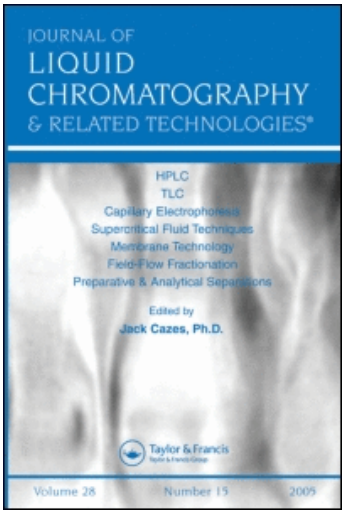

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HPLC Analysis of Bucillamine by Derivatization with *N*-(1-Pyrenyl)Maleimide in Human Blood

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ABSTRACT

Bucillamine, *N*-(2-mercapto-2-methylpropionyl)-*L*-cysteine, is a novel disease-modifying anti-rheumatic drug containing two sulfhydryl groups. We investigated high performance liquid chromatographic (HPLC) analysis of bucillamine by derivatization with *N*-(1-pyrenyl)maleimide (NPM) in human blood. Human blood samples were immediately mixed with NPM at room temperature for 15 min and injected into an HPLC. The linearity was displayed for bucillamine concentrations ranging from 3 to 3000 nM ($r = 0.999$). The lower limit of detection of bucillamine derivative was 2.5 nM. Bucillamine derivative remained stable at -4°C for at least 2 weeks with less than 5% variation. The coefficients of variation for intra-day and inter-day assays were less than

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4.2% and 5.3%, respectively. No endogenous and exogenous compounds (*L*-cysteine, glutathione, *N*-acetyl-*L*-cysteine, captopril, and *D*-penicillamine), possessing a sulfhydryl group in these structures, were found to interfere with the peak of the bucillamine derivative. These results indicate that HPLC assay of bucillamine by derivatization with NPM is rapid, sensitive, and reproducible for determining bucillamine in human blood. This method may be applied to pharmacokinetic studies of bucillamine in humans.

Key Words: HPLC; Bucillamine; *N*-(1-pyrenyl)maleimide; Derivatization.

INTRODUCTION

Bucillamine, *N*-(2-mercapto-2-methylpropionyl)-*L*-cysteine, is a novel disease-modifying anti-rheumatic drug containing two sulfhydryl groups.[1–3] After prolonged treatment, bucillamine induces undesirable side effects including nephrotic syndrome and agranulocytosis.[4–6] Therefore, it is necessary to detect blood levels of bucillamine in order to assess the degree of possible toxicity.[4–6]

Despite its frequent use in medicine, the determination and pharmacokinetic study of bucillamine is carried out by gas chromatography–mass spectrometry (GC–MS) and gas chromatography–electron capture detection (GC–ECD).[7,8] However, GC–MS and GC–ECD systems are too specialized or expensive to be generally available in a clinical setting where therapeutic drug monitoring is carried out.

Bucillamine has no prominent absorption properties in the UV spectrum and no fluorescence properties. Therefore, the use of a derivatization reagent, which is reactive toward sulfhydryl groups, would seem suitable for determination of bucillamine concentration in blood.

In general, fluorescence detection is superior to UV detection in terms of sensitivity. Some authors formed sulfhydryl compound adducts with *N*-(1-pyrenyl)maleimide (NPM), which showed fluorescence properties to enhance sensitivity.[9,10] For example, *D*-penicillamine, which is useful in the treatment of rheumatoid arthritis as well as heavy metal poisoning and contains one free sulfhydryl group, is determined by derivatization with NPM using high performance liquid chromatography (HPLC).[10] In addition, captopril, an angiotensin-converting enzyme inhibitor, cysteine and glutathione also possess one free sulfhydryl group in the structure, and determination of these compounds has been performed by derivatization with NPM using HPLC.[9,11]

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In the present study, NPM was used to form a fluorescent derivative with bucillamine according to the reaction shown in Fig. 1. We have utilized this derivatization technique and reversed-phase HPLC to measure bucillamine in human blood.

EXPERIMENTAL

Equipment

The HPLC system comprised a model L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne injection valve (Cotati, California) with a 20 μL, and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The HPLC column (KANTO Chemical Co., Inc., Tokyo, Japan) was 150 × 4.6 mm i.d. with 5 μm particles of C_{18} packing material.

Reagents

Bucillamine was obtained from Santen Pharmaceutical Co., Ltd. (Osaka, Japan). *N*-(1-pyrenyl)maleimide, *L*-cysteine and *D*-penicillamine were purchased from Aldrich Chemical. Co. (Milwaukee, WI). Acetonitrile and ethanol for HPLC, glutathione (reduced form), *N*-acetyl-*L*-cysteine, captopril, and other general reagents were supplied by Wako Pure Chemical Industries (Osaka, Japan).

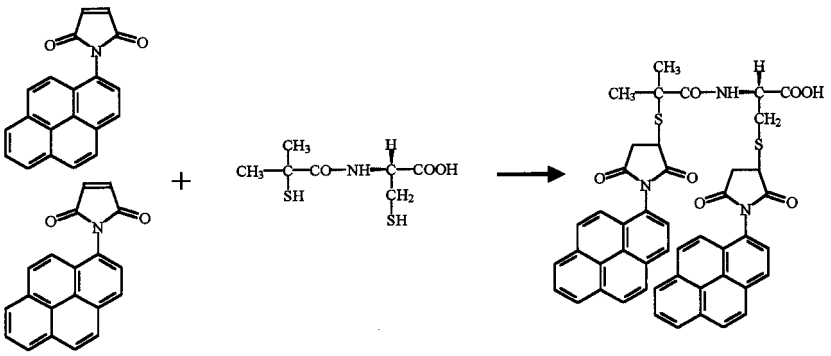

Figure 1. Formation of fluorescent bucillamine derivative.

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Chromatographic Conditions

Quantitation of the peaks was performed with a Chromatopac, Model CR-3A integrator (Shimadzu, Kyoto, Japan). The mobile phase was prepared by addition of ethanol (250 mL) to a solution containing 42% acetonitrile, 0.1 (v/v)% *o*-phosphoric acid, and 0.1 (v/v)% acetic acid in H_2O (500 mL). The NPM derivatives were eluted from the column at a flow rate of 0.5 mL min^{-1}.

Derivatization of Bucillamine

Human blood samples (50 μL) were added to polyethylene tubes containing 50 μL of 10 mM *tris*(hydroxymethyl)aminomethane–hydrochloric acid (Tris–HCl) buffer (pH 8.0) with 1 mM ethylenediaminetetraacetic acid, disodium salt. NPM (0.167 mM) in acetonitrile solution (300 μL) was immediately added to the blood. The resulting solution was vortexed and incubated at room temperature for 15 min before acidification with 5 μL of 0.167 M HCl to stop the reaction. After centrifugation (GL Sciences, Tokyo, Japan) at 12,000 rpm for 2 min, the supernatant was decanted to a new tube and injected into the HPLC system.

Drug Standard

The standard solution for bucillamine was prepared in Tris-HCl buffer (pH 8.0). A 50 μL volume of bucillamine (0, 3, 6, 10, 30, 60, 100, 300, 600, 1,000, and 3,000 nM) in Tris-HCl buffer (pH 8.0) and 50 μL of human blood were mixed. Then, a series of methods from the derivatization of bucillamine to the injection into the HPLC system was carried out according to the above description.

RESULTS AND DISCUSSION

Reaction Time Course of Bucillamine Derivative

Figure 2 shows the reaction time course of bucillamine derivative with NPM. Bucillamine (100 nM) was derivatized with NPM, the peak area of bucillamine derivative reached a maximum at 15 min. There were no significant differences in bucillamine derivative peak areas when a sample was incubated from 15 to 40 min. It was considered that it should be noted that the reaction is complete at 15 min, but must be stabilized with the addition of HCl within 40 min.

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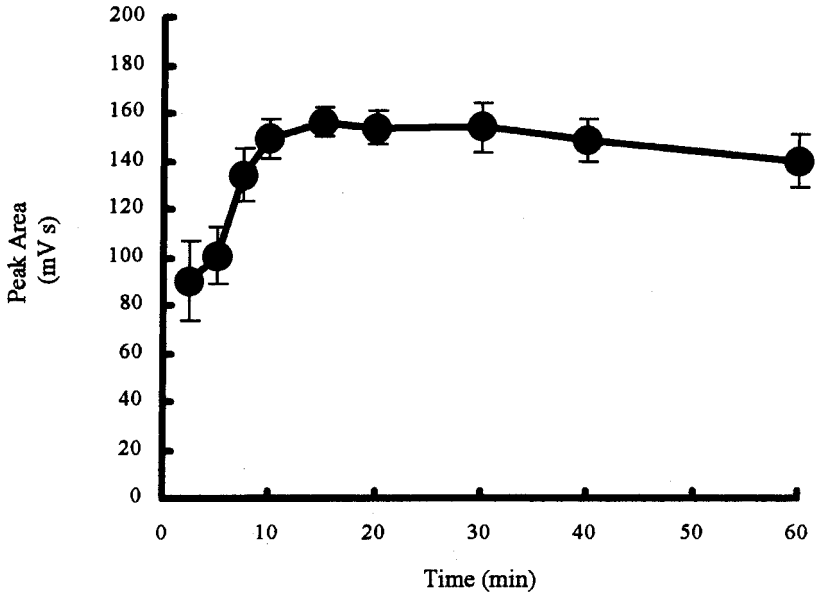

Figure 2. Reaction time course of bucillamine derivative. Peak areas are reported as mean ± S.D. of three experiments.

Chromatograms

Figure 3 illustrates a representative chromatogram of blank blood (A) and blood with 100 nM of bucillamine (B). Drug-free pooled human blood yielded relatively clean chromatograms with no significant interfering peaks. Retention time (*Rt*) of the bucillamine derivative was 10.1 min.

Linearity and Sensitivity

A standard curve of bucillamine is shown in Fig. 4. The standard curve was constructed by plotting integrated peak area vs. bucillamine concentrations. The linearity was displayed for bucillamine concentrations ranging from 3 to 3000 nM. The regression coefficient was 0.999 ($y = 1.526x - 20.576$). The lower limit of detection for bucillamine utilizing this method was established at 2.5 nM (signal-to-noise ratio of 3 : 1). The lower limit of quantitation (LOQ) of bucillamine derivative was 3 nM. Lower limit of quantitation of *D*-penicillamine derivative, shown by Yusof et al.,[10] was 4 nM. The sensitivity of bucillamine derivative was better than that of the *D*-penicillamine derivative. It was guessed

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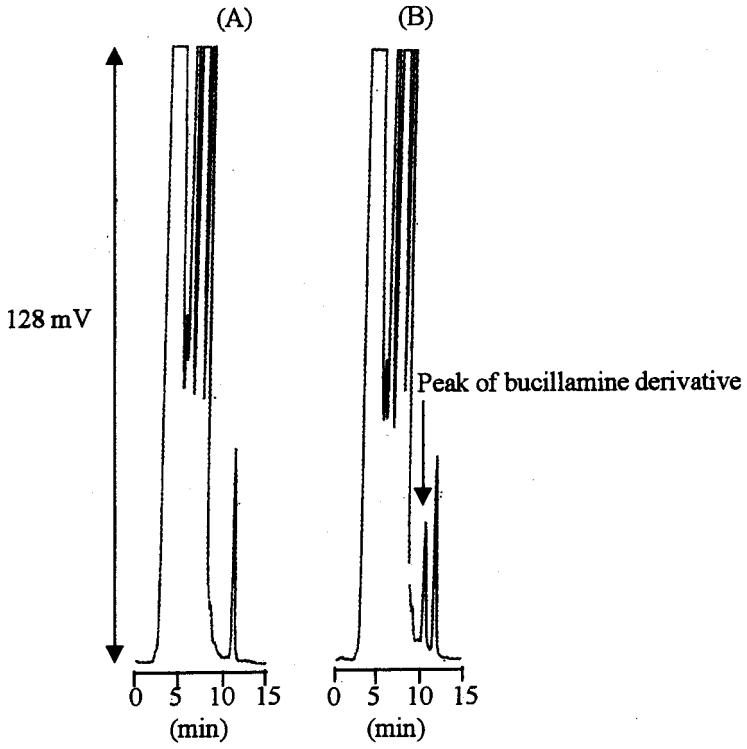

Figure 3. Chromatograms of blank human blood and bucillamine derivative. (A) Chromatogram obtained from a blood sample (no bucillamine peak). (B) Standard chromatogram containing a peak from the bucillamine derivative (100 nM). The attenuation for all chromatograms is 128 mV/full scale.

that the sensitivity of bucillamine might reach 2 nM because of two sulfhydryl groups in the structure of bucillamine. However, it did not reach that level. While the effective concentration range of bucillamine in blood is not determined, we estimate that our method may be suitable for analysis of bucillamine pharmacokinetics in humans.

Storage Stability

The storage stability of bucillamine in human blood was examined by the analysis of human blood containing known amounts of analytes stored at −4°C for 2 weeks, and the data are listed in Table 1. Tested samples (10, 100,

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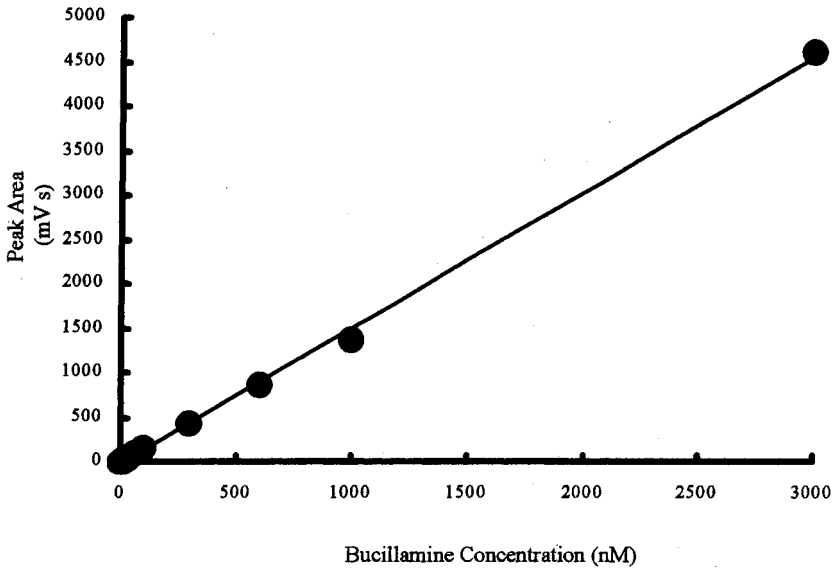

Figure 4. Standard curve of bucillamine derivative.

and 1000 nM) were immediately mixed with NPM and allowed to stand at room temperature for 15 min, and then stored at −4°C for 2 weeks. The stabilities of bucillamine at 10, 100, and 1,000 nM were 95.7, 95.5, and 96.7%, respectively. It was ascertained, that the stability of bucillamine derivatives and precision and accuracy of the measurement by HPLC were satisfactory.

Precision and Accuracy

Precision and accuracy for intra-day and inter-day assay of bucillamine derivative are shown in Table 2. In the intra-day assay, the range of standard

Table 1. Stability data for bucillamine derivative stored at −4°C for 2 weeks.

Bucillamine concentration (nM)	Measured (nM) mean ± S.D. ($n = 5$)	CV (%)	Recovery (%)
10	9.57 ± 0.42	4.4	95.7
100	95.5 ± 2.4	2.5	95.5
1,000	967 ± 38	3.9	96.7

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Table 2. Intra- and inter-day assay reproducibility for determination of bucillamine.

Bucillamine concentration (nM)	Measured (nM) mean ± S.D.	CV (%)	Recovery (%)
Intra-day assay ($n = 5$)			
10	9.77 ± 0.41	4.2	97.7
100	101.7 ± 3.4	3.3	101.7
1,000	1,049 ± 32	3.1	104.9
Inter-day assay ($n = 5$)			
10	9.73 ± 0.52	5.3	97.3
100	103.8 ± 3.9	3.8	103.8
1,000	1,035 ± 40	3.9	103.5

deviation to the average was within 3.1–4.2%, and the recovery was within 97.7–104.9%. In inter-day assay, the range of coefficient variation was within 3.8–5.3%, and the recovery was within 97.3–103.8%.

Interference

Furthermore, we examined the interference of endogenous and exogenous compounds (*L*-cysteine, glutathione, *N*-acetyl-*L*-cysteine, captopril, and *D*-penicillamine, each 1,000 nM) on bucillamine (100 nM) with NPM, as shown in Table 3. Because the peaks of NPM derivatives of these compounds and the blank blood peak overlapped, these *Rt*s were not determined. Namely, *Rt*s of these peaks were different from that of bucillamine derivatives. Therefore, the recovery of bucillamine derivatives was satisfactory. These results

Table 3. Interference of endogenous and exogenous compounds on the assay of bucillamine.

Added	Measured (nM) mean ± S.D. ($n = 5$)	CV (%)	Recovery (%)
Bucillamine (100 nM)			
+*L*-Cysteine (1,000 nM)	101.3 ± 3.8	3.8	101.3
+Glutathione (1,000 nM)	101.8 ± 3.6	3.5	101.8
+*N*-Acetyl-*L*-cysteine (1,000 nM)	97.0 ± 4.7	4.8	97.0
+Captopril (1,000 nM)	99.8 ± 5.0	5.0	99.8
+*D*-Penicillamine (1,000 nM)	102.2 ± 4.4	4.3	102.2

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indicate that the blood bucillamine levels can be precisely measured when these compounds and bucillamine are co-administered to humans.

CONCLUSIONS

Except for the derivatization of sulfhydryl groups with NPM, Nakashima et al. have provided a method for the determination of sulfhydryl groups with *N*-[4-(6-dimethylamino-2-benzofuranyl)-phenyl] maleimide.[12] However, this method requires specific reaction conditions (60°C) and the samples are only stable for 24 h after derivatization. Although the fluorometric HPLC method is good for obtaining high sensitivity, the method using 5-dimethylaminonaphthalene-1-sulfonylaziridine and *N*-[*p*-(2-benzoxazolyl)phenyl]-maleimide as derivatizing agents requires lengthy incubation periods overnight at 37°C.[13,14] The GC assays are not only laborious and time consuming, but also, they are not sensitive enough to detect the presence of low levels of sulfhydryl compounds.[15,16] In contrast, the NPM method was shown to be reproducible, sensitive, specific, and easy to use.

In conclusion, our HPLC assay of bucillamine by derivatization with NPM is rapid, sensitive, and reproducible for determining bucillamine in human blood, and this method may be applied to the pharmacokinetic studies of bucillamine in humans. We expect that our method will become popular and enable the assessment or prediction of severe side effects, such as nephrotic syndrome and agranulocytosis, based on blood bucillamine levels.

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