Journal of Chromatography B, 877 (2009) 2130–2134

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of a reversed-phase fluorescence HPLC method for determination of bucillamine in human plasma using pre-column derivatization with monobromobimane

Kang Choon Lee[a], Young Goo Chun[a], Insoo Kim[c], Beom Soo Shin[b], Eun-Seok Park[a], Sun Dong Yoo[a], Yu Seok Youn[c,*]

[a] College of Pharmacy, SungKyunKwan University, 300 Chonchon-dong, Jangan-ku, Suwon 440-746, Republic of Korea
[b] College of Pharmacy, Catholic University of Daegu, 330 Geumrak 1-ri, Hayang Eup, Gyeongsan si, Gyeongbuk 712-702, Republic of Korea
[c] College of Pharmacy, Pusan National University, 30 Jangjun-dong, Geumjeong-gu, Busan 609-735, Republic of Korea

ARTICLE INFO

Article history:
Received 7 February 2009
Accepted 5 June 2009
Available online 13 June 2009

Keywords:
Pre-column fluorescence derivatization
RP-HPLC
Monobromobimane
Bucillamine
Pharmacokinetics

ABSTRACT

A simple, specific and sensitive derivatization with monobromobimane (mBrB) and the corresponding HPLC-fluorescence quantitation method for the analysis of bucillamine in human plasma was developed and validated. The analytical procedure involves a simple protein precipitation, pre-column fluorescence derivatization, and separation by reversed-phase high performance liquid chromatography (RP-HPLC). The calibration curve showed good linearity over a wide concentration range (50 ng/mL to 10 μg/mL) in human plasma (r^2 = 0.9998). The lower limit of quantitation (LLOQ) was 50 ng/mL. The average precision and accuracy at LLOQ were within 6.3% and 107.6%, respectively. This method was successfully applied to a pharmacokinetic study after oral administration of a dose (300 mg) of bucillamine to 20 healthy Korean volunteers.

1. Introduction

Bucillamine, *N*-(2-mercapto-2-methylpropionyl)-L-cysteine, is a new class of disease-modifying anti-rheumatic drug with immunological effect similar to D-penicillamine [1]. It decreases the blood levels of rheumatic factor and the erythrocyte sedimentation rate [2], and it is reported to have potential to attenuate damage during myocardial infarction and cardiac surgery [3]. It has not been approved by the FDA, but it is widely used in Japan and Korea. Since this compound is highly soluble and has two sulfhydryl groups in its structure, organic phase liquid–liquid extraction is not feasible. Moreover, it may be strongly bound to serum proteins with cysteine moieties. Therefore, specific quantitative analysis of bucillamine is relatively difficult.

The first approaches for determining bucillamine content in biological samples included gas chromatography-electron capture detection [4,5] or gas chromatography–mass spectrometry [6,7] of the pentafluorobenzyl and methyl acrylate derivatives, respectively. Several methods to enhance specificity and sensitivity were also introduced, as well as use of reversed-phase high-performance liquid chromatography (RP-HPLC) [8,9]. In these methods, isobutylacrylate [8] and *N*-(1-prenyl)maleimide [9] were used for bucillamine derivatization, and the resulting bucillamine derivatives were determined by using atmospheric pressure chemical ionization (APCI)–liquid chromatography–mass spectrometry and fluorescence detection, respectively. Additionally, a unique quantitative bucillamine determination method from its pharmaceutical formulation was developed using FT-IR spectrometry of conventional KBr-disks [10].

In this study, a simple quantitation method for bucillamine, which have good sensitivity and wide concentration range, involving pre-column fluorescence derivatization using monobromobimane (mBrB) was developed and validated. This proposed method was applied to a clinical pharmacokinetic study of bucillamine in healthy male volunteers.

2. Experimental

2.1. Materials and reagents

Bucillamine (hereafter termed Buc) was supplied by YooYoung Pharm. Co. Ltd. (Seoul, Korea). deALP [1-((S)-3-mercapto-2-methylpropanoyl)-L-prolyl-L-phenylalanine] was provided by the Korea Institute of Science and Technology (Seoul, Korea). Monobromobimane (mBrB) and 2-mercaptoethanol were purchased from Fluka (Milwaukee, WI, USA) and Sigma–Aldrich Co. (St. Louis, MO,

* Corresponding author. Tel.: +82 51 510 2800; fax: +82 51 513 6754.
E-mail address: ysyoun@pusan.ac.kr (Y.S. Youn).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.06.010

USA), respectively. Methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA) was purchased from Sigma–Aldrich Co. All other chemicals and solvents, unless otherwise stated, were of analytical or HPLC grade. Drug-free human plasma was obtained from 20 healthy volunteers, using sodium heparin as the anticoagulant.

2.2. Preparation of stocks, calibration standards, and quality control samples

Primary stock solutions were prepared by dissolving Buc and deALP (internal standard: IS) (10 mg/mL of each) in methanol. Working standard solutions of Buc were prepared by diluting the primary solution with methanol. The working solution for IS (200 μg/mL) was prepared by diluting an aliquot of stock solution with methanol. All Buc and IS solutions were stored at 4 °C in polypropylene bottles in the dark when not in use, and were stable for at least 7 days.

Human plasma calibration standards of Buc (10, 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 μg/mL) were prepared by spiking appropriate amounts of the working standard solutions into a pool of 20 lots of drug-free human plasma. Quality control (QC) samples (0.05, 0.25, 2.5, and 7 μg/mL) were prepared in bulk by adding 100 μL of the appropriate working standard solutions (1, 5, 50, and 140 μg/mL) to drug-free human plasma (1900 μL). The QC samples were aliquoted (100 μL) into polypropylene tubes and stored at −20 °C until analysis.

2.3. Optimization of fluorescence derivatization

The derivatization of Buc with mBrB was monitored and optimized by varying the mBrB concentration and the reaction time. The basic protocols for derivatization and HPLC detection were the same as those described below. In this set of experiments, Buc concentration was fixed at 10 μg/mL, and mBrB concentration in methanol was varied from 0.5 to 8 mg/mL to obtain a curve for concentration vs. derivatization degree. Separately, at a fixed concentration of 4 mg/mL of mBrB, Buc derivatization was monitored at various reaction times. The degree of Buc-mBrB formation was evaluated by considering the integrated peak areas (mV s). Data represent three individual measurements and are presented as means ± S.Ds.

2.4. Stability of Buc-mBrB derivative

An aliquot (2 mL) of a standard solution (10 μg/mL) was mixed with 1 mL of 50 mM 2-mercaptoethanol, and kept for 20 min. An aliquot (1 mL) of the supernatant obtained by serial precipitation and centrifugation was mixed with 1 mL of mBrB solution (4 mg/mL, in methanol) and 5 mL of 50 mM phosphate buffer (pH 7.5). Three aliquots (2 mL) were transferred to brown amber vials and then kept at room temperature for 3 days. At predetermined times, each aliquot (50 μL) was injected onto the HPLC for fluorescence analysis. The amount remaining was expressed as the percent of initial, and data are presented as means ± S.Ds.

2.5. Sample preparation and procedure

An aliquot (100 μL) of standard solutions or blank plasma was mixed with 50 μL of 50 mM 2-mercaptoethanol and 5 μL of IS working solution at room temperature. After 20 min, 0.3 mL of ice-cold methanol was added; the mixture was thoroughly vortexed for 30 sec to induce protein precipitation, and further centrifuged at 10,000 rpm for 5 min. An aliquot (20 μL) of the resulting supernatant was mixed with 20 μL of mBrB solution (4 mg/mL, in methanol) and 100 μL of 50 mM phosphate buffer (pH 7.5) and maintained for 20 min. Aliquots (50 μL) of the mixture were also injected onto the HPLC for fluorescence analysis. Drug concentrations were determined from peak area ratios of Buc and IS.

2.6. Apparatus and data analysis

HPLC analyses were performed on Shimadzu equipment consisting of a model SCL-10A system controller, LC-10AT pump, SIL-10A autosampler 234, RF-10AXL fluorescence detector (150 W Xenon arc lamp), and CTO-10A column oven. Data were acquired and processed with a chromatography station for Windows (CSW 32) from DataApex (Prague, Czech Republic). Compounds were separated on a Capcellpak analytical column (250 mm × 4.6 mm, 5 μm) and guard column (4 mm × 3 mm, 5 μm, Phenomenex). The mobile phase was 0.1% TFA (trifluoroacetic acid)/acetonitrile: 0.1% TFA/deionized water (Puris Ultrapure Water system, Korea) (70:30, v/v); before use, this solution was passed through a 0.22-μm membrane filter and degassed by ultrasonication under vacuum. The mobile phase flow rate was maintained at 1.2 mL/min, and the column temperature was 30 °C. The excitation and emission wavelengths were 270 and 474 nm, respectively.

2.7. Assay validation

The assay validation was validated according to the FDA guidance for industrial bioanalytical method validation (2001) [11]. Linearity was determined using linear least-square regression with a weighting index of $1/x$, which was performed on the peak area ratios of Buc and IS vs. Buc concentrations in the five human plasma standards. The sensitivity of the method was expressed as the lower limit of quantitation (LLOQ) that could be determined with acceptable accuracy and precision. The accuracy and precision were assessed by analyzing eight theoretical concentrations from five validation batch samples and five different concentrations of QC samples (0.05, 0.25, 2.5, and 7 μg/mL). Precision was expressed as a percentage of coefficients of variation (CV), and accuracy was expressed as the mean percentage of analyte recovered in the assay.

2.8. Pharmacokinetic application

The developed fluorescence HPLC method was applied to a pharmacokinetic study as a bioequivalence test, which had been approved by the Korean Food and Drug Administration (KFDA). Twenty healthy male volunteers, fasted for 12 h, received a single oral dose (300 mg) of three Bucillamine tablets with 240 mL of water. Blood samples (2 mL) were withdrawn via catheter cannula from the forearm vein at 0, 20, 40, 60, 80, 100, 120, 150, 180, 240, 300, and 360 min post-dosing, transferred to fresh tubes containing sodium heparin (BD, NJ, USA) and then centrifuged (12,000 rpm for 5 min). Plasma samples were transferred to polypropylene tubes and stored at −20 °C prior to analysis. Drug concentrations were determined as the mean of duplicate samples. Pharmacokinetic parameters were determined from the time vs. plasma concentrations of Buc by the 2002 version of K-BE test software program supplied by KFDA. The parameters included peak concentration (C_{max}), time to reach peak concentration (T_{max}), area under the plasma concentration–time curve from zero to last time (AUC_{last}), area under the plasma concentration–time curve from zero to infinite (AUC_{inf}), elimination half-life ($t_{1/2}$), systemic clearance from zero time to infinite (Cl/F), and volume of distribution from zero time to last time (V/F). The protocol was approved by the Institutional Review Board on human studies at the Research Institute of Pharmaceutical Sciences in SungKyunKwan University, and informed consent was obtained from the subjects after explaining the nature and purpose of the study in accordance with the Korean Guidelines for Bioequivalence Test.

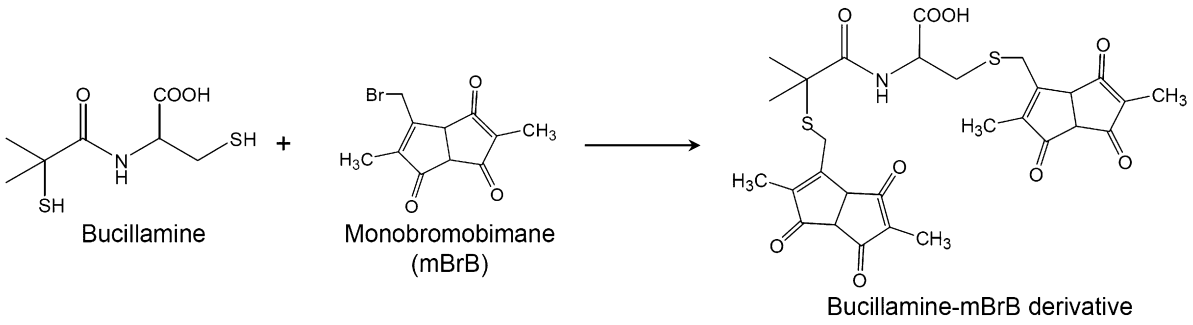

Fig. 1. Derivatization reaction of bucillamine with monobromobimane.

3. Results and discussion

3.1. Separation and optimization of mBrB derivatization

As shown in Fig. 1, this compound has a carboxylic group, two sulfhydryl groups, and an amide bond, which produce great hydrophilic properties. Thus a liquid–liquid extraction method using organic solvents was not feasible, despite acidic treatment. Therefore, a protein precipitation method was carried out, and the resulting supernatant containing Buc was used for analysis. Like *o*-phthaldialdehyde (OPA), mBrB is reviewed as one of the most available agents for fluorescent labeling of the SH group [12,13]. Since plasma contains numerous cysteine-containing molecules that react with mBrB, blank human plasma had a variety of endogenous peaks until 8 min. However, under the isocratic mobile conditions employed, there were no significant interfering peaks at the retention times of Buc-mBrB and IS-mBrB (9.32 and 11.89 min, respectively) (Fig. 2A) in blank plasma spiked with IS and 5 μg/mL Buc (Fig. 2B), blank plasma spiked with IS and 50 ng/mL Buc (LLOQ) (Fig. 2C), and plasma obtained from a subject at 40 min after oral administration of 300 mg Buc spiked with IS (Fig. 2D).

According to the previous finding [12], the optimal pH for mBrB derivatization was 7.5. At this pH, the Buc derivatization with mBrB was optimized by considering mBrB concentration and reaction time. As shown in Fig. 3A, the reaction degree gradually increased with increasing mBrB concentration up to 4 mg/mL, but at higher concentrations, there was no further increase. Also the reaction was achieved very rapidly, and almost all (>93%) Buc fraction was derivatized with mBrB within 3 min (Fig. 3B). Based on such findings, the mBrB concentration (4 mg/mL) and reaction time (20 min) were considered acceptable for Buc derivatization.

3.2. Stability

The stability of Buc-mBrB derivative in the final reaction media was briefly investigated at room temperature for 3 days. This was performed in order to assess the peak integrity and to identify degradation products, which may disturb the analysis. As shown in Fig. 3C, the molecular integrity of the Buc-mBrB derivative was maintained very well until 3 days, and no significant degradation peaks were observed in the chromatogram.

3.3. Method validation

The specificity of the fluorescence HPLC method was assessed by analyzing five different batches of blank human plasma. As

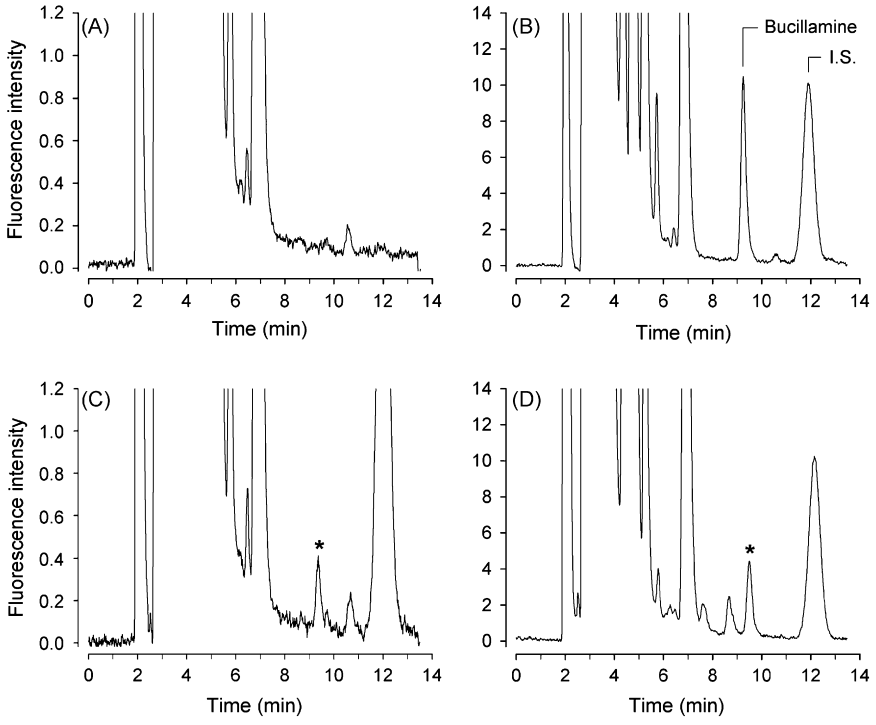

Fig. 2. Chromatograms of (A) blank human plasma, (B) blank plasma spiked with IS and 5 μg/mL Buc, (C) blank plasma spiked with IS and 50 ng/mL Buc (LLOQ), and (D) plasma obtained from a subject at 40 min after oral administration of 300 mg Buc spiked with IS.

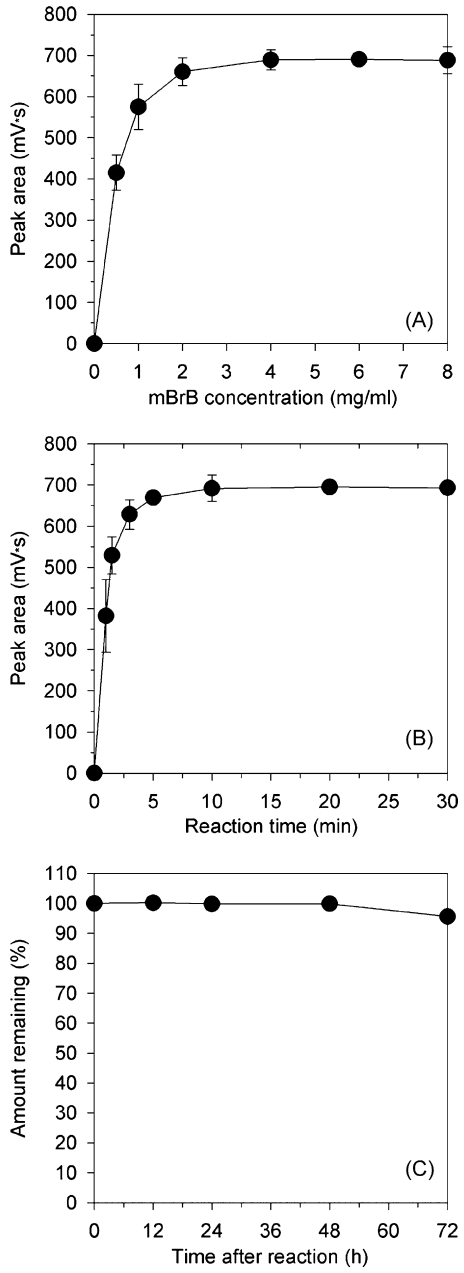

Fig. 3. Monitoring and optimization of Buc derivatization with mBrB based on (A) mBrB concentration and (B) reaction time. (C) Short-term stability of Buc-mBrB derivative in reaction media.

mentioned, all plasma samples had no interference at the retention times of the analyte and IS. The eight-point calibration curve obtained by least-squares linear regression showed excellent linearity within the range of 50 ng/mL to 10 μg/mL, which includes the concentrations typically found in human plasma after oral administration of Buc in the pharmacokinetic study. The calibration curve for Buc had a slope of 0.00017, an intercept of 0.0013 and an r^2 value of 0.9998.

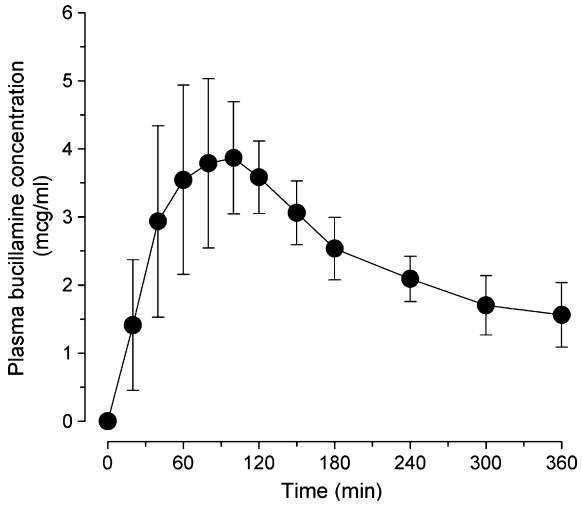

Fig. 4. Mean plasma concentration–time curve of Buc in 20 healthy volunteers after oral administration of 300 mg Buc (mean ± S.D., $n = 20$).

The lower limit of quantitation (LLOQ) for Buc was 50 ng/mL (signal-to-noise ratio of >10), and the minimum detectable level (LOD) was 20 ng/mL (signal-to-noise ratio of ~3). As seen in Table 1, the intra- and inter-day precision value at the LLOQ were 6.3% and 5.1%, respectively, and the intra- and inter-day accuracies were 107.6% and 106.8%, respectively.

Intra- and inter-day precision and accuracy were also determined by analyzing QC samples (50 ng/mL, LLOQ; 0.25 μg/mL, low QC; 2.5, medium QC; and 7 μg/mL, high QC) against a calibration curve on the same day ($n = 5$) and on different days ($n = 5$). The intra- and inter-day precision values were 2.7–7.3% and 3.3–6.9%, respectively, and the intra- and inter-day accuracies were 93.1–101.7% and 95.9–104.1%, respectively.

3.4. Pharmacokinetics

The proposed method was applied to the determination of Buc levels in 480 plasma samples for the pharmacokinetic and bioequivalence study of three Buc tablets (300 mg) administered to 20 healthy Korean volunteers. The mean (±S.D.) plasma concentration–time profile of Buc after oral administration of bucillamine is shown in Fig. 4. The C_{max} and T_{max} values were 4.5 ± 1.1 μg/mL and 89.5 ± 27.0 min, respectively. The AUC_{last} and AUC_{inf} values were 867.8 ± 155.6 and 1410.9 ± 428.9 μg min/mL, respectively. The half-life of drug elimination during terminal phase was 229.5 ± 86.1 min. The CL_{inf}/F and V_{last}/F values were 0.0008 ± 0.0002 mL/min and 0.2357 ± 0.0507 mL, respectively (Table 2).

Table 1
Precision and accuracy of bucillamine in calibration standards prepared in human plasma ($n = 5$).

		Theoretical concentrations (ng/mL)								Slope	Intercept	R^2
		10000	5000	2000	1000	500	200	100	50			
Intra-day ($n = 5$)	Mean	10231.5	4905.6	1897.3	962.4	499.0	209.2	104.2	53.8	0.00017	0.0013	0.9998
	CV (%)	3.0	8.0	3.7	5.9	4.5	5.1	3.4	6.3			
	RE (%)	2.3	−1.9	−5.1	−3.8	0.0	4.6	4.2	7.6			
Inter-day ($n = 5$)	Mean	10411.2	5108.6	1982.8	996.2	511.6	207.3	99.3	53.4			
	CV (%)	5.3	4.2	2.5	5.3	2.1	4.7	3.2	5.1			
	RE (%)	4.1	2.2	−0.9	−0.4	2.3	3.7	−0.7	6.8			

Table 2
Pharmacokinetic parameters of bucillamine after an oral administration of a dose (300 mg) of bucillamine tablets to 20 healthy Korean volunteers ($n = 20$).

Pharmacokinetic parameters	Values (mean ± S.D.)
AUC_{last} (μg min/mL)	867.8 ± 155.6
AUC_{inf} (μg min/mL)	1410.9 ± 428.9
C_{max} (μg/mL)	4.5 ± 1.1
T_{max} (min)	89.5 ± 27.0
CL/F (mL/min)	0.0008 ± 0.0002
V/F (mL)	0.2357 ± 0.0507
$t_{1/2}$ (Half-life, elimination phase)	229.5 ± 86.1

4. Conclusion

A simple fluorescence HPLC method for bucillamine after pre-column monobromobiman derivatization in human plasma was developed and validated. The developed assay demonstrated acceptable specificity, precision, accuracy, sensitivity, stability, and linearity over a therapeutically relevant concentration range. Particularly, comparing with the previous methods [8,9], our method shows several advantages of better linear correlation, wide practical concentration range, and faster derivatization time. Moreover, this validated method was also successfully applied to assay the human plasma bucillamine levels in a clinical pharmacokinetic study of bucillamine in tablet dosage form. To our knowledge, this is the first report on fluorescence HPLC method of bucillamine validated by a clinical pharmacokinetic study.

Acknowledgments

This work was supported by the 2008 Specialization Project Research Grant funded by the Pusan National University, and by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (no. R01-2008-000-20197-0). The authors thank YooYoung Pharm. Co. Ltd., for the kind supply of bucillamine tablets.

References

[1] H. Matsuno, E. Sugiyama, A. Muraguchi, T. Nezuka, T. Kubo, K. Matsuura, H. Tsuji, Int. J. Immunopharmacol 20 (1998) 295.
[2] H. Matsuno, T. Kitano, I. Matsushita, et al., Drug Under Exp. Clin. Res. 19 (1993) 205.
[3] L.D. Horwitz, Cardiovasc. Drug Rev. 21 (2003) 77.
[4] H. Takashina, M. Horiuchi, S. Miyagi, O. Uemura, T. Iso, Yakugaku Zasshi 105 (1985) 671.
[5] M. Horiuchi, H. Takashina, T. Iwatani, et al., Yakugaku Zasshi 105 (1985) 665.
[6] K. Matsuura, K. Murai, H.J. Takashina, Mass Spectrom. Soc. 46 (1998) 25.
[7] K. Matsuura, H. Takashina, J. Chromatogr.: Biomed. Appl. 616 (1993) 229.
[8] F. Beaudry, D. Proulx, M. Furtado, Biomed. Chromatogr. 18 (2004) 805.
[9] Y. Higashi, M. Yamashiro, R. Yamamoto, Y. Fujii, J. Liq. Chromatogr. Related Technol. 26 (2003) 3265.
[10] A.A. Bunaciu, H.Y. Aboul-Enein, S. Fleschin, II Farmaco 60 (2005) 685.
[11] FDA/CDER/CVM, Guidance for Industry (2001).
[12] F. Tache, A. Farca, A. Medvedovici, V. David, J. Pharm. Biomed. Anal. 28 (2002) 549.
[13] R. Koka, J. Visser, F. Moolenaar, D. de Zeeuw, D. Meijer, J. Chromatogr. B 693 (1997) 181.