Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1073 (2005) 263–267
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analysis of guanidine in high salt and protein matrices by cation-exchange chromatography and UV detection

Jinshu Qiu*, Hans Lee, Chengfeng Zhou

Department of Analytical Science, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320, USA

Available online 26 November 2004

Abstract

A simple and sensitive HPLC method for quantitative determination of guanidine in high salt and protein matrices was developed. The HPLC system consisted of an Agilent 1100 pump with an online degasser, a UV detector, an autosampler, and Dionex CS 14 cation-exchange guard (4 mm × 50 mm) and analytical (4 mm × 250 mm) columns. The mobile phase was 3.75 mM methanesulfonic acid (MSA) with a flow rate of 1 mL/min. The other analysis parameters were: 50 μL injection volume, 195 nm UV detection, and 21 min runtime. The limit of quantitation (LOQ) for guanidine HCl was determined to be 0.25 mg/L and the standard curve ranged from 0.25 mg/L to 10 mg/L. Sample preparation was required for the samples containing high protein concentrations. Proteins were removed by centrifuging a sample in a 30 K NanoSep centrifugal filter at 15,300 × *g* for 20 min. The method could determine guanidine accurately in sample matrices containing up to 200 mM sodium ion or up to 50 mM potassium ion. The method can be used for clearance testing of guanidine in biopharmaceutical products.

Keywords: Guanidine HCl; Reagent clearance testing; Biopharmaceutical products

1. Introduction

Guanidine HCl is a strong chaotropic agent that can solubilize insoluble or denatured proteins such as inclusion bodies. Guanidine HCl is commonly used at the first step of protein purification processes for refolding proteins or enzymes into their active form. The concentration used can be as high as 6M. Clearance studies are required to demonstrate the removal of guanidine by biopharmaceutical purification processes and its absence from drug products [1].

Several methods have been developed for determination of guanidine with different instrumental techniques. Jones and Thompson developed a method for the detection of guanidine compounds on paper chromatograms [2]. Guanidine reacted with ninhydrin in alkaline media to form highly fluorescent products, which could be determined on exposure to UV light. A GC procedure was developed to determine methylguanidine, guanidine and agmatine as their hexafluoroacetylacetonates [3]. The acetyl derivatives were separated with a chromosorb glass column and measured with an electron capture detector or FID.

Bird and Smith separated amines, guanidines and hydroxycinnamic acid amides by ion-exchange chromatography [4]. The compounds were detected by fluorescence after reaction with *o*-phthalaldehyde. All the above methods require a derivatization procedure.

Burrows et al. developed an ion chromatographic (IC) procedure that allowed direct determination of guanidine and substituted guanidines in water [5]. Guanidine was separated as a guanidinium ion using a cation-exchange column and measured by a suppressed conductivity detector. The detection limit was 0.5 mg/L guanidine HCl. The procedure was adapted to determine guanidine in protein matrices using a modern ion chromatograph (unpublished results). Though the IC method with suppressed conductivity detection is rapid and sensitive, it is unable to determine guanidine at a trace level in solutions containing more than 100 mM salt because of significant tailing of the salt peak. The objective of the present study was to develop a simple and sensitive method for determination of guanidine in high salt and protein matrices.

* Corresponding author. Tel.: +1 805 447 3183; fax: +1 805 480 1752.
E-mail address: jqiu@amgen.com (J. Qiu).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.10.045

2. Experimental

An HPLC system included an Agilent (Wilmington, Delaware, USA) 1100 binary pump with an online degasser, an autosampler, and a UV detector. Dionex (Sunnyvale, California, USA) CS 14 cation-exchange guard (4 mm × 50 mm) and analytical (4 mm × 250 mm) columns were employed for guanidine separation. Guanidine HCl (J.T. Baker, Phillipsburg, New Jersey, USA) and methanesulfonic acid (Fluka, Buchs, Switzerland) were used directly after purchase. The mobile phase was a methanesulfonic acid solution with a flow rate of 1 mL/min. The separated guanidine was detected at a wavelength of 195 nm and the runtime was 21 min.

Samples were obtained from different protein purification steps and purified proteins. In-process samples were diluted with Milli-Q water when guanidine concentration was higher than the highest standard. For the samples containing high protein concentrations, 30 K NanoSep centrifugal filters (Pall Life Sciences, East Hills, New York, USA) were used for removing the proteins prior to HPLC analysis. An aliquot (0.5 mL) of the samples was transferred into a 30 K NanoSep centrifugal filter and centrifuged at 15,300 × *g* for 20 min. The filtrate (50 μL) was injected to the HPLC system for analysis.

3. Results and discussion

3.1. Absorbance wavelength

The guanidine molecule contains a single C=N bond that can absorb UV light at the short wavelength end of the UV spectrum. The UV spectrum of guanidine ranging from 190 nm to 400 nm is shown in Fig. 1. Guanidine almost does not absorb any UV light at 210 nm and above. The UV absorbance increases dramatically as the wavelength decreases from 200 nm to 190 nm. The strong UV absorbance at the short wavelength provides the possibility to detect guanidine. The linear response of the UV absorbance by guanidine at 195 nm and 200 nm was compared (Fig. 2). The slope of the

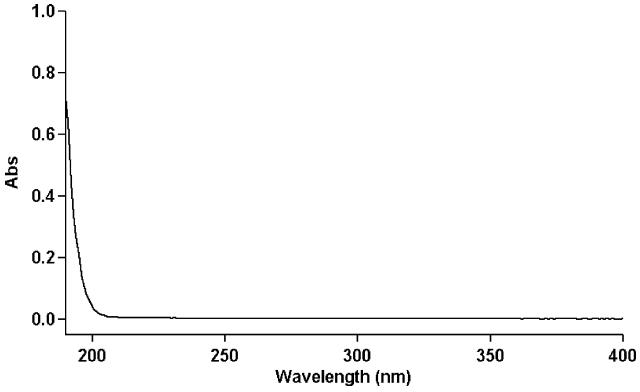

Fig. 1. Spectrum of guanidine. The concentration of the guanidine HCl solution used for obtaining the spectrum was 10 mg/L.

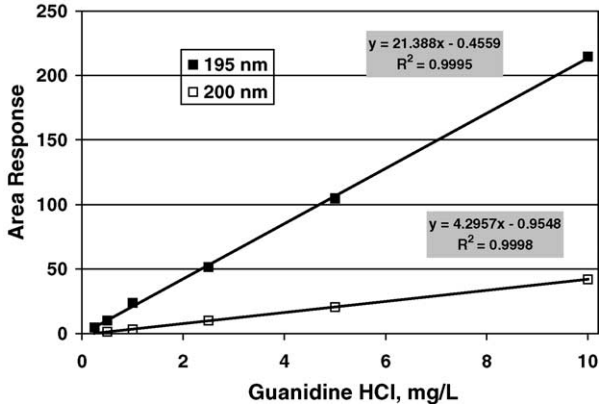

Fig. 2. Comparison of guanidine absorbance at 195 nm (■) and 200 nm (□). The calibration curves were linear at both wavelengths. However, the slope at 195 nm was five times as high as that at 200 nm.

regression equation at 195 nm was five times as high as that at 200 nm, suggesting that high sensitivity could be obtained at 195 nm.

3.2. Mobile phase strength

The IonPac CS14 column is a hydrophilic, carboxylate-functionalized cation exchanger and employs macroporous particles consisting of ethylvinylbenzene crosslinked with 55% divinylbenzene with carboxylic acid functional groups to provide separation of cations [6]. A dilute acid such as MSA is a common eluent used for this type of column. The MSA concentration in the mobile phase had a significant impact on the retention time of guanidine on the cation-exchange column (Table 1). The guanidine retention time reduced from 36.4 min to 13.3 min when the MSA concentration increased from 1.25 mM to 3.75 mM. Significant reduction in the retention time was due to a decrease in ionization of the carboxyl group at a higher MSA concentration (i.e., more acidic) and a consequent decrease in the ion-exchange affinity of the resin [7]. Since an unknown peak always eluted at 21.5 min at the different mobile phase strengths, the run time could not be shortened further. Therefore, the 3.75 mM MSA solution was used as the mobile phase for the rest of studies.

3.3. LOD and LOQ

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by the signal to noise ratio approach. The ratio of 3:1 and 10:1 was used for determining LOD

Table 1
Effect of the mobile phase strength on the guanidine retention time on the cation-exchange column

Mobile phase MSA concentration (mM)	Guanidine retention time (min)
1.25	36.4
2.50	19.0
3.75	13.3

and LOQ, respectively. At a wavelength of 195 nm, LOD and LOQ for guanidine HCl were 0.08 mg/L and 0.25 mg/L, respectively.

3.4. Linearity and range

Since this is a reagent clearance assay focusing on the low concentration range close to the method's LOQ value, a narrow range from 0.25 mg/L to 10 mg/L was used for the calibration curve. The linear regression equation of the calibration curve was $y = 21.388x - 0.456$ with a determination coefficient (r^2) of 0.9995 (Fig. 2).

3.5. Precision and accuracy

The precision was determined by injecting an in-process sample five times. It was expressed as the relative standard deviation (R.S.D.) of the five injections. The intermediate precision was determined from four analyses during a three-week period. The R.S.D. value was 0.4% for both precision and intermediate precision. For the accuracy determination, an aliquot (200 μL) of 5 mg/L guanidine standard was spiked into an in-process or purified protein sample. The spike recovery was then calculated by dividing the value found in the sample by the expected value and multiplying by 100%. The results are shown in Table 2. The recoveries obtained from two types of proteins ranged from 98% to 113%. The results demonstrate that the method can accurately measure guanidine in those samples.

Table 2
The spiked recoveries of guanidine in in-process and purified protein samples of two types of proteins

Sample	Recovery of guanidine (%)	
	Protein A	Protein B
In-process sample 1	99	98
In-process sample 2	113	107
In-process sample 3	110	100
Purified protein	105	99

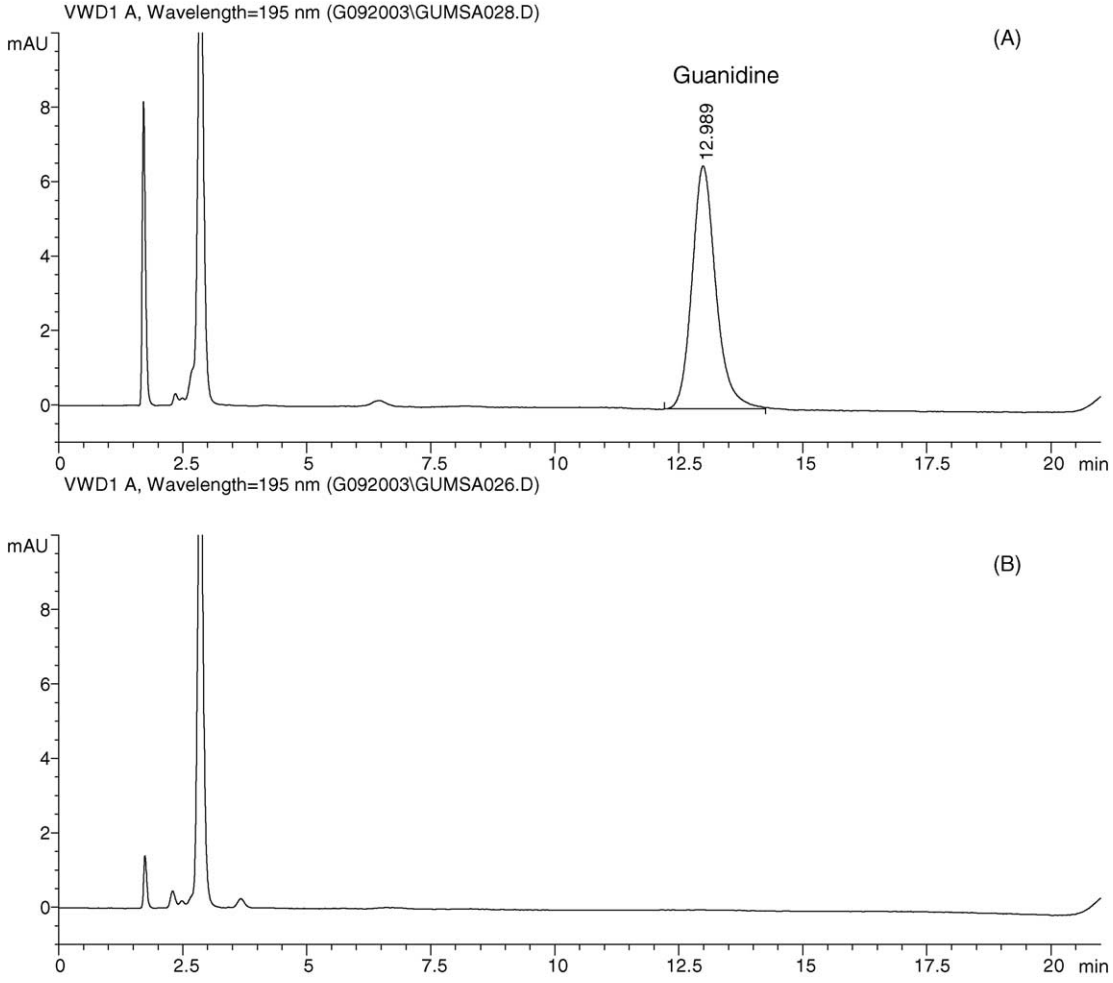

Fig. 3. Chromatograms of eight compounds with guanidine (Panel A) and without guanidine (Panel B). Eight compounds commonly used in protein purification did not interfere with the guanidine peak.

3.6. Specificity

Eight ions or compounds at a concentration of 20 mg/L each that are commonly used in protein purification, including sodium, potassium, 2-(*N*-morpholino)ethanesulfonic acid, urea, glycerol, acetate, tris-(hydroxymethyl)amino methane (Tris) and dithiothreitol, were tested for interferences with the guanidine peak. No interference was found from those compounds (Fig. 3). Among the eight ions or compounds, sodium, potassium and Tris have retention on a carboxylate-functionalized cation-exchange column, but they are separated from the guanidine peak.

3.7. Effects of sodium and potassium concentrations in the sample matrix on the retention time and area of the guanidine peak

To study their effects, the sample matrix was mimicked with a solution consisting of 10 mg/L guanidine HCl and either sodium chloride or potassium chloride at a concentration of 10, 50, 100, 150 or 200 mM. The solutions were analyzed using the developed method, and the retention time and area of the guanidine peak were measured. The experiments were replicated three times. The results are shown in Table 3. The guanidine peak areas are similar at all the sodium ion levels, but its retention time increased slightly as sodium ion concentration increased from 10 mM to 200 mM. Potassium ion concentration had significant effects on the retention time of the guanidine peak. The retention time increased from 13.4 min to 16.6 min as potassium ion concentration in the solution increased from 10 mM to 200 mM. The peak area was unaffected at potassium levels equal to and less than 50 mM. When the potassium ion concentrations in the sample matrix were greater than 50 mM, the apparent peak area decreased significantly due to occurrence of a flat and long front tail of the guanidine peak. On a carboxylate-functionalized cation-exchange column, sodium ions elute earlier than potassium ions, farther away from the guanidine peak. This could be one

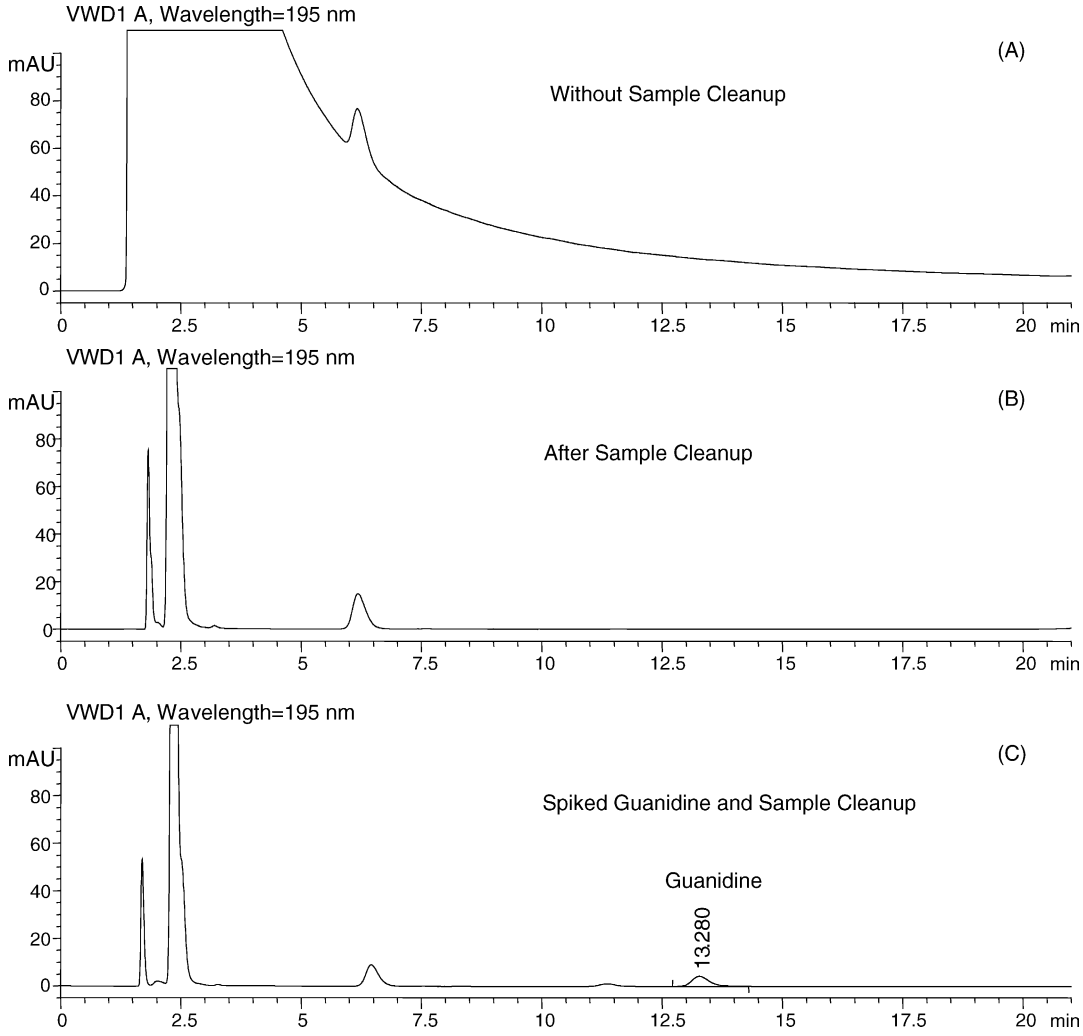

Fig. 4. Chromatograms of purified protein samples with and without sample cleanup. A high protein sample was directly injected into HPLC (Panel A), the same sample was cleaned up with a centrifugal filter (Panel B), and the same sample was spiked with 5.0 mg/L guanidine HCl and then cleaned up with a centrifugal filter (Panel C).

Table 3
Effects of sodium and potassium on the retention time and area of the guanidine peak

Sample	Retention time (min)		Peak area (mAU × S)	
	Average ($n = 3$)	Standard deviation	Average ($n = 3$)	Standard deviation
Guanidine	13.15	±0.02	215	±5
10 mM NaCl	13.21	±0.02	218	±2
50 mM NaCl	13.34	±0.01	220	±2
100 mM NaCl	13.47	±0.02	221	±5
150 mM NaCl	13.57	±0.01	217	±4
200 mM NaCl	13.66	±0.02	220	±2
10 mM KCl	13.40	±0.02	216	±3
50 mM KCl	14.46	±0.02	210	±1
100 mM KCl	15.41	±0.02	173	±3
150 mM KCl	16.07	±0.04	151	±2
200 mM KCl	16.58	±0.03	135	±2

The guanidine peak had a long and flat front tail at 100, 150 and 200 mM KCl levels.

of the reasons why potassium ions had more profound effect than sodium ions. An appropriate amount of sodium chloride and/or potassium chloride may be added into standards to minimize the retention time difference between standards and samples. The method should not be used if sample matrices contain more than 50 mM potassium ions.

3.8. Protein interference with guanidine

All proteins have a strong absorbance at 195 nm and some proteins at high concentrations cannot be separated from the guanidine peak by a cation-exchange column. Sample cleanup is necessary for samples with high protein concentrations. Fig. 4 shows the chromatograms with and without sample cleanup. The interference from proteins was removed completely after the protein samples were cleaned up with a centrifugal filter.

4. Conclusions

A method was developed to determine guanidine quantitatively in high salt and protein matrices by HPLC cation-exchange separation and UV detection at 195 nm. The method is rapid, simple and sensitive with a minimal sample preparation. Centrifugal filters were used to remove proteins that might interfere with the guanidine peak. The method could determine guanidine concentration accurately in sample matrices containing up to 200 mM sodium ion or up to 50 mM potassium ion. It may be necessary to add an appropriate amount of potassium or sodium ions into the standards for matching the retention time of the guanidine peak in samples.

Acknowledgement

The authors sincerely thank Dr. Janice Davis, Associate Director of Analytical Sciences, for her support, suggestions, and editing the manuscript.

References

[1] ICH Harmonized Tripartite Guideline, Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (Q6B), ICH Steering Committee, 10 March 1999.
[2] A.S. Jones, T.W. Thompson, J. Chromatogr. 10 (1963) 248.
[3] T. Kawabata, H. Ohshima, T. Ishibashi, M. Matsui, T. Kisuwa, J. Chromatogr. 140 (1977) 47.
[4] C.R. Bird, T.A. Smith, J. Chromatogr. 214 (1981) 263.
[5] E.P. Burrows, E.E. Brueggeman, S.H. Hoke, J. Chromatogr. 294 (1984) 494.
[6] Dionex Corporation, IonPac CS14 Cation Exchange Column, 1997, http://www.dionex.com.
[7] J.S. Fritz, D.T. Gjerde, Ion Chromatography, third ed., Wiley–VCH, Weinheim, 2000, p. 141.