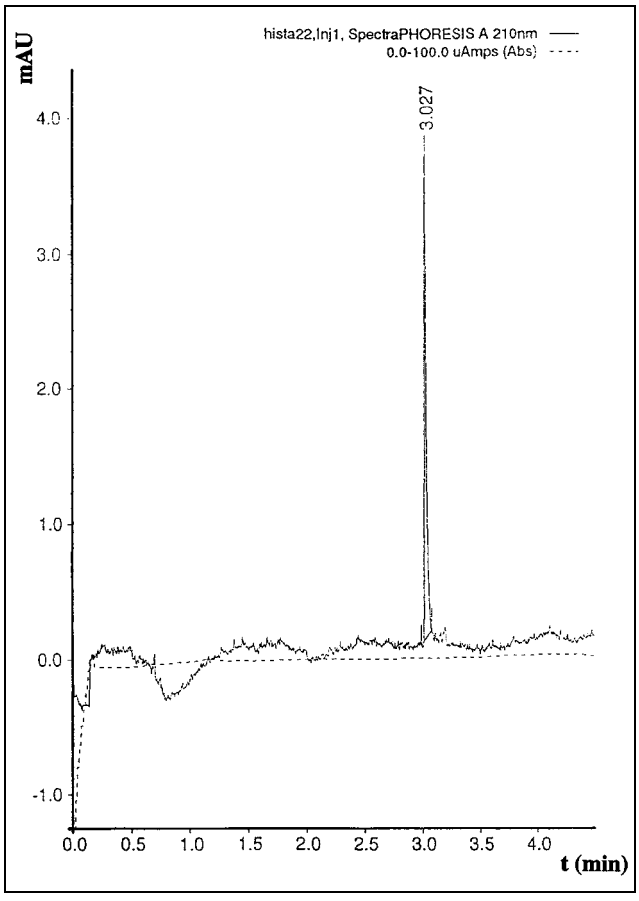

Fig. 1: Electropherogram obtained by injection of 10 μg/ml histamine in trypsine (1 mg/ml), $t_{m\ histamine}$ 3.027 min, trypsine peak not shown, trypsine was washed from capillary by limiting the run time to 4.5 min and rinsing with buffer

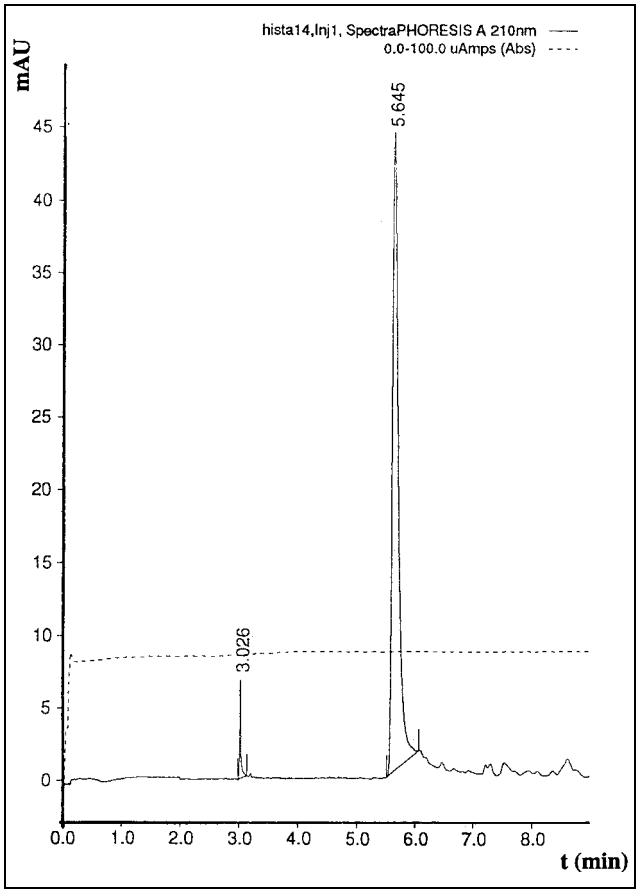

Fig. 2: Electropherogram obtained by injection of 20 μg/ml histamine in trypsine (1 mg/ml), $t_{m\ histamine}$ 3.026 min, $t_{m\ trypsine}$ 5.645 min

sine was accelerated to a migration time of 5.6 min. A noticeable tailing occurred at higher histamine concentrations beginning at approximately 20 μg/ml (Figs. 1 and 2).

The relationship of peak area versus histamine concentration is linear (Fig. 3) and can be described with the eq. $y = 432.91x - 94.404$. The correlation coefficient obtained is $R^2 = 0.9906$. The critical value of the determination is 27.46 μAU · s. The resulting detection limit is 0.3 μg/ml. The resulting registration limit is 0.6 μg/ml. The resulting determination limit is 1.0 μg/ml ($n = 18$, $\alpha = 0.01$, $k = 4$) [15]. The standard deviation of the slope is ±2.4%. The intercept is not significantly different from zero ($\alpha = 0.01$) considering the standard deviation of ±63.1%. The mean precision was ±0.19 μg/ml histamine

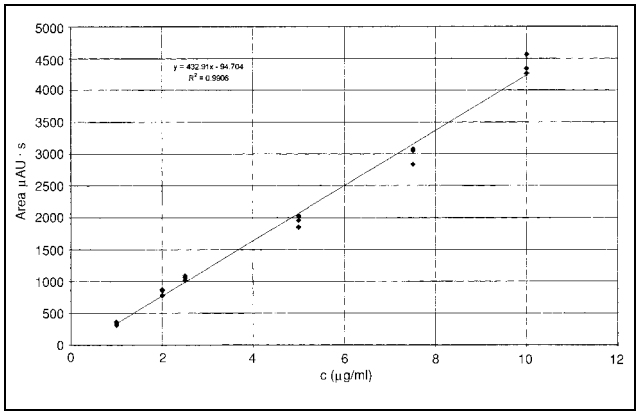

Fig. 3.: Calibration curve peak area versus histamine concentration, obtained by capillary electrophoresis separation of histamine in trypsine (1mg/ml)

over the whole calibration range (standard deviation of determined amounts). The mean accuracy of the determination was ±1.13% (recovery of the spiked amounts of standard concentrations). On testing a blank injection of water, buffer and trypsine solution no histamine peak could be detected. After injection of trypsine solutions made of 10 year-old material no peak occurred. The concentrations were equivalent to the calibrated solutions 1 mg/ml. No histamine could be detected after injection of higher concentration solutions up to 50 mg/ml trypsine. An comparison of histamine injection with and without a trypsine matrix led to the conlusion that the trypsine matrix creates no change of response.

3. Discussion

The maximum histamine content in trypsine is limited in the monograph to 1 μg (calculated as base) per 0.2 microkatal enzyme activity. The lowest possible limiting value is 2.5 μg histamine base in 1 mg trypsine (on a mass basis), corresponding to the minimum permitted enzyme activity of 0.5 microkatal per 1 mg trypsine [2]. A higher histamine contamination (with respect to the mass of the substance) is permitted with a higher enzyme activity of the trypsine examined. The determination limit of the method should be lower than 2.5 μg/mg trypsine. The final value necessary depends on the concentration of trypsine in the solution examined; e.g. a solution of 50 mg/ml trypsine requires a determination limit of lower than 125 μg/ml histamine. Conversely a low determination limit of an analytical method results in a higher safety margin if high concentration solutions are examined. The method developed allows the determination of histamine in trypsine in a range between 1 μg/ml and 10 μg/ml. This has been tested for a trypsine matrix concentration of 1 mg/ml in accordance with the requirements of the monograph of the European Pharmacopoeia. A lower limit of histamine content appears to be possible, since no interference with the determination occurs at higher trypsine concentration. Improved precision could be achieved by use of an internal standard and a peak area correction by migration time.

In conclusion, determination of histamine in trypsine by capillary zone electrophoresis is possible with a rapid method and a low determination limit. This method offers the potential of an alternative method to the animal experiment described in the Pharmacopoeia. A similar method could possibly be developed as a histamine impurity test for chymotrypsine and aprotinine.

4. Experimental

The capillary electrophoresis apparatus was the Spectra Phoresis 500™ including the PC 1000™ software version 2.6 (TSP Thermo Separation Products, Darmstadt, Germany). All separations were performed with a fused silica capillary ID 75 μm, total length 70 cm integrated in a capillary cassette. Glass sample vials (2 ml) with caps made of polypropylene were used (all TSP Thermo Separation Products Darmstadt). For the 1000 μg/ml stock solution of histamine (calculated as base) 167.2 mg substance (Histamindihydrochloride, Synopharm Pharmazeutische Feinchemikalien, Barsbüttel, Germany 156340-000) was diluted in 100.0 ml purified water (in-house-high-purity-water system >16 MΩ). A second stock solution of 100 μg/ml was made by diluting 1.00 ml of the 1000 μg/ml stock solution in 10.0 ml purified water. 200.0 mg trypsine (bovine pancreas trypsine Boehringer Mannheim GmbH, Mannheim, Germany 14211429-89, 06/96) was diluted in 20.0 ml purified water for the 10 mg/ml stock solution of trypsine. This was used for the calibration procedure. 100.0 and 500.0 mg of 10 year-old trypsine (bovines pancreas trypsine Boehringer Mannheim GmbH, Mannheim, Germany 10908728-73, 08/89) was diluted in 10.0 ml purified water for the investigation of possible degradation. The calibration solutions were made by adding different amounts of the histamine stock solution (10 μl, 20 μl; 25 μl, 50 μl, 75 μl and 100 μl of the 1000 μg/ml

stock solution) as well as 1.00 ml of the trypsine stock solution in a 10,0 ml flask and filling it up to the mark with purified water (calibration range 1 μg/ml to 10 μg/ml histamine, calculated as base). The blank solution was made in the same way without adding histamine. The 100 μg/ml solution was used for identification of histamine. A 20 mmol/l citric acid buffer solution was made up from 384.2 mg anhydrous citric acid (Riedel de Haën Laborchemikalien GmbH & Co KG Seelze, Germany) diluted in 1.0 l purified water. It was used as the electrolyte for the electrophoretic separation. The pH-value of 2.5 was adjusted with 1 N NaOH (Sodium hydroxide, Riedel de Haën Laborchemikalien GmbH & Co KG Seelze, Germany). The capillary conditioning procedure was performed by washing for 5 min with 1 N NaOH at 30 °C, 10 min with high-purity H_2O at 30 °C, and 15 min with citric acid buffer solution at 30 °C. Between each injection a capillary rinse of 2 min with citric acid buffer solution was made. The separations were performed at 30 °C and at a field strength of 285 Vcm^{-1} (20 kV) or at 428 Vcm^{-1} (30 kV). The detection wavelength was 210 nm. The injections were performed hydrodynamically for 5 s. The calculation was made by peak area and by peak height (results not shown but equivalent) versus concentration with Microsoft® Excel 97.

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