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Determination of Acetyl-Tryptophan in Human Albumin by Reversed-Phase High Performance Liquid Chromatography

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A reliable reversed-phase high performance liquid chromatography combined with perchloric acid deproteinization is presented for the determination of acetyl-tryptophan in human albumin. HPLC was achieved on an Accucore XL-C_{18} column by gradient elution of acetonitrile and phosphate buffered solution (pH 2.3) at a flow rate of 0.7 mL min^{-1} and detection was by UV adsorption at 220 nm. The standard curve of acetyl-tryptophan was linear over the range of 1-60 μg mL^{-1}. The limits of quantitation and detection for acetyl-tryptophan were 0.167 and 0.050 μg mL^{-1}, respectively. The recoveries of acetyl-tryptophan in human albumin were 90.5-96.8 % with the relative standard deviations (n = 3) of 1.4-4.3 %. This method was found to be superior to the current UV method and other liquid chromatographic methods.

Keywords: Acetyl-tryptophan, Human albumin, High performance liquid chromatography, Perchloric acid.

INTRODUCTION

Human albumin is a common therapeutic preparation obtained from human plasma. During production, human albumin is heated at 60 °C for 10 h to inactivate hepatitis viruses. A stabilizer, such as acetyl-tryptophan or sodium caprylate or a combination of these two, is usually added to enhance the thermal stability of human albumin and minimize denaturation in the heating procedure so that the product is safe for intravenous use[1-4]. Chinese Pharmacopoeia (2010) requires that 0.16 mM sodium caprylate alone or alternatively 0.08 mM sodium caprylate together with 0.08 mM acetyl-tryptophan be added for each gram of human albumin and that acetyl-tryptophan be quantitated by means of ultraviolet-visible spectrophotometry (UV), that is, absorption coefficient method[3]. By the UV method, human albumin sample is diluted with normal saline and then perchloric acid is used to precipitate the protein, followed by centrifugation. The absorbance of the supernatant is measured at 280 nm[5]. However, the obtained reading by UV is the total absorbance of the sample solution. The determined content of acetyl-tryptophan may exceed the true value if the albumin fails to precipitate completely or if the sample solution contains oligopeptides or free amino acids such as tryptophan, tyrosine and the like, which absorb UV light at about 280 nm but refuse to precipitate[6,7]. There are no analytical methods for acetyl-tryptophan in human albumin recorded in current United States Pharmacopoeia and European Pharmacopoeia. Wei *et al.*[8] proposed a size exclusion chromatographic (SEC) method for determination of acetyl-tryptophan in human albumin. Acetyl-tryptophan was separated from albumin and other proteins on a gel column, detected with a UV-visible detector at 280 nm and assayed using external standard method. The size exclusion chromatographic method can separate acetyl-tryptophan and proteins by molecule size, thus avoiding the procedure of precipitation. However, free amino acids and small peptides with similar molecular weight in human albumin might not be separated completely from acetyl-tryptophan[9], which likely results in an over-evaluation of acetyl-tryptophan content. Nelis *et al.*[10] developed a more specific reversed phase liquid chromato-graphic (LC) method for determination of acetyl-tryptophan in human albumin. However, methanol, chosen as a protein-denaturing agent prior to liquid chromatography analysis, is not as effective as perchloric acid[11]. Therefore, to ensure that human albumin is safer for intravenous use, it is necessary to develop a novel assay for acetyl-tryptophan that is more accurate, reliable and convenient. It has been reported that high performance liquid chromatography (HPLC) is able to separate and determine acetyl-tryptophan and tryptophan

in other matrices[7,12]. The present paper is aimed at establishing a reversed phase HPLC protocol including deproteinization with perchloric acid for acetyl-tryptophan in human albumin.

EXPERIMENTAL

HPLC analysis was performed on an Alliance 2695 separations module equipped with an on-line degasser, a quaternary pump, an auto-sampler injector, a 2996 photodiode array detector (PDA) and an Empower chromatography manager system (Waters, Milford, MA, USA). Comparative UV analysis was performed on a DU-800 ultraviolet-visible spectrophotometer (Beckman, Fullerton, CA, USA). Sample solutions were centrifuged by a DT5-3 low speed auto-balancing centrifuge (Beijing Era Beili Centrifuge Co., Ltd, China). The pH of phosphate buffer solution was adjusted *via* a PHS-3C pH meter (Shanghai Precision Scientific Instrument Co., Ltd, China). Degasification of mobile phase and dispersion of sample solution were aided by a KQ-250B ultrasonic bath (Kunshan Ultrasonic Instruments Co., Ltd, Suzhou, China).

Human albumin with a protein content of 20 % for injection was kindly provided by National Institutes for Food and Drug Control of China (Beijing, China). Reference substance (RS) of acetyl-tryptophan was purchased from European Directorate for the Quality Control of Medicines and RS of tryptophan was kindly provided by Jiangsu Institute for Food and Drug Control (Nanjing, China). Acetonitrile (HPLC grade) was purchased from J.T. Baker (USA). Perchloric acid of guaranteed reagent grade was purchased from Jinlu Chemical Co., Ltd (Shanghai, China). Sodium chloride of analytical reagent grade was obtained from Nanjing Chemical Reagent Co., Ltd (Nanjing, China). Phosphoric acid and sodium dihydrogen phosphate (both for HPLC) were purchased from Fluka (USA) and Acros (USA), respectively. The water was ultrapure water (18 MΩ cm) prepared by a PURELAB Ultra ultrapure water purifier (ELGA LabWater, Marlow, UK).

Preparation of solutions

Standard solutions: 50 mg of acetyl-tryptophan RS and 40 mg of tryptophan RS were weighed separately and dissolved in acetonitrile-water solution (1:9, v/v) in 50 mL volumetric flasks, serving as 1.00 mg mL^{-1} acetyl-tryptophan and 0.80 mg mL^{-1} tryptophan stock solutions, respectively. A series of mixed standard solutions were prepared by mixing certain volumes of acetyl-tryptophan stock solution and tryptophan stock solution.

Phosphate buffer solution: 10 volumes of sodium dihydrogen phosphate solution, prepared by dissolving 3.90 g of sodium dihydrogen phosphate in 1000 mL of water, was mixed with 7 volumes of 2.9 g L^{-1} phosphoric acid solution. Additional phosphoric acid solution was added to acquire a pH of 2.3. The mixed solution was then filtered with a 0.22 μm microporous membrane for aqueous solution and degassed in ultrasonic bath.

HPLC conditions: Chromatographic parameters referred to the HPLC separation conditions for acetyl-tryptophan and tryptophan described in the issue of Relative impurities in Tryptophan of the European Pharmacopoeia version 6.0[12]. Adjustment was made according to the actual situation of human albumin. Chromatographic separation was carried out on an Accucore XL-C_{18} column (150 mm × 4.6 mm, 4 μm) at a column temperature of 40 °C. The mobile phase consisted of acetonitrile and phosphate buffer solution and the flow rate was 0.7 mL min^{-1}. The gradient elution program is described in Table-1. The injection volume was 20 μL and the detection wavelength was 220 nm.

TABLE-1
GRADIENT ELUTION PROGRAM

Time (min)	Acetonitrile (v %)	Phosphate buffer solution (v %)
0→4	11.5	88.5
4→6	11.5→20.0	88.5→80.0
6→12	20.0	80.0
12→15	20.0→35.0	80.0→65.0
15→35	35.0	65.0
35→36	35.0→11.5	65.0→88.5
36→60	11.5	88.5

Sample analysis: Human albumin sample was pretreated following the procedure of UV assay for acetyl-tryptophan in Chinese Pharmacopoeia (2010)[3]. Human albumin with a protein content of 20 % was diluted with normal saline (0.9 % sodium chloride solution) until its protein content was reduced to 5 %. Afterwards, 0.30 mL of normal saline and 3.60 mL of 0.30 mol L^{-1} perchloric acid were added to 0.10 mL of 5 % human albumin. After thorough mixing, the mixture was laid aside for 10 min and centrifuged for 20 min at 3500 rpm. The supernatant was filtered with a 0.22 μm microporous membrane prior to analysis. The blank solution was prepared in the same way except that in the absence of human albumin 0.40 mL of normal saline was mixed with 3.60 mL of 0.30 mol L^{-1} perchloric acid. Sample and blank solutions were respectively injected into HPLC and the standard curve method was used for quantitation of acetyl-tryptophan and tryptophan. In addition, the content of acetyl-tryptophan in human albumin sample was also determined with the UV method[3] for comparative purpose.

RESULTS AND DISCUSSION

System suitability: 20 μL of mixed standard solution of acetyl-tryptophan and tryptophan and 20 μL of human albumin sample solution were separately injected for HPLC analysis. Corresponding chromatograms are shown in Figs. 1a and 1b. It is indicated that there was no interference peak around acetyl-tryptophan peak in the chromatogram of sample solution. The numbers of theoretical plates for acetyl-tryptophan and tryptophan were approximately 9100 and 12000, respectively. The resolution (Rs) between acetyl-tryptophan and tryptophan was much greater than 1.5, hence meeting quantitative requirements.

Standard curves and limits of detection: The standard curves were obtained by plotting the peak area (Y) against the concentration (X) of acetyl-tryptophan or tryptophan. Acetyl-tryptophan exhibited good linearity over the range of 1.00-60.00 μg mL^{-1} with the regression equation Y = 256278.29X - 16326.43 and the correlation coefficient r = 0.9999. Tryptophan displayed linearity over the range of 0.80-48.00 μg mL^{-1} with the regression equation Y = 211545.62X + 62121.24 and the correlation coefficient r = 0.9996. A mixed standard solution

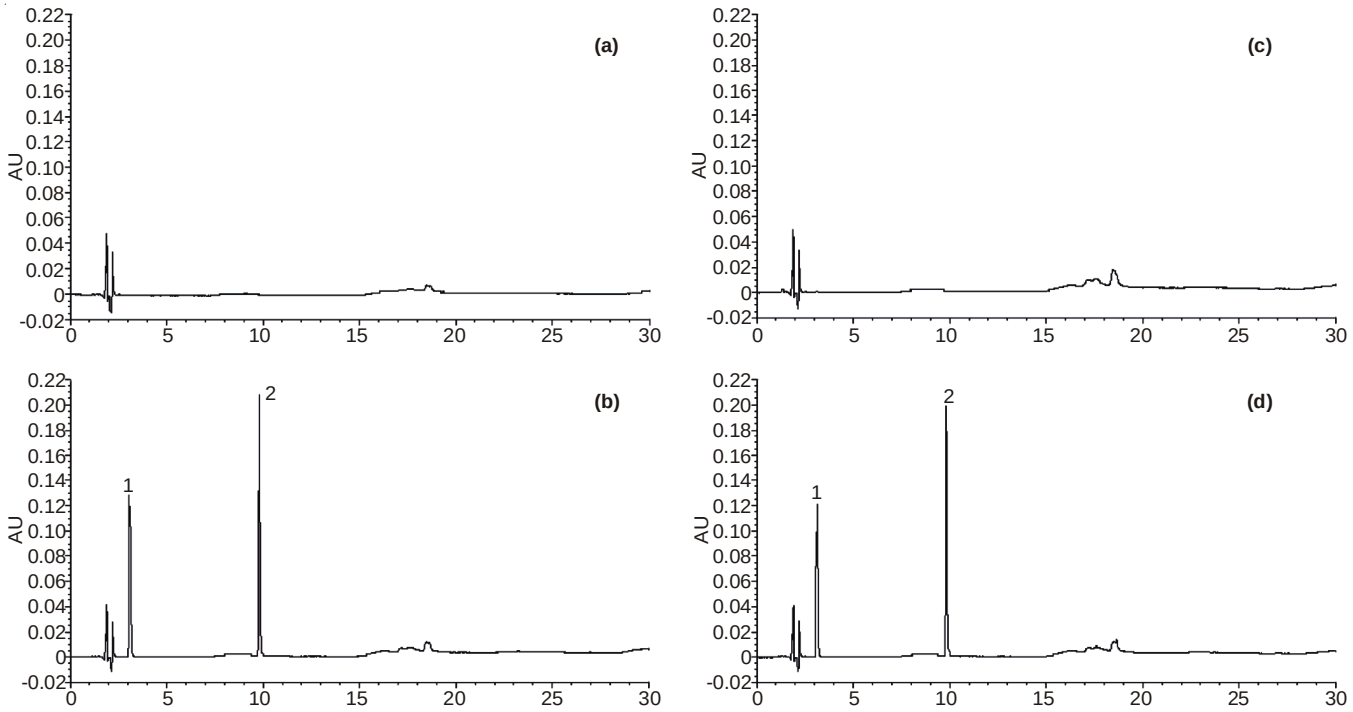

Fig. 1. HPLC chromatograms (a) Blank solution; (b) Mixed standard solution; (c) Sample solution; (d) Spiked sample solution. Peaks: 1. Tryptophan; 2. Acetyl-tryptophan

containing 25 μg mL^{-1} acetyl-tryptophan and 20 μg mL^{-1} tryptophan was injected for six times in succession and the relative standard deviations (RSDs) of the peak areas of acetyl-tryptophan and tryptophan were 0.66 % and 0.86 %, respectively. The limits of detection (LODs) of acetyl-tryptophan and tryptophan, determined based on a signal-to-noise ratio (S/N) of 3:1, were 0.050 μg mL^{-1} and 0.020 μg mL^{-1}, respectively. The limits of quantitation (LOQs) of acetyl-tryptophan and tryptophan, determined based on a signal-to-noise ratio of 10:1, were 0.167 μg mL^{-1} and 0.067 μg mL^{-1}, respectively.

Recoveries: No acetyl-tryptophan was detected by HPLC in the human albumin sample (Fig. 1b). The sample solution was spiked with mixed standard solutions of acetyl-tryptophan and tryptophan (Fig. 1c). Table-2 lists the recoveries corresponding to the sample solutions with different spiking levels. The recoveries of acetyl-tryptophan and tryptophan in human albumin were 90.5-96.8 % and 96.2-105.7 %, respectively, with RSDs of 1.4-4.3 % and 2.0-5.3 %.

TABLE-2
TEST RESULTS FOR RECOVERIES (n = 6)

Component	Background value (μg mL^{-1})	Spiking levels (μg mL^{-1})	Average recoveries (%)	RSD (%)
Tryptophan	ND	4.00	105.7	2.8
		20.00	98.0	5.3
		40.00	96.2	2.0
Acetyl-tryptophan	ND	5.00	90.5	2.2
		25.00	96.8	4.3
		50.00	93.7	1.4

Stability test for sample solution: The spiked sample solution of 25 μg mL^{-1} acetyl-tryptophan and 20 μg mL^{-1} tryptophan was chromatographed in different storage time after preparation. The peak areas of acetyl-tryptophan and tryptophan were recorded. The RSDs of areas were 1.0 % and 1.6 % during 72 h for acetyl-tryptophan and tryptophan, respectively, indicating good stability of the sample solution within at least 72 h.

Comparison of HPLC and UV: Whatever analytical method is adopted to determine acetyl-tryptophan in human albumin, potential interfering substances are or derived from other free amino acids and residual oligopeptides, or proteins not precipitated. The UV-visible absorption spectra of tryptophan and acetyl-tryptophan are very similar. Their characteristic absorption wavelengths are both approximately 220 nm and 280 nm, at which interference is difficult to avoid if UV method is used. By contrast, HPLC can first separate acetyl-tryptophan and tryptophan (Rs > 1.5), thus more resistant to the wavelength based interference than UV method. In addition, as comparative experiment showed, the LOD (S/N = 3) and LOQ (S/N = 10) of UV method for acetyl-tryptophan were 0.12 μg mL^{-1} and 0.40 μg mL^{-1}, respectively, both of which were higher than the HPLC counterparts. This demonstrates high sensitivity of HPLC in comparison to UV method. As for SEC method, despite its simpler procedures free from protein precipitation, it is unable to separate acetyl-tryptophan from other amino acids and small peptides and less interference-resistant than HPLC.

Conclusion

The proposed reversed phase HPLC method in combination with perchloric acid deproteinization can efficiently separate acetyl-tryptophan from not only albumin but also oligopeptides and free amino acids like tryptophan. It guarantees the authenticity of analytical results and fits better into drug testing practice than the current UV and other liquid chromatographic methods do.

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