Determination of Hemocoagulase Agkistrodon in a Pharmaceutical Preparation by High-Performance Liquid Chromatography with Pre-Column Derivatization and Fluorescence Detection

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Currently, there is no analytical method for the quantification of hemocoagulase agkistrodon (HCA) in pharmaceutical preparations. This study presents a pre-column derivatization method for the quantification of HCA, a compound extracted from the venom of Agkistrodon acutus, in a pharmaceutical preparation (trade name Suling). In the proposed method, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate was used to tag the HCA substrate, and the derivatives were analyzed by high-performance liquid chromatography with fluorescence detection. Complete and homogeneous derivatization of HCA was confirmed by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry analysis. The specificity of the method was validated by forced degradation, and interference was assessed using a placebo. Under the optimum chromatographic conditions, the calibration curve was linear over a range of 10 to 500 ng/mL, featuring a correlation coefficient of 0.9999. The limits of detection and quantification of the method were 0.57 and 1.6 ng/mL, respectively. The percentage recovery of HCA in quality control samples ranged from 97.49 to 99.15%. Overall, this novel method can be applied to the quantitative determination of HCA in pharmaceutical preparations.

Introduction

Hemocoagulase agkistrodon (HCA), a thrombin-like enzyme from the venom of the Chinese moccasin snake (Deinagkistrodon acutus), has been isolated and purified to homogeneity by ion exchange chromatography on DEAE-SepharoseFF (1). HCA is a double chain glycoprotein with a molecular weight of 29 kDa, containing 252 amino acid residues (2). Prior studies have shown that HCA decreases the clotting time of whole blood and decreases bleeding time, as determined by using a mouse tail-snip model (3). Classified as a national class 1 drug in China, HCA has been completely sequenced, and has been used as a coagulant for more than two years. HCA has a very high specific activity, permitting common doses of only one to two units per day. The HCA pharmaceutical preparation of interest contains a large amount of dextran 20 as an excipient. At present, no adequate analytical method has been established to determine HCA content of this preparation, because it contains a relatively low concentration of HCA compared with dextran 20, and the two compounds share a similar molecular weight. These conditions lead to excipient interference when trying to evaluate HCA content.

According to prior research reports, derivatization methods can effectively improve the detectability of proteins (4–7). One of the key challenges of such methods is to homogenize derivatize the proteins. However, many publications have demonstrated that fluorescent labeling reagents such as 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and 9-fluorenyl methylchloroformate (FMOC-Cl) can be used as pre-column derivatization reagents to form a single, homogeneously derivatized product in which all reactive sites are tagged (8–12).

The present article reports a fluorescent pre-column derivatization method and a reversed-phase high-performance liquid chromatography (HPLC) method for the quantitative analysis of HCA. The method was successfully validated based on the International Conference on Harmonization (ICH) guidelines for pharmaceutical quality assurance (13). The proposed method was successfully applied to the determination of HCA in a pharmaceutical preparation.

Materials and Methods

Materials and reagents

The HCA reference substance (assigned purity, 97.0%) was obtained from the National Institutes for Food and Drug Control (NIFDC, China). HCA for injection (one unit per ampoule) was supplied by Beijing Kangchen Pharmaceutical Co. (Beijing, China). The derivatization reagent kit, including AQC and the labeling buffer (0.2M borate, pH 8.8), was purchased from Waters (Milford, MA). The proteomass peptide, protein matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS) calibration kit and 3,5-dimethoxy-4-hydroxycinnamic acid were obtained from Sigma-Aldrich (St. Louis, MO). Zip-Tips (C18) was purchased from Millipore (Billerica, MA). All chemicals and reagents were analytical grade or better. Water was purified using a Milli-Q water purifying system (Millipore, Bedford, MA).

Instruments

Derivatized HCA samples were analyzed using a Kratos Axima-CFR plus mass spectrometer (Kratos Analytical, Manchester, UK) equipped with a 337 nm N2 laser. HPLC analysis was performed on a Shimadzu LC-10A system (Kyoto, Japan), consisting of two Shimadzu LC-10ADVP pumps, an
RF-10AXL fluorescence detector, an SCL-10AVP system controller, an SIL-10ADVP auto-injector and a degasser module. HPLC data were acquired and processed by Shimadzu CLASS-VP 6.12 SP2 software. Chromatographic separations were accomplished on a reversed-phase Phenomenex (Torrance, CA) Jupiter C18 column (250 × 4.6 mm, 5 μm, 300).

**Reference substance and sample preparation**

The standard stock solution of HCA (2 μg/mL) was prepared in a 500 mL volumetric flask by dissolving 1,000 μg of reference substance in water. The standard solution (200 ng/mL) was prepared by diluting 5 mL of standard stock solution to 50 mL in a volumetric flask with water. All solutions were stored at 4°C before use.

One vial of HCA for injection, containing one unit, was transferred into a 10 mL volumetric flask and diluted to volume with water. This produced a 200 ng/mL concentration.

**Pre-column derivatization**

HCA was derivatized by using the following protocol. Ten microliters of 0.1 M sodium dodecyl sulfate (SDS) was added to 40 μL of analyte solution, and the reaction mixture was incubated at 95°C for 10 min to denature the HCA protein. The mixture was cooled to room temperature, and 110 μL of labeling buffer was added. Then, 40 μL of reconstituted AQC reagent (10 mM in acetonitrile) was added and the mixture was immediately vortexed for several seconds. The solution was covered and incubated for 1 min at room temperature. Finally, the solution was heated to 55°C for 10 min to complete the derivatization.

**Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry conditions**

All samples were diluted to the desired concentrations with water. A saturated sinapinic acid solution [10 mg/mL in 50% acetonitrile (ACN)–0.1% trifluoroacetic acid] was used as the matrix. Before the MS analysis, each sample was mixed with an equal volume of matrix, and 1 μL of the mixture was deposited onto a stainless steel target-plate and allowed to dry at ambient temperature. The running conditions were as follows: mode of MS operation, linear; polarity, positive; accelerating voltage, 20 kV; number of laser shots, 150/spectrum; calibration type, external; low mass gate, 500–5000.

**HPLC conditions**

The mobile phase consisted of solvent A (16.4 g sodium acetate, 1.72 g TEA, 1.0 mg EDTA in 1,000 mL water, pH adjusted to 4.95 with phosphoric acid) and solvent B (60% solvent A in ACN by volume). Before use, the mobile phase was filtered through a 0.45 μm Millipore membrane filter and degassed. The HPLC system was operated isocratically at a flow rate of 1.0 mL/min and at a controlled temperature of 37°C, using a mobile phase ratio, A/B, of 88/12. Fluorescence detection was conducted with excitation at 250 nm and emission at 395 nm. The injection volume was 20 μL. For regeneration, the column was washed for 10 min with 100% of mobile phase B, and then equilibrated for 15 min with 88% of mobile phase A (12% of B).

**Method Validations**

Validation of the method was performed by establishing specificity, linearity, intra-day and inter-day precision, accuracy, recovery, limit of detection (LOD) and limit of quantitation (LOQ), according to related requirements of ICH guidance.

**Specificity**

In this experiment, a placebo solution (excipient only) was used to test the specificity of the method. The absence of interference was confirmed using the proposed method.

Evaluating HCA degradation was performed under various stress conditions: 1 M NaOH, 1 M HCl, heating to 80°C, 3% H2O2, or photolytic degradation, in which 200 μL of 1 M NaOH was added to the 200 μL sample solution (200 ng/mL) and kept at 80°C for up to 3 h. The same procedure was applied for acid hydrolysis testing using 1 M HCl. Both solutions were refluxed at 80°C for 3 h, cooled and neutralized with acid or base, as necessary. Oxidative degradation was induced by storing the sample solutions in 3% H2O2 at ambient temperature for 6 h (protected from light). In the case of photolysis, the samples were exposed to 254 nm light for up to 8 h. For the thermal degradation study, the sample solutions were incubated at 80°C for up to 6 h.

All samples were derivatized according to the previously mentioned method, following degradation. The specificity of the method was established by studying the resolution of the HCA peak from the nearest resolved peak, and also among all other peaks.

**Linearity**

Six concentrations of HCA, 10, 50, 100, 200, 400 and 500 ng/mL, were prepared from a stock solution and used for linearity testing. A standard curve was generated by plotting peak area against concentration, and fitting the data using linear regression analysis (least-squares method).

**LOD and LOQ**

The LOD and LOQ for HCA were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentrations (n = 3).

**Precision and accuracy**

Intra-day and inter-day precision were assessed by the analysis of six replicate injections of the three levels of quality controls (10, 200 and 400 ng/mL), within a day, or during three different days. The relative standard deviation (RSD) obtained for intra-day and inter-day assays was used to assess repeatability and intermediate precision, respectively.

Accuracy was assessed by determining the recovery of the method at three different concentrations (corresponding to 50, 100 and 200% of the test solution concentration) by adding known amounts of standard to the placebo preparation (10 mg/mL of dextran 20).

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Figure 1. Effect of different factors on the derivatization reaction: pH (A); AQC concentration (B); reaction time (C); temperature (D).
Stability of derivatives
Sample solutions of the HCA–AQC derivatives were kept in tightly capped volumetric flasks for 48 h at room temperature, and analyzed using freshly prepared mobile phase. The stability of the AQC derivatized samples was tested by comparing the chromatographic patterns of the samples at different time points during the 48 h period.

Results and Discussion
Pre-column derivatization
HCA–AQC (derivatized compound) is a highly fluorescent derivative with excitation and emission maxima of 250 and 395 nm, respectively. The experimental parameters affecting derivatization were optimized to produce a robust, yet specific, peak area resulting from the HCA–AQC derivative (Figure 1). The pH was varied over the range of 7–10, and the maximum fluorescence signal was obtained at pH 8.0 using a 0.2M borate buffer. The optimized concentration of SDS for denaturing HCA was 20 mM. SDS should always be present before the actual derivatization reaction. The maximal reaction yield was obtained using an AQC concentration of 2 mM. The influence of temperature and time on the reaction of HCA and AQC was studied using a water bath. The effect of heating time at different temperatures (40–80°C) was assessed for AQC derivatives. By varying time, pH and substrate concentrations, the most suitable reaction conditions were obtained. The most efficient reaction occurred at 55°C for 10 min at pH 8.0.

MALDI–TOF-MS measurement of tagged amino groups of HCA
For analytical methods of proteins, especially those used for quantitative analysis, homogeneity (single species present) and ubiquitous protein tagging are desirable characteristics. Each HCA molecule has 20 possible reaction sites with AQC. To obtain completely derivatized products, a denaturing process is
Figure 3. Representative chromatograms: placebo solution (A); HCA standard solution (B); basic-degraded HCA solution (C); acid-degraded HCA solution (D); oxidative degraded HCA solution (E); photo-degraded HCA solution (F); thermal-degraded HCA solution (G).
necessary to expose all of the reactive sites. In this method, an SDS solution (optimized concentration, 20 mM) was used as denaturing buffer. MALDI–time-of-flight (TOF)-MS was used to demonstrate the formation of a fully tagged derivative of HCA. Underivatized HCA and its derivatized product were analyzed by MALDI–TOF-MS. The measurement principle was based on the fact that one AQC tag to an amino group of a protein would result in an increase of 170 Da in MW of the derivatized molecule. The numbers of the tagged amino groups were then determined before and after derivatization. The MALDI–TOF-MS spectra of underivatized (A) and derivatized HCA (B) are shown in Figure 2A; the peak with an MW of 29,202.9 Da corresponds to the underivatized HCA. The 15 kDa peak is believed to be the peak of the doubly protonated HCA.

As shown in Figure 2B, the measured MW of derivatized HCA is 32,603.1 Da. No other peaks were observed in the mass range of 25,000–35,000 Da. The number of amino groups that were tagged with AQC was estimated at $20 \pm 0.3$ ($n = 3$), which is very close to the number of total amino groups on HCA. These results indicated that in the derivatization conditions mentioned previously, all of the available binding sites can be completely derivatized.

**Specificity and forced degradation studies**

The specificity of the proposed method was evaluated by analyzing a placebo sample (without HCA). The chromatograms showed no interference with the HCA peak (Figures 2A and
Additionally, forced degradation studies were conducted to confirm the specificity of the proposed method using the HCA reference substance.

When submitted to basic conditions (Figure 3C), acidic conditions (Figure 3D) and oxidizing conditions (Figure 3E), major degradation occurred and the HCA was degraded by approximately 72, 31 and 78%, respectively. Only minor degradation occurred under photolytic conditions (Figure 3F) and thermal conditions (Figure 3G), with degradation of HCA approximately 2 and 3%, respectively. In all chromatograms, the peaks of the degradation products were well separated from the HCA peak with appropriate resolution ($R_s > 2$). Excessive derivative agent can lead to complete hydrolysis of the substrate; however, because hydrolysis is a slow reaction, the hydrolysis product did not interfere with the analysis.

**Linearity**

Linearity measurements were obtained over the calibration ranges tested (i.e., 10–500 ng/mL). Each point was prepared in triplicate. The correlation coefficient, slope, and intercept values were 0.9999, 204153 and 92137, respectively ($n = 3$).

**LOD and LOQ**

The LOD and LOQ were 0.57 and 1.6 ng/mL, respectively, which were confirmed experimentally, indicating the sensitivity of the method.

**Precision and accuracy**

The method led to favorable precision and accuracy. Intra-day and inter-day precision and accuracy for HCA quantification are shown in Table I. The RSD of HCA during the intra-day study was within 1.50%, and the RSD in the inter-day study was within 2.20%. The results confirm the repeatability of the method.

To obtain the accuracy of the method, recovery experiments were conducted at three concentrations. Known amounts of HCA standard (100, 200 and 400 ng/mL) were added to a placebo sample and the amount of HCA was measured in the presence of the placebo interference. The percentage recovery of HCA in quality control samples ranged from 97.49 to 99.15%. Recovery values are shown in Table II. These results confirmed that the proposed method is accurate.

**Stability of derivatives**

The stability of HCA–AQC derivatives was examined, and no chromatographic changes were observed over 48 h at room temperature. These results indicated that the AQC derivatives of HCA were stable at ambient temperature for at least 48 h.

**Method application**

An assay of three batches of HCA for injection was performed, and the measured amount was within 95.82–104.74% of the labeled amount. The results obtained from the validation studies meet the quality inspection requirements of HCA in a pharmaceutical preparation (95.0–105.0%).

**Conclusion**

To summarize, a pre-column derivatization HPLC method with fluorescence detection was developed and validated for the analysis of HCA in a pharmaceutical preparation. The pre-column derivatization conditions were optimized. A complete and homogeneous derivatization of HCA was confirmed by MALDI–TOF-MS analysis. Regarding sensitivity and reliability, the established method is suitable and can be recommended for the analysis and routine quality control of HCA in pharmaceutical preparations.

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