Short communication

HPLC determination of chlorpropamide in human serum by fluorogenic derivatization based on Suzuki coupling reaction with phenylboronic acid

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Abbreviations: PBA, phenylboronic acid; HPLC, high-performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; RSD, relative standard deviation

Abstract

A fluorogenic derivatization method for the determination of chlorpropamide in human serum was developed by means of high-performance liquid chromatography (HPLC) with fluorescence detection. Suzuki coupling reaction with a non-fluorescent reagent, phenylboronic acid (PBA), was employed to convert chlorpropamide into highly fluorescent biphenyl derivative. Chlorpropamide was extracted from human serum by liquid-liquid extraction with toluene after addition of hydrogen chloride, and subsequently reacted with PBA. Because the fluorogenic derivatization was highly selective for aryl halide, the proposed method allowed sensitive and selective detection of chlorpropamide with a detection limit (at a signal to noise ration of 3) of 0.5 ng/mL. The sensitivity of our method was from 4- to 100-times better than HPLC-UV, gas chromatography- and LC-mass spectrometry.

Keywords: fluorogenic derivatization; phenylboronic acid; chlorpropamide; high-performance liquid chromatography

Introduction

Chlorpropamide is one of sulphonylurea derivatives that are widely used as an oral hypoglycemic drug for the treatment of non-insulin-dependent diabetes mellitus [1]. Chlorpropamide can stimulate pancreatic beta-cell insulin production, which results in the reduction of glucose levels in blood. However, excess dose of chlorpropamide can cause side effects including hypoglycemic symptoms and coma [2] because it possibly stimulates excessive insulin production [3]. Therefore, methods for monitoring therapeutic blood levels are required for the management of side effects of chlorpropamide.

Several chromatographic methods have been reported for the analysis of chlorpropamide in biological samples, such as high-performance liquid chromatography (HPLC) and micellar electrokinetic chromatography with UV detection [4-8]. However, owing to low selectivity of UV detection, some interfering peaks appeared on the chromatogram. Also, methods employing gas chromatography (GC) or LC with mass spectrometry (MS) were developed [6, 9]; however, MS equipment is very complicated and is not available in many research laboratories.

On the other hand, we previously developed selective fluorescence derivatization techniques for aryl halides using aryl boronic acid as a reagent [10-14]. The derivatization techniques are based on Suzuki coupling reaction [6], which is a palladium-catalyzed cross-coupling reaction of aryl halides with aryl boronic acids. The techniques can change drugs containing aryl halides in their structures into fluorescent derivatives even in the presence of other biological components. Among these techniques, the fluorogenic derivatization method using non-fluorescent phenylboronic acid (PBA) as a reagent provides clean chromatogram because the interference derived from the reagent can be negligible [14]. The aim of this study is to develop a highly

sensitive and selective HPLC method for the determination of chlorpropamide in human serum by fluorogenic derivatization using PBA (Fig. 1). Chlorpropamide can be converted to a fluorescent biphenyl derivative after reaction with PBA because it has aryl chloride moiety in the structure (Fig. 1), and the derivative can be determined sensitively by fluorescence detection.

Materials and Methods

Chemicals

PBA and *p*-chlorobenzaldehyde (internal standard (IS)) were purchased from Tokyo Chemical Industries (Tokyo, Japan). Palladium (II) acetate ($Pd(OAc)_2$), potassium fluoride (KF) and *N*,*N*-dimethylformamide (DMF) were from Nacalai Tesque (Kyoto, Japan). Acetonitrile, *n*-hexane, toluene, chloroform, diethyl ether and dichloromethane were from Kanto Chemical (Tokyo). Sodium carbonate (Na_2CO_3), potassium carbonate (K_2CO_3), and trisodium phosphate (Na_3PO_4) were from Wako Pure Chemicals (Osaka, Japan). Water was distilled and passed through a Pure Line WL21P system (Yamato, Tokyo). All other chemicals were the highest purity and quality available.

Apparatus

The HPLC system consisted of a pump LC-6A (Shimadzu, Kyoto), a Shimadzu RF-550 fluorescence detector, a 7125 injector with a 5-μL loop (Rheodyne, Cotati, CA, USA), and a FBR-1 recorder (Tosoh, Tokyo). Chromatographic separation was performed on a Daisopak ODS-AP (250 x 4.6 mm, i.d., Daiso, Osaka) by an isocratic elution with a mixture of acetonitrile and 1% acetic acid (50:50, v/v %) at a flow rate of 1.0 mL/min. The fluorescence (FL) was monitored at

λex 260 nm and λem 325 nm.

Assay procedure for chlorpropamide in human serum

To a 200-μL portion of the serum spiked with known concentration of chlorpropamide, 20 μL of IS (2.5 ng/mL in DMF/water (60:40, v/v %)) and 30 μL of 1 M hydrogen chloride were added and vortexed briefly, after which 1 mL of toluene was added. The mixture was vortexed for 2 min and centrifuged at 1400x*g* for 15 min. The organic layer was carefully taken and evaporated to dryness using centrifugal evaporator. The residue was reconstituted in 50 μL of DMF/water (60:40, v/v %) and subjected to the fluorogenic derivatization as follows: 50-μL portion each of 5 mM PBA in DMF, 0.5 mM $Pd(OAc)_2$ in DMF, and 50 mM Na_2CO_3 in water were added to the reconstituted residue. The mixture was vortexed for 10 s and deoxygenated by N_2 purge for 5 s. The reaction mixture was heated at 100 °C for 20 min. The resultant mixture was passed through a membrane filter (0.5 μm DISMIC-3, Toyo Roshi, Tokyo). A 5-μL portion of the filtrate was injected into the HPLC-FL system.

Result and Discussion

Optimization of fluorogenic derivatization conditions

The fluorogenic derivatization conditions were optimized using a standard solution of chlorpropamide and IS. The concentration of PBA was investigated over the range 1-6 mM. As shown in Fig. 2, since the maximum peak height for the fluorescent derivative of chlorpropamide was obtained at a concentration 5 mM, 5 mM PBA was chosen for further experiments. In the Suzuki coupling reaction, $Pd(OAc)_2$ and base are used as catalysts. The effects of KF, K_2CO_3,

Na_3PO_4, and Na_2CO_3 were compared to determine which base would work best in the reaction (Fig. 3). Among the examined bases, Na_2CO_3 was the most effective and gave maximum and constant peak heights with a concentration of more than 40 mM; 50 mM was thus chosen for further experiments. $Pd(OAc)_2$ concentration was investigated over the range 0.1-3 mM, and the maximum peak heights were observed at concentrations over 0.5 mM; 0.5 mM $Pd(OAc)_2$ was selected. The temperature and time also influenced on the derivatization reaction. The reaction cannot occur efficiently when temperature is less than 100 °C. The maximum peak heights were achieved by heating for more than 20 min at 100 °C.

Optimization of sample preparation conditions

For the effective extraction of chlorpropamide and IS from serum samples, commonly used liquid-liquid extraction was investigated as a sample treatment by using 200-μL serum spiked with 300 ng/mL chlorpropamide. *n*-Hexane, toluene, chloroform, diethyl ether and dichloromethane were compared as extraction solvent and maximum recovery was obtained with toluene. The effect of the volume of toluene was also examined. Maximal and constant recoveries were almost attained for 1 mL of solvent. Therefore, 1 mL of toluene was selected as extraction solvent. Furthermore, 30 μL of hydrogen chloride was added to extraction solvent to improve the extractability. Under the optimized conditions, the recoveries of chlorpropamide and IS were 78.0% and 40.4%, respectively.

Figure 4(a) and (b) show typical chromatograms obtained with the extracts from drug-free human serum and serum spiked with chlorpropamide, respectively. The peak of the chlorpropamide derivative could be detected clearly at 13 min without any interfering peaks from

the serum components.

Calibration curves, detection limit, and repeatability

Calibration curves were prepared for human serum spiked with the standard solution of chlorpropamide. A linear relationship (r=0.999) between the peak height ratio and the concentration of chlorpropamide was obtained in the range from 2.5 to 300 ng/mL. The detection limit (S/N=3) of chlorpropamide in human serum was 0.5 ng/mL, which was 100-times, 10-times, and 4-times more sensitive than HPLC-UV [4, 5], GC-MS [6], and LC-MS [9], respectively.

The method was validated using two levels of spiked serum (25 and 100 ng/mL) for within-day (n = 5) and between-day (n = 5) repeatabilities. Relative standard deviation (RSD) in the within- and between-day were less than 2.7% and 8.2%, respectively.

Conclusion

A sensitive and selective HPLC method for the determination of chlorpropamide was developed by using fluorogenic derivatization based on Suzuki coupling reaction. The proposed method was successfully applied to the determination of chlorpropamide in human serum without any interference. This method should be useful for therapeutic drug monitoring of chlorpropamide in patients.

References

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Figure captions

Fig. 1 Fluorogenic derivatization of chlorpropamide with PBA based on Suzuki coupling reaction.

Fig. 2 Effect of PBA concentration on the reactivity. Conditions of the derivatization reaction are described in Materials and Methods section, except for PBA concentration. Sample concentration: chlorpropamide (300 ng/mL) and IS (250 ng/mL). Peak height at 5 mM of PBA was defined as 100%.

Fig. 3 Reactivity of chlorpropamide and IS with PBA in the presence of various bases (50 mM). Conditions of the derivatization reaction are described in Materials and Methods section, except for base. Sample concentration: chlorpropamide (300 ng/mL) and IS (250 ng/mL). Peak height obtained by Na_2CO_3 was defined as 100%.

Fig. 4 Chromatograms for extract from (a) human serum and (b) human serum spiked with standard chlorpropamide (150 ng/mL) and IS (250 ng/mL).

Fig. 1

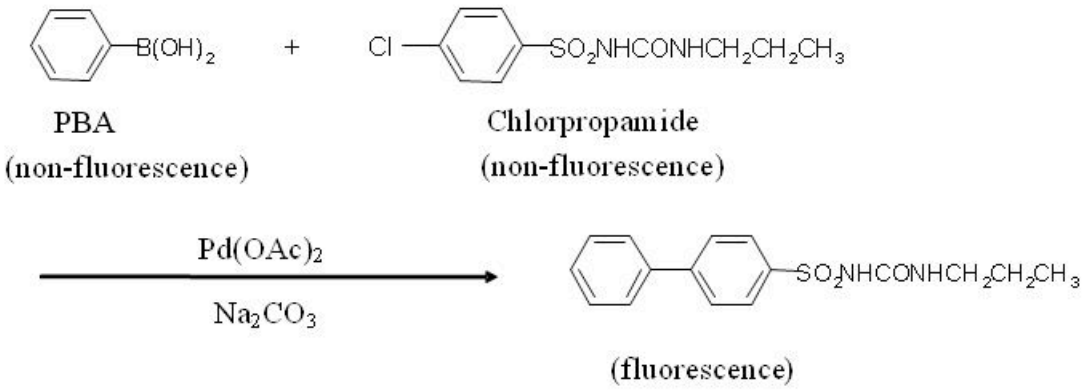

B(OH)2
+
Cl
SO2NHCONHCH2CH2CH3
PBA
(non-fluorescence)
Chlorpropamide
(non-fluorescence)
Pd(OAc)2
Na2CO3
SO2NHCONHCH2CH2CH3
(fluorescence)

Fig. 2

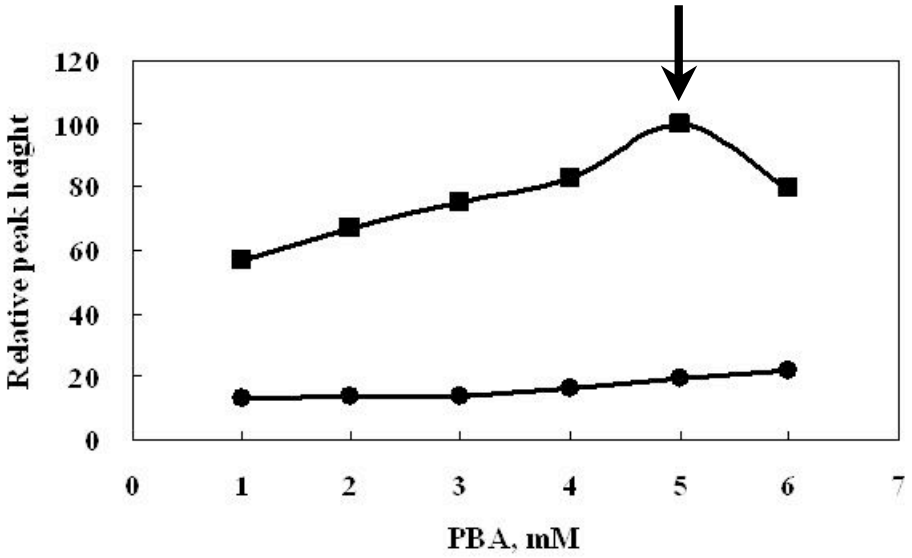


Fig. 3

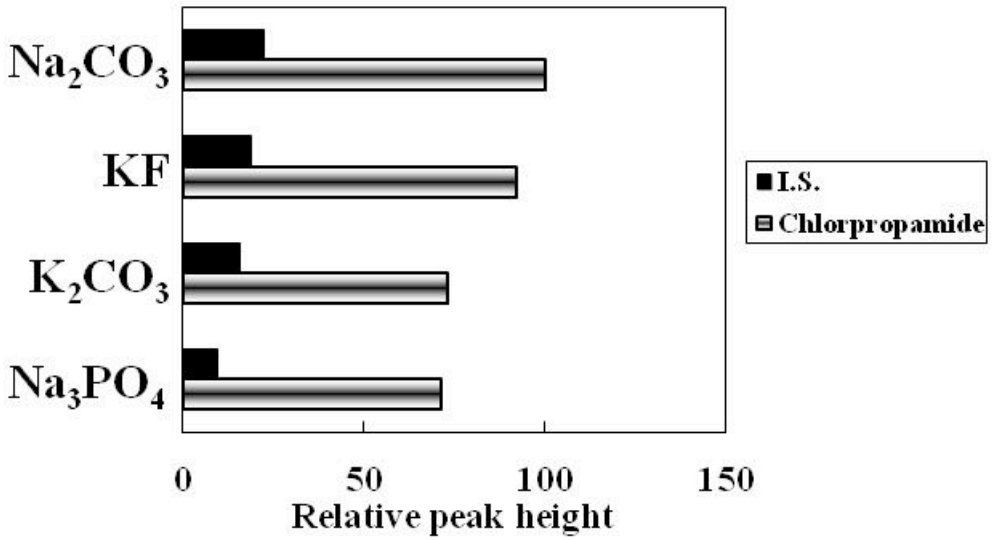
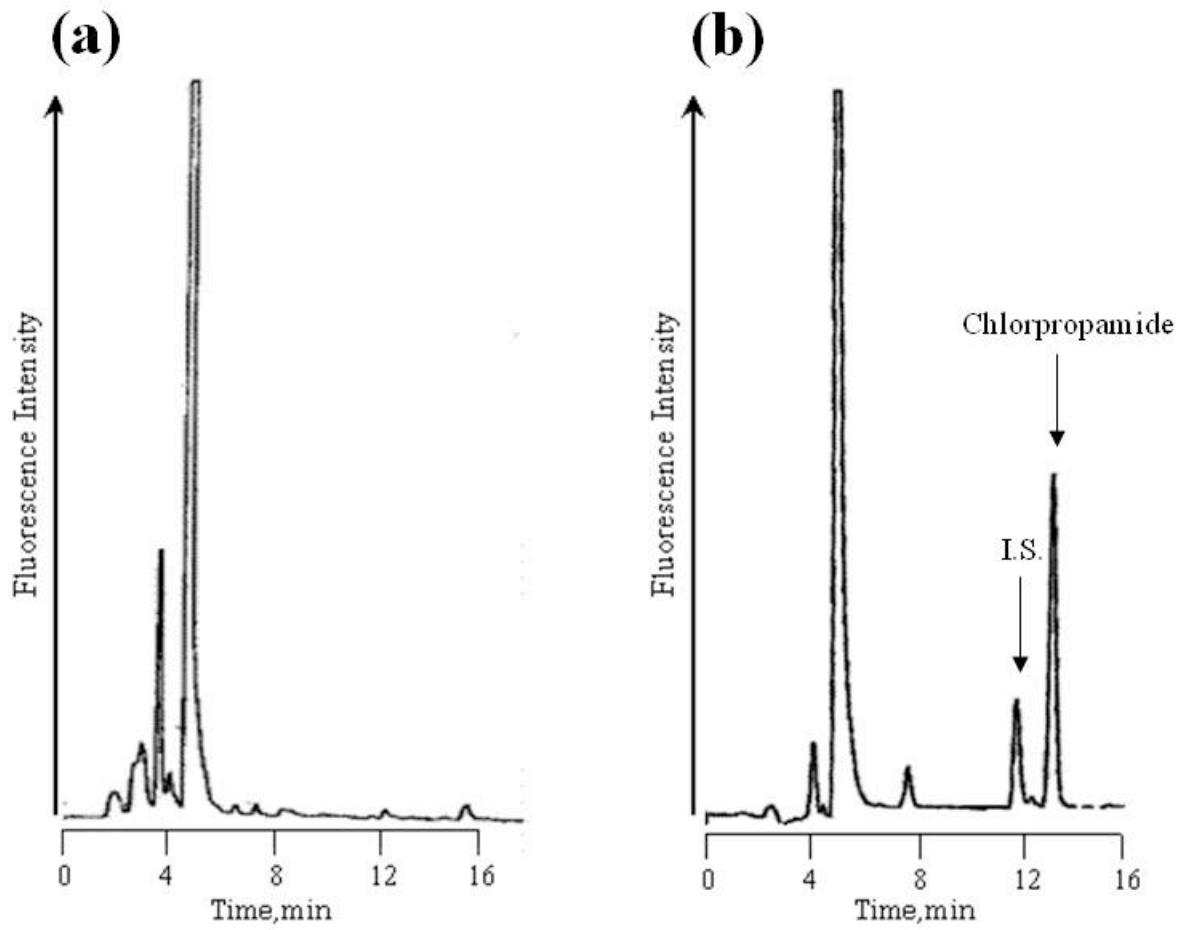

Na2CO3
KF
K2CO3
Na3PO4
I.S.
Chlorpropamide
0
50
100
150
Relative peak height

Fig. 4


(a)
Fluorescence Intensity
0
4
8
12
16
Time,min
(b)
Fluorescence Intensity
Chlorpropamide
I.S.
0
4
8
12
16
Time,min