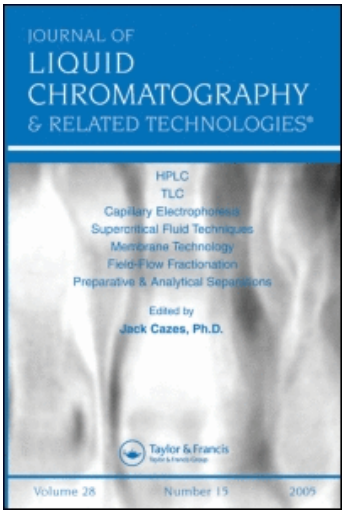

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FLUOROMETRIC DETERMINATION OF INDOMETHACIN IN SERUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING IN-LINE OXIDATION WITH HYDROGEN PEROXIDE

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ABSTRACT

A fluorometric method has been developed for the determination of indomethacin in serum by high performance liquid chromatography. Indomethacin after deproteinization with acetonitrile was separated by reversed-phase chromatography with a neutral mobile phase (pH 7.0, 35% acetonitrile in phosphate buffer) containing hydrogen peroxide as a fluorogenic reagent, and was fluorometrically detected (Ex 358nm and Em 462nm) via in-line oxidation at high temperature (180 °C). The calibration curve was linear over the range of 2.5-15.0μg/ml by injecting a volume of 20μl of deproteinized serum. The detection limit (signal-to-noise ratio=3) for indomethacin in serum was 0.5μg/ml using a 20-μl aliquot of deproteinized serum.

INTRODUCTION

Indomethacin (IDM) is a non-steroidal anti-inflammatory drug that is used in the treatment of arthritic disease [1,2], patent ductus arteriosus [3,4] and idiopathic orthostatic hypotension [5]. Therefore, the determination of IDM in serum is expected to be indicative of the therapeutic effects.

A number of methods by high performance liquid chromatography (HPLC) with spectrophotometric [6-12] and fluorophotometric [13-17] detection have been described for the determination of IDM in blood . The spectrophotometric determination lacks selectivity and sensitivity. The fluorophotometric determination was performed by postcolumn alkaline hydrolysis [13-16] or by on-line oxidation using hydrogen peroxide and ultraviolet irradiation [17]. For the hydrolysis method, an additional pump is required to deliver the alkaline reagent. For the oxidation method, the photochemical reactor is necessary.

This report describes a HPLC method using the neutral mobile phase containing hydrogen peroxide. IDM was separated by reversed-phase chromatography using an Inertsil ODS-2 column and the effluent from the column was subjected to in-line oxidation with hydrogen peroxide at high temperature.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Model 501G solvent delivery pump (Waters Chromatography Division of Millipore, Milford, MA,

USA), a Model U6K injector (Waters), and a SOMA S-3810 reaction system (Soma Optics, Tokyo, Japan) equipped with a stainless-steel reaction coil (15m x 0.5mm i.d.) in a heating bath (180°C) and a stainless-steel coil (3m x 0.5mm i.d.) in a cooling box (15°C). The Inertsil ODS-2 column (5μm,150mm x 4.6mm i.d., GL Sciences, Tokyo, Japan) was used for separation of IDM. A SOMA S-3350 FL fluorometer (Soma Optics) was used for detection at an excitation wavelength of 358nm and an emission wavelength of 462nm. A PTEE tube (10m x 0.25mm i.d.) was connected to the detector as a pressure restrictor. The detection signal was recorded with a HITACHI Recorder 056 (Hitachi, Ltd., Tokyo, Japan).

Reagents and Chemicals

IDM was purchased from Sigma (St. Louis, MO, USA). Water and acetonitrile used were of liquid chromatographic grade. All other chemicals were of reagent grade.

The mobile phase was prepared to contain 30mM hydrogen peroxide and 35% acetonitrile in 25mM phosphate buffer, adjusted to pH7.0 with 1N sodium hydroxide.

Sample Preparation

Twenty μl of serum in a 1.5ml tapered polypropylene tube was vortex-mixed with 20μl of acetonitrile for a few seconds. The mixture was centrifuged at 10,000 G for 2min, and a 20μl aliquot of the supernatant was injected into the chromatograph. Standard sera supplemented with various known amounts of IDM (2.5-15μg/ml) were prepared and analyzed. Peak height measurements were performed to construct the calibration curve. Every serum was analyzed in duplicate and the results were averaged.

Optimization of the Reaction

The optimum reaction conditions for the in-line oxidation with hydrogen peroxide were studied by injecting 20μl of a standard solution containing IDM (5.0μg/ml). The flow rate of the mobile phase was 1.0ml/min.

RESULTS AND DISCUSSION

The basis of this method was the formation of a fluorophor by in-line oxidation with hydrogen peroxide at high temperature. The excitation and the emission maximum of the fluorophor of IDM in the heat treated effluent were at 358nm and 462nm, respectively. These data agreed well with those reported by Mawatari et al. using hydrogen peroxide and photooxidation[17]. Therefore, these wavelengths were used for detection.

Figure 1 shows the effect of pH of mobile phase containing 20mM hydrogen peroxide at temperature of 100°C on the fluorescence intensity. The maximum fluorescence intensity occurred at pH7.0. An mobile phase of pH higher than 7.0 can not be used for octadecylsilica columns for a long period of time because it would cause a deterioration of column stability. And hydrogen peroxide is unstable in the mobile phase. Therefore, the mobile phase of pH 7.0 was used.

The effect of reaction temperature was studied in the range of 130°C to 200°C (Figure 2). The fluorescence intensity increased gradually up to the temperature of 180°C followed by a decrease at 200°C. The optimal final temperature was chosen 180°C. Connection of

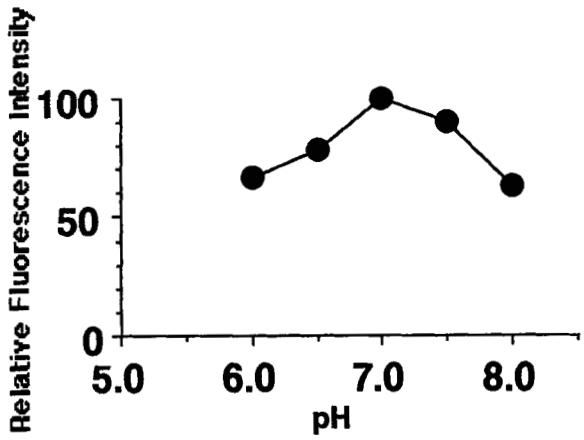

FIGURE 1
Effect of pH of mobile phase on the fluorescence intensity using the mobile phase containing 20mM hydrogen peroxide at 100°C.

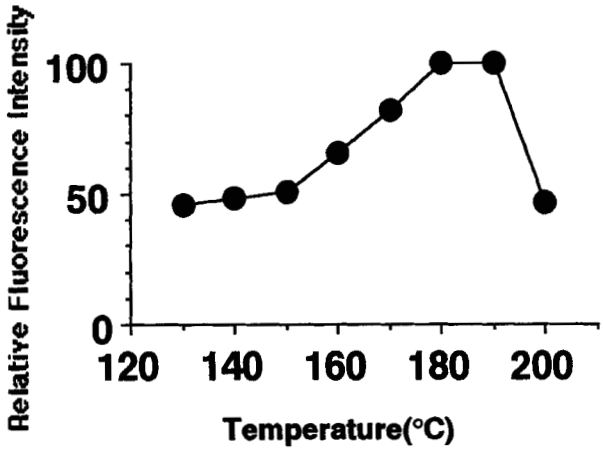

FIGURE 2
Effect of reaction temperature on the fluorescence intensity using the mobile phase containing 20mM hydrogen peroxide at pH 7.0.

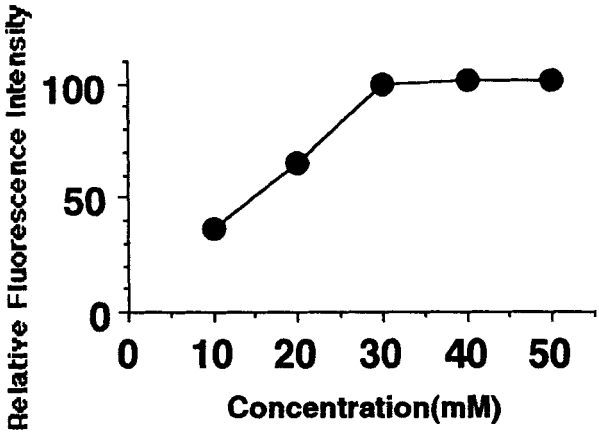

FIGURE 3
Effect of hydrogen peroxide concentration on the fluorescence intensity using the mobile phase of pH 7.0 at 180°C.

the pressure restrictor to the detector outlet was necessary because, without the restrictor, bubbles would form in the detector cell due to the high temperature.

Figure 3 shows the effect of hydrogen peroxide concentration on the fluorescence intensity. The fluorescence intensity increased gradually up to the concentration of 30mM and appeared to plateau between 30mM and 50mM. Therefore, the concentration of hydrogen peroxide was used 30mM in the mobile phase. The hydrogen peroxide in the mobile phase did not influence the separation of IDM.

Reaction coils (0.5mm I.d.) of various lengths were studied to give optimal fluorescence intensity for IDM. Figure 4 shows the effect of the coils on the fluorescence intensity. The fluorescence intensity increased up to the length of 15m. Therefore, the coil of the length of 15m was used. The residence time of IDM in the reaction coil is 3 min.

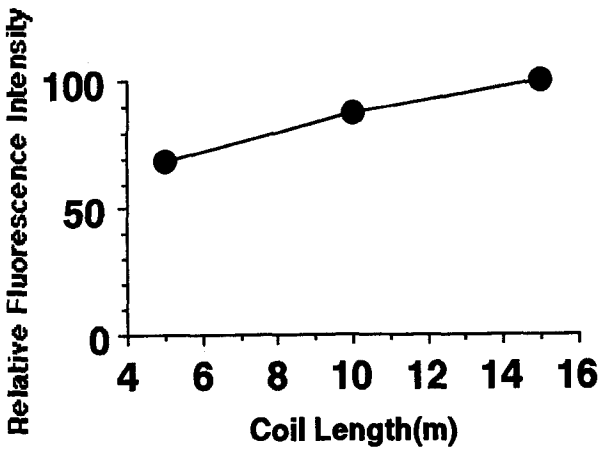

FIGURE 4
Effect of reaction coil length on the fluorescence intensity using the mobile phase of pH 7.0 containing 30mM hydrogen peroxide at 180°C.

A linear regression analysis of the calibration curve obtained from the standard sera (2.5-15μg/ml) yielded the equation, Y=0.047X + 0.108 (r=0.999). A high linearity was obtained between peak heights(Y) and IDM concentration(X) in serum, even though no internal standard was used. Within-run and day-to-day precision were obtained from two serum pools containing IDM, 1.0μg/ml and 5.0μg/ml. The relative standard deviation values of within-run (n=10) for 1.0μg/ml and 5.0μg/ml were 4.56% and 2.12%, respectively. Those of day-to-day (n=6) for 1.0μg/ml and 5.0μg/mil were 3.05% and 2.10%, respectively. The recovery of IDM from serum was determined by comparison of the peak height of spiked serum (1.0μg/ml) and that of spiked water (1.0μg/ml) using the same sample preparation. The recovery of IDM was 95.0%.

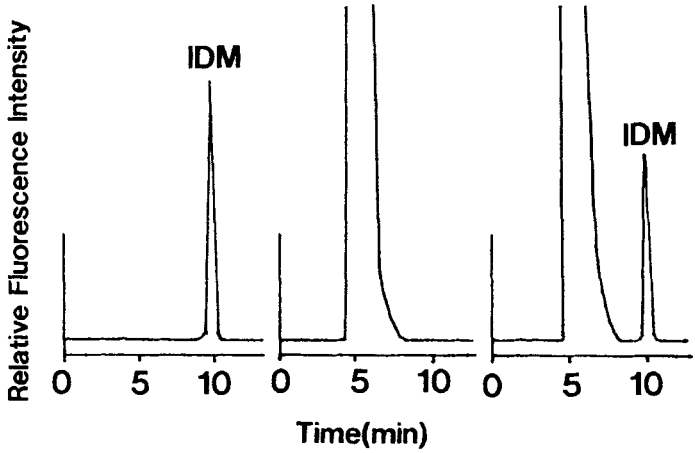

FIGURE 5
Chromatograms obtained from (A) standard solution; (B) indomethacin-free serum; (C) volunteer serum.
IDM:Indomethacin, (Conditions see **Experimental**)

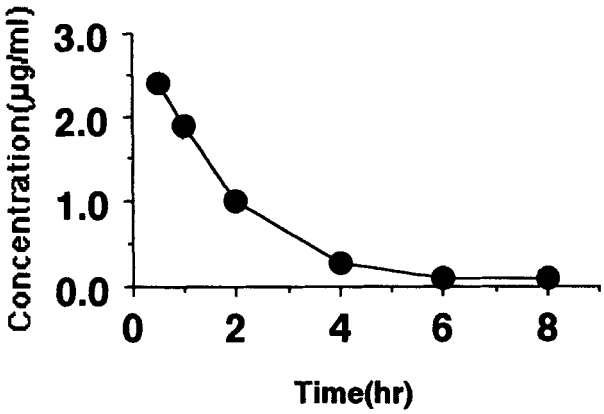

FIGURE 6
Serum concentration-time curve for indomethacin after oral administration of a 25mg capsule.
Volunteer:48 yrs., Male, 66kg.

The detection limit (signal-to-noise ratio=3) was determined to be 0.5μg/ml using a 20-μl aliquot of deproteinized serum. If necessary, the sensitivity can be heightened by increasing the injection volume.

Figure 5 shows typical chromatograms obtained from the standard solution of IDM, IDM-free serum, and volunteer serum. IDM-free serum shows no peaks that would interfere with determination of IDM. The retention time of IDM was 10mim.

Figure 6 shows a serum concentration-time profile following oral administration of an IDM capsule (25-mg). The concentration-time curve rose up immediately after the administration. The curve suggests that the volunteer corresponding to it showed rapid gastric absorption of IDM.

This method is sensitive and specific enough to estimate IDM in serum and can be used for routine therapeutic monitoring of IDM in serum. This method, using the in-line oxidation with hydrogen peroxide at high temperature, could be used also for the determination of other compounds that are oxidized with hydrogen peroxide.

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