Determination of 4'-Carbomethoxyphenyl 4-Guanidinobenzoate Mesylate by HPLC and Identification of its Related Substances by LC–MS–MS

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Abstract

4'-Carbomethoxyphenyl 4-guanidinobenzoate mesylate (4'-CMGB) is a sperm acrosin inhibitor. A validated reversed-phase isocratic high-performance liquid chromatographic (HPLC) method for its assay and related substances detection is described. The chromatographic system consists of a C_{18} Shimpack column (150 mm × 4.6 mm, 5 μm) with a mobile phase composition of methanol–0.2% ammonium acetate–triethylamine–glacial acetic acid (470:524:1:5, v/v/v/v) and UV detection at 266 nm. The method shows good chromatographic separation for 4'-CMGB and its related substances. The method is found to be linear in the range of 2.0–100 µg/mL, precise, and sensitive (limit of detection of 15 ng/mL). The major related substances are resolved and identified by HPLC and LC-tandem mass spectrometry as *p*-guanidino benzoic acid, methyl *p*-guanidino benzoate, and methyl *p*-hydroxybenzoate.

Introduction

4'-Carbomethoxyphenyl 4-guanidinobenzoate mesylate (4'-CMGB)(Figure 1) is a candidate vaginal contraceptive drug developed by the Department of Medicinal Chemistry of Second Military Medical University (Shanghai, China)(1). It functions as a potent inhibitor of sperm acrosin, and the contraceptive effects are achieved not by immobilizing spermatozoa, as in the case of spermicidal contraceptives, but by preventing the fusion of spermatozoa with ova by inhibiting the sperm enzymes necessary for this process (2–4). It is more potent and less toxic or irritating to the vagina than nonoxynol-9, the most commonly used active spermicidal ingredient in today's vaginal contraceptive preparations (5). The preliminary study results indicate that 4'-CMGB is suitable for long-term use.

The pharmaceutical development of 4'-CMGB necessitated the quality control technique to safeguard the quality and, subsequently, the efficacy of the product. So far there has been no report about the assay or impurity test of this compound or any other aryl 4-guanidinobenzoate (6). Only several analytical methods have been reported on the determination of similar compounds, camostat mesylate [*N,N*-dimethyl carbamoylmethyl-4-(4-guanidinobenzoyloxy) phenylacetate methanesulfonate](CM), nafamostat mesylate (6-amidino-2-naphthyl-4-guanidinobenzoate dimethanesulfonate)(NM), and gabexate mesylate [ethyl-4-(6-guanidino hexanoyloxy) benzoate methanesulfonate](GM). CM, NM, and GM are protease inhibitors and have been used clinically in the treatment of acute pancreatitis (7). Most of the quantitation determination of CM, NM and GM in drug preparations or biological fluids have been performed by reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection (8–17). Chemically bonded C18 or CN phase was used as the solid phase. The mobile phase was the mixture of organic solvent (methanol or acetonitrile) and different aqueous solutions, such as potassium phosphate (8–10), sodium acetate (11), trifluoroacetic acid (12–13), and ion-pairing reagents (14–17).

This paper describes the development and validation of a simple, specific and precise RP-HPLC method for the quality control of 4'-CMGB, and a HPLC coupled to tandem mass spectrometry (LC–MS–MS) method for the identification and elucidation of the structures of three major impurities of 4'-CMGB.

Experimental

Chemicals and reagents

4'-CMGB drug substances and 4'-CMGB reference substance (99.7 %) were provided by Department of Medicinal Chemistry of Second Military Medical University (Shanghai, China). HPLC-grade methanol was purchased from Merck (Merck Company,

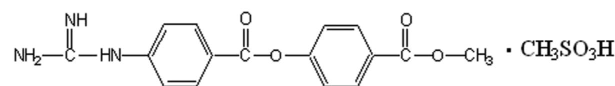

Figure 1. Chemical structure of 4'-CMGB.

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Darmstadt, Germany). Analytical-grade ammonium acetate, triethylamine, glacial acetic acid, and other chemicals were all purchased from Nanjing Chemical Reagent No.1 Factory (Nanjing, China). Water was purified by redistillation before use.

Compounds which might be impurities of 4'-CMGB were supplied by Department of Medicinal Chemistry of Second Military Medical University (Shanghai, China). They are *p*-guanidino benzoic acid, methyl *p*-hydroxybenzoate, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and methyl *p*-guanidino benzoate. All these chemicals are of reagent grade.

Sample preparation

Approximately 50 mg of 4'-CMGB reference substance was accurately weighed, dissolved in the mobile phase [(methanol–0.2% ammonium acetate–triethylamine–glacial acetic acid (470:524:1:5, v/v/v/v)], and diluted quantitatively to obtain standard calibration solutions at concentrations of 2, 10, 20, 30, 50, and 100 µg/mL. 4'-CMGB drug substance solutions were also prepared in the mobile phase at concentration of 20, 100, and 200 µg/mL. Possible impurity reference substances were dissolved in the mobile phase at concentration of 10 µg/mL.

Apparatus and operating conditions

HPLC–UV detection

Analysis of 4'-CMGB drug substance was performed with a HPLC system consisting of a LC-10A binary pump equipped with a 20 µL fixed injection loop (Shimadzu Technologies, Kyoto, Japan). Analytes were detected at 266 nm with a SPD-10A UV detector (Shimadzu Technologies). Data was acquired and processed with N2000 work station (Zhejiang University Zhida Information Engineering Co. Ltd., Hangzhou, China).

Chromatographic separations were performed on a C_{18} Shimpack column (150 mm × 4.6 mm, 5 µm, Kyoto, Japan) at 30°C. The mobile phase was methanol–0.2% ammonium acetate–tri-ethylamine–glacial acetic acid (470:524:1:5, v/v/v/v). The flow rate was 1.0 mL/min.

LC–Electrospray ionization-MS/MS

Mass spectral identification of related substances of 4'-CMGB was carried out with a LC–MS/MS system consisted of Surveyor LC pump, Surveyor auto-sampler, TSQ Quantum Ultra AM triplequadrupole tandem mass spectrometer, and Xcalibur 2.1 software (Thermo Finnigan, San Jose, CA). The separation was performed on a C_{18} Shimpack column (150 mm × 4.6 mm, 5 µm). Separation conditions were as follows: auto-sample temperature, 4°C; column temperature, 30°C; sample injection volume, 20 µL. The analysis was isocratic at 1.0 mL/min flow with methanol–0.1% ammonium acetate (48:52, v/v) as mobile phase. The effluent was split in a ratio of 3:1 before being introduced into the inlet of the mass spectrometer.

The electrospray ionization (ESI) (Thermo Finnigan) was used as the interface. The mass spectrometer was operated in both the positive and negative ionization modes, full scan from *m/z* 50 to 350. The spray voltage was 4.5 kV, heated capillary temperature was 350°C, nitrogen sheath, and auxiliary gas were 240 kPa and 34.5 kPa, respectively. In MS/MS determination, the collision energy of 10–30 eV was used with argon at a pressure of 0.2 Pa for collision-induced dissociation. Different collision energies were used to get a distinct fragmentation for certain analyte. The product scan mass spectrum range was set from *m/z* 50 to 350.

Method validation

The HPLC method was validated with respect to the following parameters: specificity, linearity, precision, stability of sample solution, and limits of detection (LOD) and quantification (LOQ).

4'-CMGB drug substance solutions of different concentrations (10-100 µg/mL) were injected into the HPLC system to find out if there were detectable impurities and whether they can be separated from 4'-CMGB. Chromatographic impurities were also evaluated in the presence of possible degradation products, which were generated by several accelerated conditions. 4'-CMGB drug substance stock solutions of 200 µg/mL were subjected to accelerated conditions as follows: heat, 1 mL of stock solution was exposed to 100°C for 10 min; acidic condition, to 1 mL stock solution, 0.5 mL of 0.1 M hydrochloric acid (HCl) was added. The acidified sample solution was stored at 100°C for 10 min before it was neutralized by 0.5 mL of 0.1 M sodium hydroxide (NaOH); alkaline condition to 1 mL stock solution, 0.5 mL of 0.1 M NaOH was added. The alkalized sample solution was stored at 100°C for 10 min before it was neutralized by 0.5 mL of 0.1 M HCl. If necessary, all samples were diluted with mobile phase to a theoretical concentration of 100 µg/mL before analysis.

The calibration curves were constructed for 4'-CMGB in the concentration range of 2–100 µg/mL. Precision of the method was determined by analysis of three replicates (n = 3) of standard solution at three concentrations: 16, 20, and 24 µg/mL. To determine intra-day precision of the method, replicates (n = 3) of the three standard solutions were analyzed, and for inter-day precision, replicates (n = 3) of freshly prepared standard solutions were analyzed on three different days. The relative standard deviations (RSDs) of the analyte peak areas were determined.

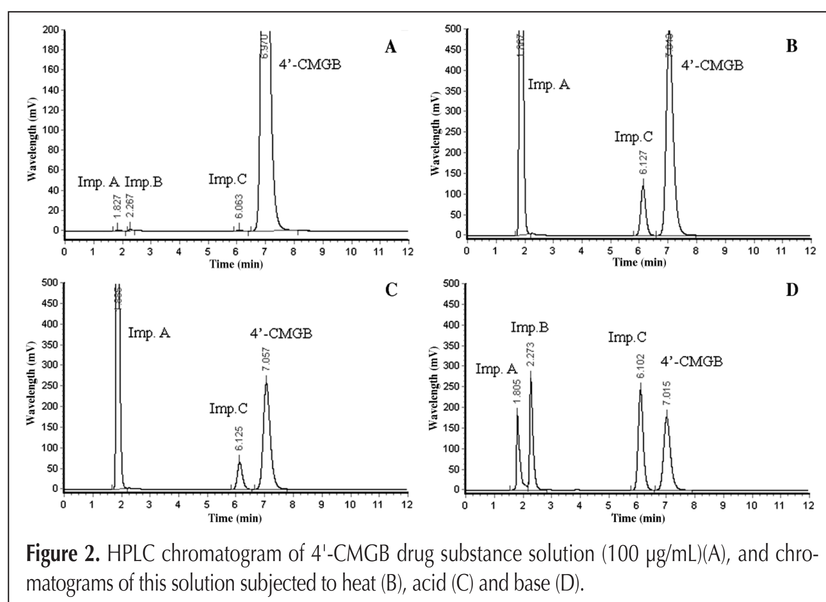

Figure 2. HPLC chromatogram of 4'-CMGB drug substance solution (100 µg/mL)(A), and chromatograms of this solution subjected to heat (B), acid (C) and base (D).

Stability test solution of 4'-CMGB (20 µg/mL) was prepared in mobile phase and aliquots analyzed at specific time intervals: 0, 2, 4, 6, 8, and 10 h at room temperature (20ºC).

LOD was established by determining the concentration of a dilute solution of 4'-CMGB that gave a signal-to-noise ratio of 3:1 while LOQ was determined as ten times of noise level.

Identification of related substances of 4'-CMGB

HPLC retention time identification

Except the main peak, any peak in the chromatogram of 4'-CMGB drug substance was possibly originated from related substances. They may be intermediates originated from the synthetic processes or degradation products generated upon storage.

Several compounds that have similarity in chemical structure with 4'-CMGB were analyzed at the same HPLC condition. These compounds are *p*-guanidino benzoic acid, methyl *p*-hydroxybenzoate (two intermediates of 4'-CMGB synthesis process), *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and methyl *p*-guanidino benzoate. Their retention times were compared with that of 4'-CMGB.

ESI-MS identification

The structures of the related substances were identified and elucidated by LC–MS–MS using a combination of full and product ion scanning techniques. Solutions of 4'-CMGB drug substance, 4'-CMGB degradation and possible related substances were analyzed. The full scan mass spectra (MS) provide the information of molecular weight, while the product ion mass spectra (MS/MS) allow for the identification of each analyte by its characteristic fragmentation pathway.

Results and Discussion

Method development

Initially, several combinations of mobile phases were tested for the HPLC method development. When different proportions of methanol–water or acetonitrile–water were used, the chromatograms showed very poor peak shapes with severe tailing and bad resolution. The presence of basic guanidino group in 4'-CMGB made it very susceptible to residual interactions with silanol groups of silica-based reversed-phase stationary phases. A silica pretreated (base deactivated) stationary phase column can solve this problem, but the cost is higher, and the ruggedness is not acceptable. Another way is to adjust the mobile phase. When a phosphate buffer was introduced into the mobile phase, little was improved. When an acetate buffer or triethylamine was added into the mobile phase, the situation became better. The separation was further optimized. Finally, the mixture of methanol–0.2% ammonium acetate–triethyl-amine–glacial acetic acid (470:524:1:5, v/v/v/v) provided the best peak shape, a satisfactory resolution, and relatively short analysis time. The presence of triethylamine in the mobile phase provided competing ammonium ions that helped to improve the peak shape of 4'-CMGB.

Method validation

Successful resolution of 4'-CMGB and its related substances was achieved. Figure 2A shows a representative chromatogram for 4'-CMGB drug substance solution of 100 µg/mL. Except the main peak, three additional peaks (impurity A, B, and C) were detected as the major related substances in 4'-CMGB. Their contents in 4'-CMGB drug substance final product were approximately 0.07%, 0.2%, and 0.1%, respectively, estimated by the peak area normalization method from the chromatogram. When 4'-CMGB drug substance was treated by the accelerated degradation test, the major decomposition products were also the same as these three impurities (Figure 2B–2D). They were all well separated from each other. Thus, the HPLC method presented in this study is selective for 4'-CMGB and its related substances.

Linear relationship between detector response and the concentration over a concentration range of 2–100 µg/mL for 4'-

Table I. Intra- and Inter-Day Assay Imprecision for 4'-CMGB

Concentration (µg/mL)	Imprecision (% RSD)*	
	Intra-day	Inter-day
16	0.1	0.9
20	0.9	1.1
24	0.7	0.7

*RSD = relative standard deviation.

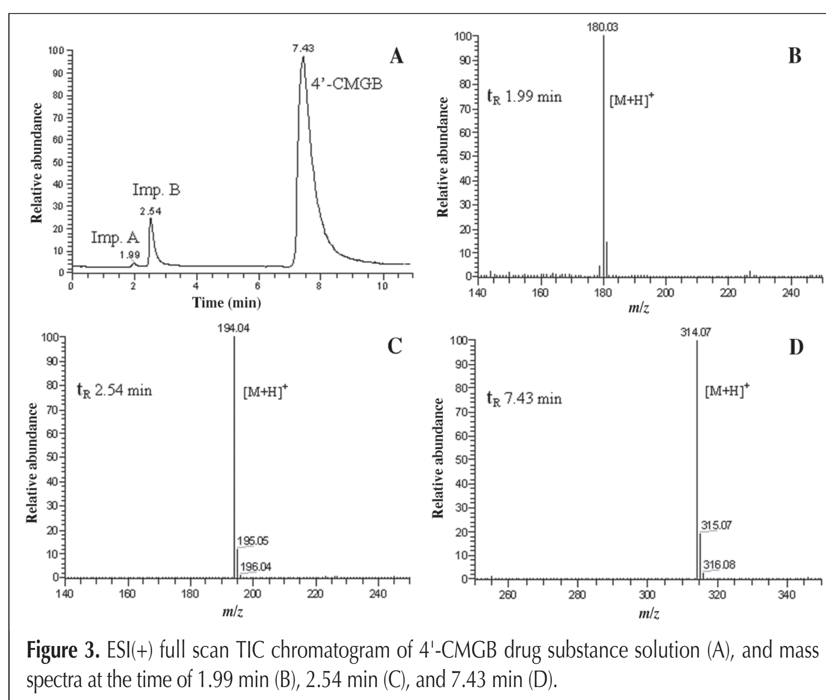

Figure 3. ESI(+) full scan TIC chromatogram of 4'-CMGB drug substance solution (A), and mass spectra at the time of 1.99 min (B), 2.54 min (C), and 7.43 min (D).

CMGB was established. A typical regression equation was *A* = 158615 *C* + 63466 (*A,* peak area; *C,* concentration) with the correlation coefficient of 0.9999. It covers the intended test concentration of the pharmaceutical quality control.

Values for the imprecision of the assay, calculated as RSD of three replicate measurements were within acceptable limits for intra-day and inter-day assay variability (Table I). This demonstrated the robustness of the method and its suitability for use in routine analysis (quality control) of the bulk substance.

The stability of 4'-CMGB in solution during analysis was determined by chromatographing the sample solution at 0–10 h and comparing the peak areas. The RSD of peak areas was less than 0.2%, and it suggested that sample solution was stable within 10 h. LOD and LOQ of the HPLC method were 15 ng/mL and 70 ng/mL, respectively.

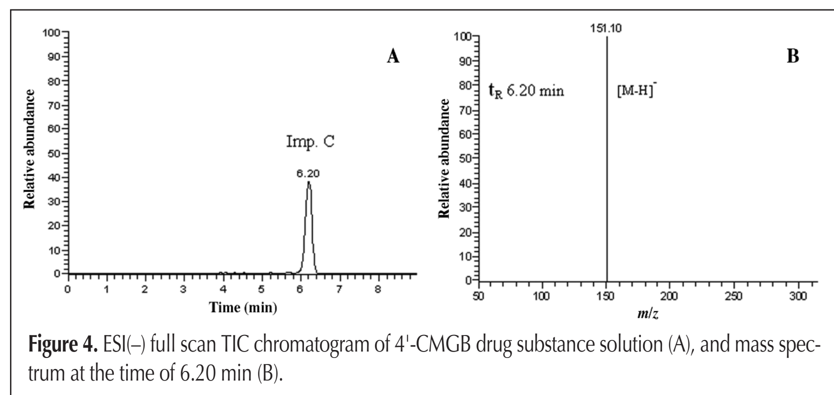

Figure 4. ESI(–) full scan TIC chromatogram of 4'-CMGB drug substance solution (A), and mass spectrum at the time of 6.20 min (B).

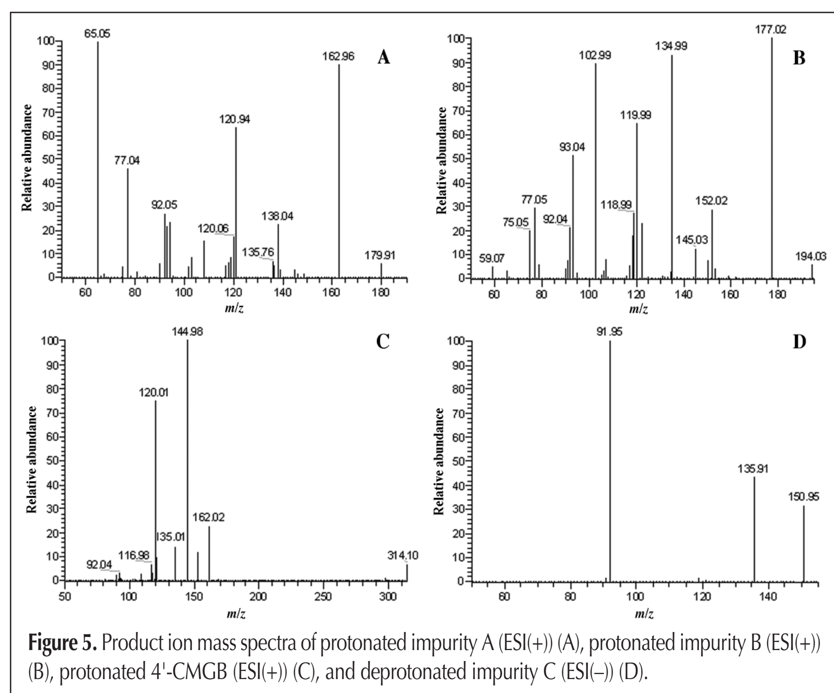

Figure 5. Product ion mass spectra of protonated impurity A (ESI(+)) (A), protonated impurity B (ESI(+)) (B), protonated 4'-CMGB (ESI(+)) (C), and deprotonated impurity C (ESI(–)) (D).

Identification of related substances of 4'-CMGB

HPLC

As was described earlier, three impurities were detected in 4'-CMGB drug substance solution. Subsequently, several compounds were analyzed under the same HPLC condition. The retention times of *p*-guanidino benzoic acid and impurity A were the same, and the retention times of methyl *p*-hydroxybenzoate and impurity C were the same, as well. The retention times of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and impurity B were similar, but when the proportion of methanol in the mobile phase decreased, they were separated.

During the study, a phenomenon drew our attention. The quantity of impurity B increased dramatically in 4'-CMGB methanol solution as days passed. It can be explained by that 4'-CMGB had the ester interchange reaction with methanol. The reaction product, methyl *p*-guanidino benzoate, was analyzed by HPLC. Its retention time matched with the retention time of impurity B. It may have also originated in the synthesis process as methanol was used as the recrystallization solvent.

Electrospray ionization mass spectrometry

In order to prove our estimation, HPLC–tandem MS was used. The mobile phase used here was a little different from the one used in the HPLC–UV to be compatible with the mass spectrometric detection. The chromatograms were similar except that the latter showed better resolution and peak shape. The analytes were assessed under both positive and negative ioniza-

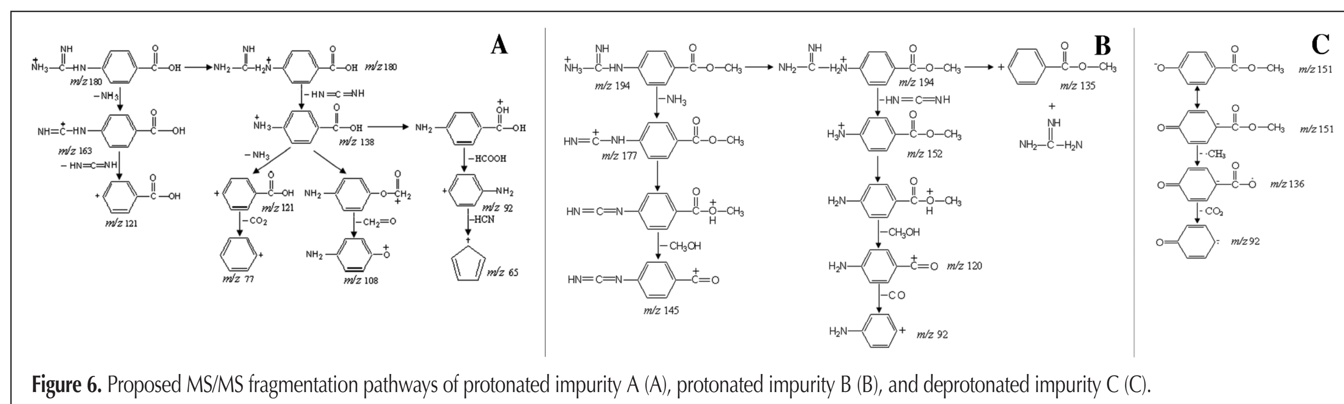

Figure 6. Proposed MS/MS fragmentation pathways of protonated impurity A (A), protonated impurity B (B), and deprotonated impurity C (C).

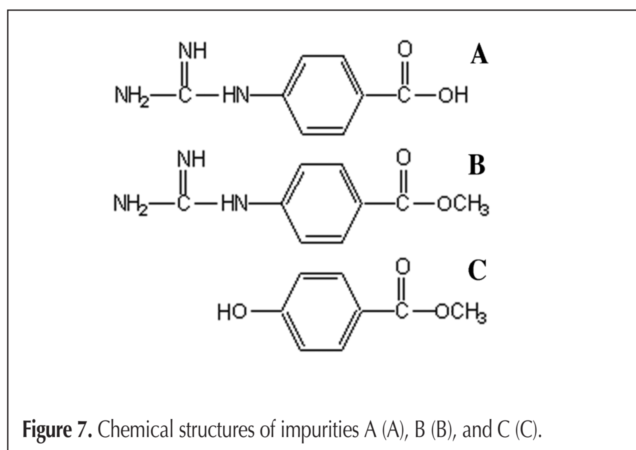

Figure 7. Chemical structures of impurities A (A), B (B), and C (C).

tion conditions in order to achieve maximum sensitivity and to obtain enough structural information.

In MS full-scan mode, the total ion current (TIC) chromatograms of 4'-CMGB drug substance solution (100 μg/mL) were acquired. The major ion detected was the protonated molecule ion, $[M+H]^+$, or the deprotonated molecule ion, $[M-H]^-$. In positive-ion mode (Figure 3), three peaks were detected at retention times of 1.99, 2.54, and 7.43 min, corresponding to the protonated impurity A, impurity B, and 4'-CMGB, respectively. Impurity A presented a $[M+H]^+$ ion at *m/z* 180, which is in agreement with the molecular weight of *p*-guanidino benzoic acid. Impurity B presented a $[M+H]^+$ ion at *m/z* 194, which is in agreement with the molecular weight of methyl *p*-guanidino benzoate. In negative-ion mode (Figure 4), one peak was obtained at retention time of 6.20 min, corresponding to the deprotonated impurity C. The $[M-H]^-$ ion was at *m/z* 151, in agreement with the molecular weight of methyl *p*-hydroxybenzoate. Because of the presence of the phenolic hydroxy group, it is easier for methyl *p*-hydroxybenzoate to form a negative ion rather than a positive one. Therefore, ESI (–) was found to be the most suitable ionization technique for methyl *p*-hydroxybenzoate and achieved higher sensitivity.

The product ion mass spectra of the protonated impurity A, B, and 4'-CMGB were obtained in positive-ion mode, while the product ion mass spectrum of the deprotonated impurity C was obtained in negative-ion mode (Figure 5). The fragmentation behaviors of these were also studied and all of them can be reasonably explained (Figure 6).

As a further confirmation of the structures, *p*-guanidino benzoic acid, methyl *p*-guanidino benzoate and methyl *p*-hydroxybenzoate were analyzed similarly. The TIC chromatograms, full-scan mass spectra and product ion mass spectra were all matched with the respective impurities.

LC–MS–MS analysis also showed no additional ions at the extract mass spectra for each compound at the time of peak upslope, apex, and downslope, which is the conformational evidence that proves the analyte peaks were homogenous, and there was no co-eluting material with each compound in this HPLC method.

From these observations, the structures of impurity A, B, and C were concluded as *p*-guanidino benzoic acid, methyl *p*-guanidino benzoate, and methyl *p*-hydroxybenzoate (Figure 7), respectively.

Conclusion

An HPLC method for the assay of 4'-CMGB drug substance and its related substances was developed and validated in this study. All the values calculated in the method validation were within the acceptable limits. This indicates that the chromatographic assay for 4'-CMGB is feasible and suitable for regulatory filing. The developed HPLC–UV method was successfully applied to the pharmaceutical quality control of 4'-CMGB drug substance and its preparation.

Moreover, the use of HPLC–MS–MS enabled the identification and elucidation of the structures of related substances. On the basis of the *m/z* values of the protonated or de-protonated molecule ions, the MS/MS fragmentations, the chromatographic retention times, and comparing with the standard compounds, three related substances of 4'-CMGB were identified. It is proven to be a simple, accurate, and rapid method for impurity identification.

References

1. J.G. Lv, CN Patent 1438217 A (2003).
2. Y.N. Zhao, Y.Z. Ji, J.G. Lv, B. Yi, Y. Sheng, and D. Jiang. Effects of acrosin inhibitor 4-guanidinobenzoate on human sperm function. *Acad. J. Sec. Mil. Med. Univ.* **21:** 1149–1151 (2000).
3. Y. Zhan, Y.N. Zhao, Z.L. Li, L. Li, and Q. Song. Effect of acrosin inhibitor KF-950 on acrosin activity and acrosome of human sperm. *Chin. J. Androl.* **8:** 92–94 (2002).
4. Y.N. Zhao, Y.Z. Ji, and Y. Sheng. Antifertility effects of intraviginal application of acrosin inhibitor KF-950 in rabbit. *Reprod. Contracep.* **20:** 294–296 (2000).
5. Y.N. Zhao, Y. Sheng, Y.Z. Ji, J.Y. Sha, Y.H. Liu, J.G. Lv, and X.T. Jiang. Acute toxic and mutagenic effects of the acrosin inhibitor KF-950. *Carcinogen. Teratogen. Mutagen.* **12:** 162–164 (2000).
6. J.M. Kaminski, L. Bauer, S.R. Mack, R.A. Anderson, D.P. Waller, and L.J. Zaneveld. Synthesis and inhibition of human acrosin and trypsin and acute toxicity of aryl 4-guanidinobenzoates. *J. Med. Chem.* **29:** 514–519 (1986).
7. M. Muramatsu and S. Fuji. Inhibitory effects of ω-guanidino acid esters on trypsin, plasmin, plasma kallikrein and thrombin. *Biochim. Biophys. Acta.* **268:** 221–224 (1972).
8. B. Göke, F. Stöckmann, R. Müller, P.G. Lankisch, and W. Creutzfeldt. Effect of a specific serine protease inhibitor on the rat pancreas: systemic administration of camostate and exocrine pancreatic secretion. *Digestion.* **30:** 171–178 (1984).
9. K. Beckh, B. Göke, R. Müller, and R. Arnold. Elimination of the low-molecular weight proteinase inhibitor camostate (FOY 305) and its degradation products by the rat liver. *Res. Exp. Med. (Berl).* **187:** 401–406 (1987).
10. I. Midgley, A.J. Hood, P. Proctor, L.F. Chasseaud, S.R. Irons, K.N. Cheng, C.J. Brindley, and R. Bonn. Metabolic fate of 14C-camostat mesylate in man, rat and dog after intravenous administration. *Xenobiotica.* **24:** 79–92 (1994).
11. M. Takeyama, T. Ishida, N. Kokubu, F. Komada, S. Iwakawa, K. Okumura, and R. Hori. Enhanced bioavailability of subcutaneously injected insulin by pretreatment with ointment containing protease inhibitors. *Pharm. Res.* **8:** 60–64 (1991).
12. P. Zlatoidsky, and T. Maliar. Synthesis and structure-activity relationship study of the new set of trypsin-like proteinase inhibitors. *Eur. J. Med. Chem.* **34:** 1023–1034 (1999).
13. M.K. Ramjee, M.J. Henderson, and B. Sheila. The kinetic and structural characterization of the reaction of nafamostat with bovine pancreatic trypsin. *Thromb. Res.* **98:** 559–569 (2000).
14. M. Kitagawa, T. Hayakawa, T. Kondo, T. Shibata, and Y. Sugimoto. Pancreatic and biliary excretion of camostat in dogs. *Digestion.* **39:** 204–209 (1988).
15. T. Uchida, N. Yasuda, and K. Matsuyama. Preparation and characterization of biodegradable or enteric-coated microspheres containing the protease inhibitor camostat. *J. Pharm. Pharmacol.* **53:** 255–261 (2001).
16. J. Yin, Y. Noda, N. Hazemoto, and T. Yotsuyanagi. Distribu-tion of protease inhibitors in lipid emulsions: gabexate mesilate and camostat mesilate determination. *Chem. Pharm. Bull.* **53:** 893–898 (2005).
17. J. Yin, Y. Noda, and T. Yotsuyanagi. Properties of poly(lactic-co-glycolic acid) nanospheres containing protease inhibitors: camostat mesilate and nafamostat mesilate. *Int. J. Pharm.* **314:** 46–55 (2006).

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