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ORIGINAL RESEARCH

Precolumn fluorescence labeling method for simultaneous determination of hydroxyzine and cetirizine in human serum

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ABSTRACT: A highly selective and sensitive method was developed for simultaneous determination of the antihistaminic drug hydroxyzine (HZ) and its pharmacologically active metabolite cetirizine (CZ) in human serum using haloperidol as internal standard. The method was based on fluorescence labeling of both drugs with a fluorescent arylboronic acid 4-(4,5-diphenyl-1*H*-imidazol-2-yl)phenyl boronic acid followed by separation on silica column using a mobile phase consisting of acetonitrile and water (90:10, v/v%) containing triethylamine and acetic acid. The labeling reaction conditions were optimized and the liquid–liquid extraction method was successfully applied to extract the both drugs from serum. The linearity range was 0.025–2.00 μg/mL for HZ and CZ. The limit of detection (S/N = 3) was 10 and 5 ng/mL for HZ and CZ, respectively. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: fluorescence labeling; Suzuki coupling reaction; antihistamines; high-performance liquid chromatography

INTRODUCTION

Like other H1-receptor antihistamines, hydroxyzine (HZ) acts by competing with histamine for sites on H1-receptor sites on effector cells. Antihistamines do not block histamine release, but can antagonize its effects. In addition to its antihistaminic effects, HZ possesses anticholinergic, sedative, tranquilizing, antispasmodic, local anesthetic, mild bronchodilative and antiemetic activities. HZ is used principally for its antihistaminic, antipruritic and sedative or tranquilization qualities (Ferrerri and Hantouche, 1998). Cetirizine (CZ) is an active metabolite of HZ and it has the advantage that it lacks the central nervous system depressant effects often encountered in antihistamines. It is a potent and well-tolerated antihistamine drug for the treatment of seasonal and perennial allergic rhinitis and chronic urticaria (De Vos *et al.*, 1987; Snyder and Snowman, 1987).

Determination of HZ and CZ in serum is necessary for pharmacokinetic studies that require a reliable method with low limit of detection to enable accurate and precise measurements. Several methods were developed for determination of HZ in serum such as high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Pehourcq, 2004) and gas chromatography (GC) (Fouda *et al.*, 1979). Most of the method recently developed are screening methods either with other antihistamines by using capillary zone electrophoresis (Capella-Peiro *et al.*, 2006) or using HPLC with tandem mass spectrometry (HPLC-MS/MS) (Gergov *et al.*, 2003). Analysis of CZ in biological fluids has been achieved using thin layer chromatography (TLC; Pandya *et al.*, 1996), GC-MS (Baltes *et al.*, 1988), HPLC-UV (Macek *et al.*, 1999; Moncrieff, 1992; Nagaralli *et al.*, 2003; Rosseel and Lefebvre, 1991) and HPLC-MS/MS (Eriksen *et al.*, 2002; Gergov *et al.*, 2003; de Jagar *et al.*, 2002). Although the HPLC-MS/MS method reached a suitable sensitivity, the instrument is somewhat complicated and not universally available due to its high cost. In this study we developed a sensitive method based on readily available chemicals and conventional instruments. This method is the first method to be used for simultaneous

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Abbreviations used: CZ, cetirizine; DCPB, 2-(dicyclohexylphosphino)biphenyl; DtPB, 2-(di-*t*-butylphosphino)biphenyl; DMF, *N*,*N*-dimethylformamide; DPA, 4-(4,5-diphenyl-1*H*-imidazol-2-yl)phenyl boronic acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; HZ, hydroxyzine; IS, internal standard; MS/MS, tandem mass spectrometry; TLC, thin layer chromatography.

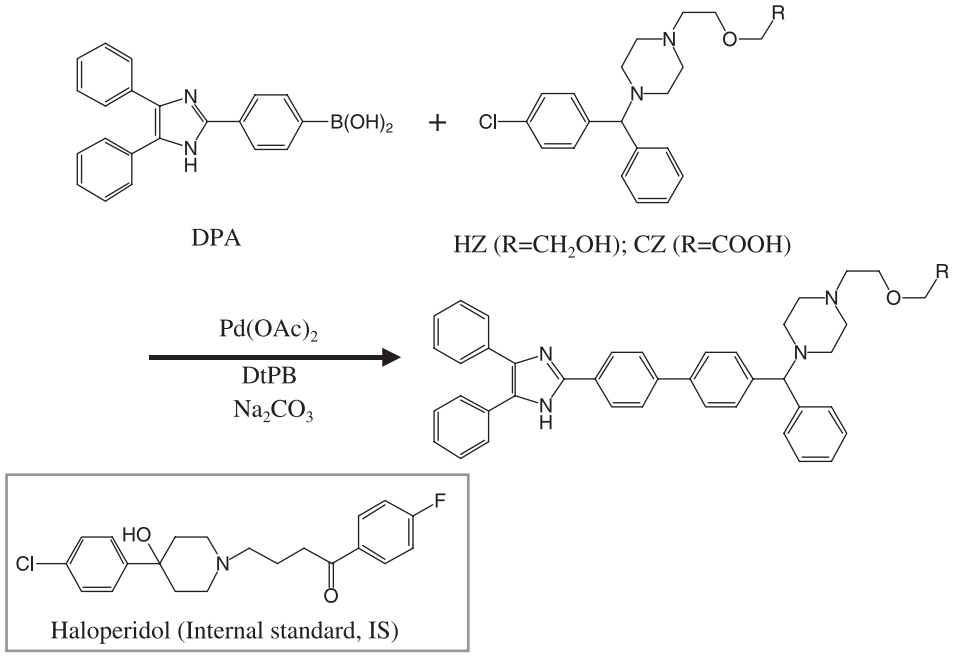

Figure 1. Scheme of Suzuki coupling reaction of HZ and CZ with DPA.

determination of both drugs using small volume of serum and it is the first method developed for their determination using the fluorescence labeling technique.

This new, sensitive and highly selective method is based on fluorescence labeling of both drugs as well as the internal standard (IS) using the Suzuki coupling reaction, as shown in Fig. 1. This reaction is one of the most versatile and utilized reactions for the selective construction of carbon–carbon bonds, in particular for the formation of biaryls (Kotha *et al.*, 2002; Miyaura and Suzuki, 1995; Suzuki, 1999) because of the availability of boronic acids and stability of the product.

For analytical application, the Suzuki coupling reaction has superior selectivity derived from the small number of interfering compounds existing in biological samples in addition to altered sensitivity due to the elimination of a halogen atom, which sometimes leads to unexpected quenching of fluorescence. Recently, we reported this newly fluorescent labeling method for the determination of aryl chloride drugs based on the Suzuki coupling reaction (Kishikawa *et al.*, 2006; Kuroda *et al.*, 2005).

Fluorescent products of both drugs were separated using a silica column with aqueous–organic mobile phase containing traces of acetic acid and triethylamine as a modifier (to control pH of the mobile phase) and the protonated form of triethtylamine functioned as a competing ion (Wu *et al.*, 2004). This separation system has the advantage that basic compounds are strongly retained on the column and easily separated from largely excess reagent.

EXPERIMENTAL

Materials. HZ hydrochloride was kindly donated by Chemical Industries Development (Giza, Egypt). CZ hydrochloride was kindly donated by Pharco Pharmaceuticals (Alexandria, Egypt). Haloperidol as IS was purchased from Sigma (St Louis, MO, USA). *N*,*N*-Dimethyl formamide (DMF) and anhydrous sodium carbonate (Na_2CO_3) were from Wako (Tokyo, Japan). Acetonitrile (HPLC grade) was from Kanto Chemical (Tokyo, Japan). 2-(Dicyclohexylphosphino)biphenyl (DCPB) and 2-(di-*t*-butylphosphino)biphenyl (DtPB) were purchased from Strem Chemicals (New-buryport, MA, USA). Palladium acetate [$Pd(OAc)_2$], triehylamine, acetic acid and dichloromethane were from Nacalai Tesque (Kyoto, Japan). DPA was synthesized in our laboratory according to the previous reported method (Kuroda *et al.*, 1999).

Chromatographic system. The HPLC system consisted of an LC-6A Pump (Shimadzu, Kyoto), a 7125 injector with a 5 μL loop (Rheodyne, Cotai, CA, USA), an RF-10 AXL fluorescence detector (Shimadzu) and an R-110 recorder (Rikadenki, Tokyo). Chromatographic separation was performed on Merck Lichrosorb Si 60 (250 × 4 mm i.d., 5 μm) column using an isocratic elution with a mixture of acetonitrile and water (90:10, v/v%) containing 0.02% triethylamine and 0.03% acetic acid at ambient temperature and a flow rate of 1.0 mL/min. The excitation and emission detection wavelengths were 320 and 410 nm, respectively.

Procedures of labeling reaction. The optimum conditions used for the labeling reaction were 50 μL of each of the two drugs and internal standard, 12.5 mM DPA, 4 mM $Pd(OAc)_2$, 2.5 mM DtPB mixture and 20 mM Na_2CO_3, all dissolved in

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DMF except Na_2CO_3, which was dissolved in water. They were mixed in an amber-colored screw-capped reaction vial. The reaction mixture was vortex-mixed and purged with N_2 for 5 s, following by heating for 30 min at 100°C. Filtering through an HLC-Disk3 (0.45 μm, Kanto Chemical), 5 μL of the reaction mixture was injected into the HPLC system.

Assay procedures for HZ and CZ in human serum. Two-hundreds microliters of serum were spiked with 50 μL of 10 μM IS aqueous solution then 0.8 mL of dichloromethane added and vortexed for 60 s followed by centrifugation at 5000***g*** for 20 min. The organic layer was transferred to a screw-capped amber-colored reaction vial and evaporated in a centrifugal evaporator for 10 min. The residue was reconstituted in 50 μL of DMF and then introduced into the labeling reaction as previously described; 5 μL was injected into the HPLC system.

RESULTS AND DISCUSSION

A typical chromatogram obtained for standard solution of HZ and CZ with haloperidol as IS is shown in Fig. 2. The DPA derivatives of both drugs as well as the IS can be separated on a silica column with aqueous–organic mobile phase. Retention times were 10.5, 13 and 17 min for HZ, IS and CZ, respectively.

Optimization of labeling reaction

Different factors affect the reaction of these aryl chloride containing drugs with DPA. The concentration of DPA was studied in the range 2.5–15 mM and the concentration selected was 12.5 mM, as shown in Fig. 3. Different solvents were also tried for derivatization reactions, such as DMF, dioxane, tetrahydrofuran, acetonitrile, isopropyl alcohol and acetone, and DMF was selected as the optimum solvent for this labeling reaction.

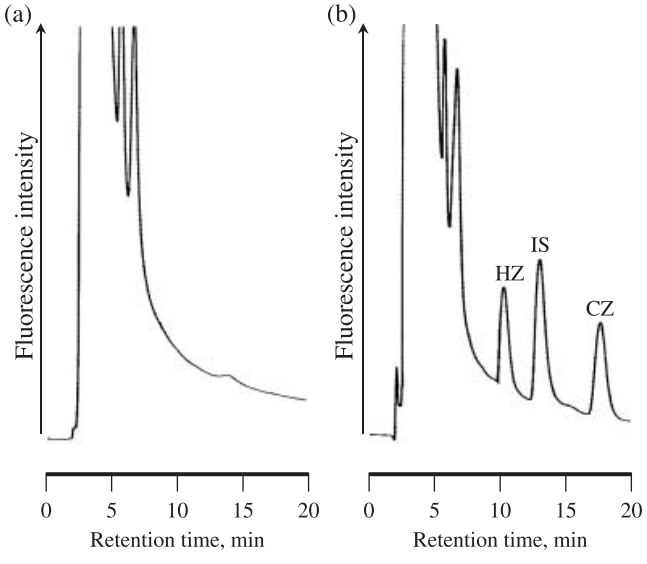

Figure 2. Chromatograms for (a) reagent blank and (b) standard solution of HZ (5 μM), CZ (5 μM) and IS (10 μM). Labeling conditions are described in the Experimental section.

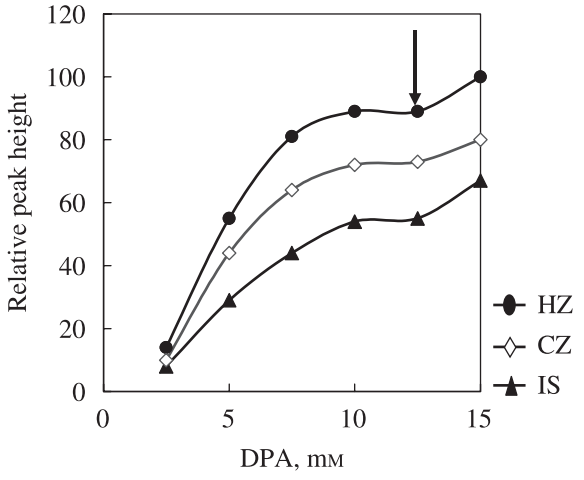

Figure 3. Effect of the concentration of DPA on the reactivity; labeling conditions as described in the Experimental section except for the concentration of DPA. Sample concentrations: HZ, 20 μM, CZ, 20 μM and IS, 10 μM.

The effect of different types of bases such as Na_2CO_3, potassium hydroxide (KOH), potassium fluoride (KF), sodium hydroxide (NaOH), trisodium phosphate (Na_3PO_4) on reactivity was investigated. Among the examined bases, Na_2CO_3 was selected because it gave reasonable peak height. The concentration of the Na_2CO_3 was changed over a range from 10 to 50 mM and 20 mM was selected, as shown in Fig. 4. Also the concentration of $Pd(OAc)_2$ catalyst was studied over a range of 1–5 mM and a concentration of 4 mM gave the best results in a

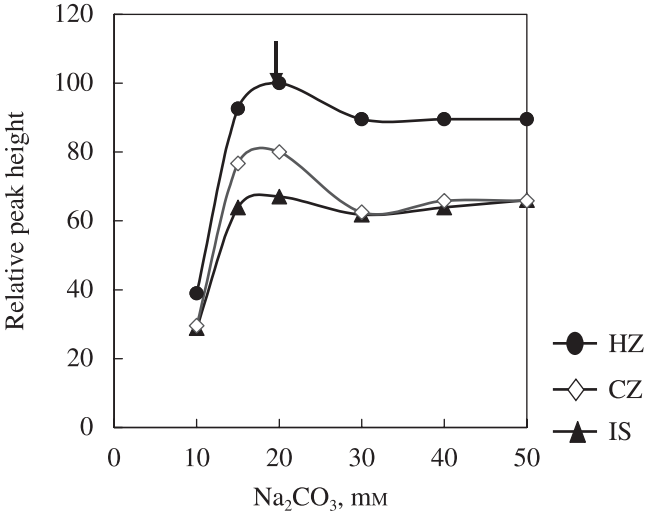

Figure 4. Effect of the concentration of Na_2CO_3 on the reactivity, labeling conditions as described in the Experimental section except for the concentration of Na_2CO_3. Sample concentrations: HZ, 20 μM, CZ, 20 μM and IS, 10 μM.

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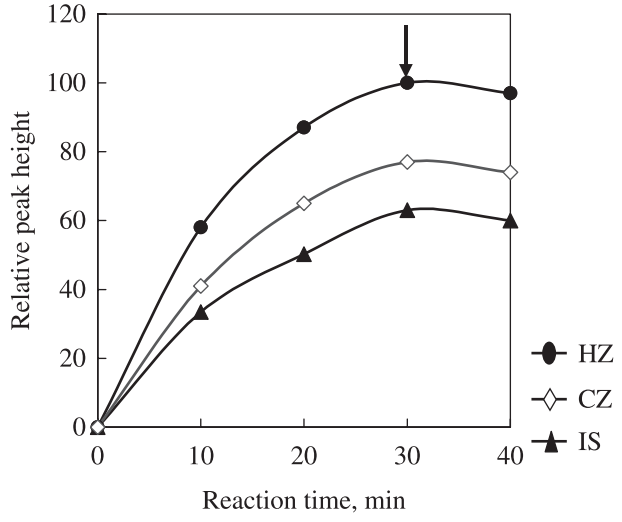

Figure 5. Effect of the reaction time on the reactivity, labeling conditions mentioned in the Experimental section except for the reaction time. Sample concentrations: HZ, 20 μM, CZ, 20 μM and IS, 10 μM.

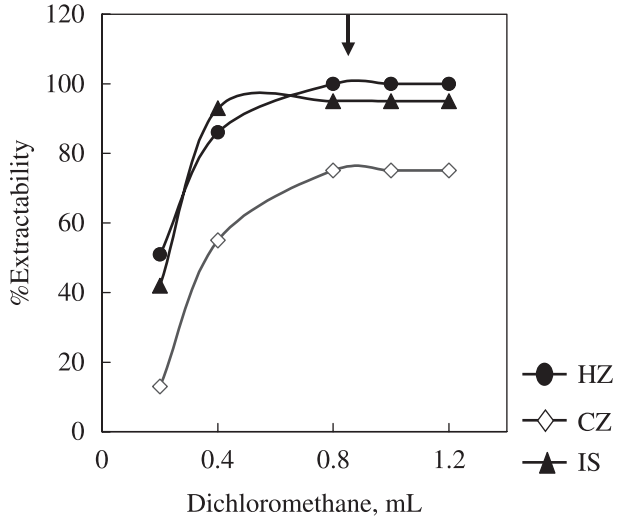

Figure 6. Effect of volume of dichloromethane on the extractability. Sample concentrations: human serum spiked with IS, 3.76 μg/mL; standard HZ, 0.50 μg/mL; and CZ, 0.50 μg/mL.

mixture with DtPB as ligand at a concentration of 2.5 mM; two types of phosphine DCPB and DtPB were tried but the latter gave the higher response. The effect of water content was studied over the range from 15 to 45%, giving the highest peak height at 25%. Reaction time was also studied, as shown in Fig. 5; we selected 30 min as the optimum reaction time.

Extraction from serum

Different solvents were tried for extraction of both drugs as well as the IS from sera such as ethyl acetate, isopropyl alcohol, chloroform, acetonitrile and dichloromethane. Dichloromethane gave the maximum extractability and, by trying different volumes, the optimum extraction was obtained using 0.8 mL, as shown in Fig. 6, with percentage extractabilities of 95, 75 and 96% for HZ, CZ and IS, respectively.

The method is highly selective with no interference from the serum, as shown in Fig. 7, which represents the chromatogram of spiked serum sample compared with blank (drug-free) serum.

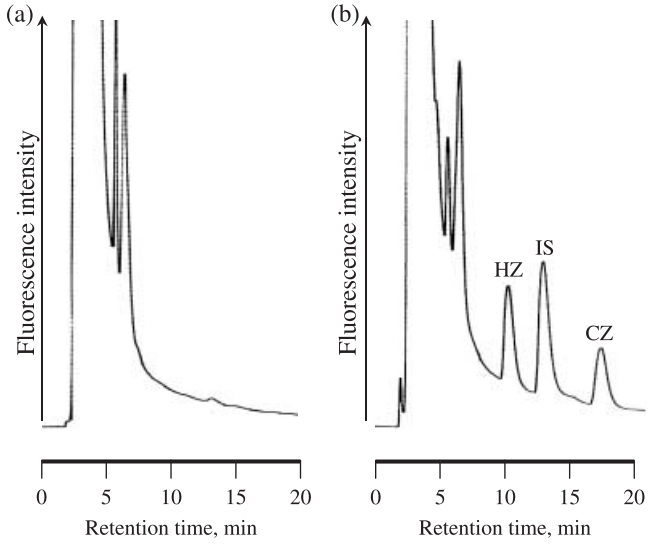

Figure 7. Chromatograms for extract from (a) human serum and (b) human serum spiked with IS (3.76 μg/mL), standard HZ (0.50 μg/mL) and CZ (0.50 μg/mL).

Validation of the method in human serum

The calibration curves, calibration ranges and detection limits are listed in Table 1. The calibration curve of HZ in serum was linear over the range 0.025–2.00 μg/mL with $r^2 = 0.999$ and that for CZ was linear over the range 0.025–2.00 μg/mL with $r^2 = 0.998$ and the lowest concentrations of these ranges represent the limits of quantitation. The proposed method can be applied to

Table 1. Calibration curves of HZ and CZ in human serum

Compound	Calibration curve[a]	Range, μg/mL	Linearity, r^2	Detection limit[b], ng/mL
HZ	$Y = 1.19X + 0.01$	0.025–2.00	0.999	10
CZ	$Y = 0.90X + 0.01$	0.025–2.00	0.998	5

[a] Y = peak height ratio; X = sample concentration (μg/mL).
[b] S/N = 3.

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Table 2. Method repeatability in human serum

Compound	Concentration, μg/mL	Precision (RSD, %)	
		Within-day (n = 5)	Between-day (n = 5)
HZ	0.15	5.1	
	0.50	1.4	4.0
	0.80	2.4	
CZ	0.15	7.3	
	0.50	6.0	9.6
	0.80	4.9	

the clinical and pharmacokinetic studies because the therapeutic levels of HZ and CZ in human are reported to be 0.09 and 1.45 μg/mL, respectively (Gergov *et al*., 2001).

The method is highly sensitive with limits of detection of 10 and 5 ng/mL for HZ and CZ, respectively, as shown in Table 1. The sensitivity of the method for CZ was 2–50 times higher than that of HPLC-UV method (Macek *et al*., 1999; Moncrieff, 1992; Nagaralli *et al*., 2003; Rosseel and Lefebvre, 1991), 10 times higher than that of TLC (Pandya *et al*., 1996) and comparable to HPLC-MS/MS (Eriksen *et al*., 2002). Although the sensitivity for HZ is almost the same as those of HPLC-UV (Pehourcq, 2004) and HPLC-MS/MS (Gergov *et al*., 2003), these methods require 1 mL of sample volume while the proposed method requires 200 μL.

Within-day precision was investigated by five-fold assay of mixtures of both drugs at three concentrations (0.15, 0.50 and 0.80 μg/mL) and the %RSDs were less than 7.3%, as shown in Table 2. Between-day precision was studied by repeating measurement five times at a concentration of 0.50 μg/mL of HZ and CZ in serum and the %RSDs for HZ and CZ were 4.0 and 9.6%, respectively.

CONCLUSION

HZ and CZ could be converted to highly fluorescent compounds by the proposed labeling reaction and detected sensitively. The developed method allowed a selective determination of HZ and CZ in human serum without any interfering peak. This method should be useful for pharmacokinetic study of these drugs in human.

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