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FLUORIMETRIC DETERMINATION OF INDOMETHACIN IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH POST-COLUMN PHOTOCHEMICAL REACTION WITH HYDROGEN PEROXIDE

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SUMMARY

A high-performance liquid chromatographic method involving post-column photochemical reaction and fluorimetric detection has been developed for the determination of indomethacin in serum. The bioanalytical set-up consisted of single-pump system for post-column photochemical reaction. The mobile phase consisted of 0.07 *M* phosphate buffer (pH 6.6)–acetonitrile (65:35, v/v) containing 180 m*M* hydrogen peroxide. The column effluent was irradiated with UV light to give fluorescence. The fluorimetric detection was monitored with excitation at 358 nm and emission at 462 nm. The calibration curve was linear over the range 0.05–30 μg/ml by injecting a volume of 10 μl of indomethacin solution. The detection limit (signal-to-noise ratio = 5) for indomethacin was 10 ng/ml using a 100-μl aliquot of deproteinized serum. The mean recovery from serum was 94.3%.

INTRODUCTION

Indomethacin (IDM) is a widely used non-steroidal anti-inflammatory agent [1,2]. IDM has also been used in the treatment of patent ductus arteriosus [3,4] and idiopathic orthostatic hypotension [5]. Consequently, the serum IDM level in biological samples is expected to be indicative of the therapeutic efficacy. A number of procedures have already been described for the measurement of IDM in biological fluids, including a radioimmunoassay [6], spectrophotometric [7], fluorimetric [8], thin-layer chromatographic [9], gas chromatographic [10] and gas chromatographic–mass spectrometric [11] methods. High-performance liquid chromatography (HPLC) has been reported, with

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spectrophotometric [12–16] and fluorimetric [17–20] detectors. The fluorimetric determination was performed with post-column alkaline hydrolysis of IDM using sodium hydroxide, thereby enabling fluorescence detection. However, the spectrophotometric detector lacks selectivity, and fluorimetric determination suffers from difficulties of maintaining the delivery of the alkaline reagent with a pump system.

In the present study, we found the reaction of IDM with hydrogen peroxide and acetonitrile to give fluorescence on irradiation with UV light. The photochemical reaction was coupled with HPLC post-column reaction on the single pump to achieve the convenient determination of serum IDM.

EXPERIMENTAL

Chemicals

Indomethacin was purchased from Sigma (St. Louis, MO, U.S.A.). Capsules of indomethacin (Idomethine) were obtained from Kouwa (Aichi, Japan) and contained 25 mg of indomethacin. Pooled serum (Consera) was obtained from Nissui Seiyaku (Tokyo, Japan). All other chemicals were purchased from Wako (Osaka, Japan).

Apparatus

Fig. 1 shows the reversed-phase HPLC system for the post-column photochemical reaction. It consisted of a Sanuki SSP pump (Model DM-3U 2047, Sanuki, Tokyo, Japan), a sample injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) fitted with a 100-μl loop, a stainless-steel column (150 mm×4.6 mm I.D.) packed with Unisil Q C_{18} (particle size 5 μm, Gasukuro, Tokyo, Japan), operated at room temperature. A Model RF-530 fluorescence HPLC monitor (Shimadzu, Kyoto, Japan) with a 12-μl flow-cell and a Shimadzu Chromatopac C-R3A data processor, recorder–integrator, were used. The photochemical reaction was carried out in a PTFE tube (7 m×0.5 mm I.D.×1.5 mm O.D.; Gasukuro), which was wound round a black light (Model FL-15BL, Nippon Denki, Tokyo, Japan). The black light emits characteristic wave-

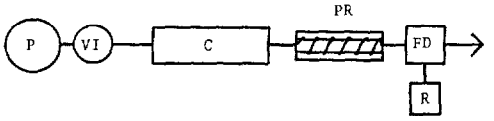

Fig. 1. Flow diagram of the HPLC system for photochemical detection of indomethacin. P = high-pressure pump, 0.07 *M* phosphate buffer (pH 6.6)–acetonitrile (65: 35, v/v) containing 180 m*M* hydrogen peroxide at a flow-rate of 1.0 ml/min; VI = valve injector; C = separation column (150 mm×4.6 mm I.D.) packed with Unisil Q C_{18} (particle size 5 μm); PR = photochemical reaction coil (PTFE tube, 7 m×0.5 mm I.D.×1.5 mm O.D.); light source, FL-15BL black light; FD = fluorescence detector (excitation 358 nm, emission 462 nm); R = recorder and integrator.

lengths between 300 and 400 nm, and has an electric power of 15 W. Though IDM was not very reactive at ca. 254 nm, it was liable to react at ca. 365 nm. Consequently, the black light was used as a light source.

Chromatography

The mobile phase, which consisted of phosphate buffer, pH 6.6 (600 ml of 0.07 *M* potassium dihydrogenphosphate and 400 ml of 0.07 *M* disodium hydrogen phosphate)–acetonitrile (65:35, v/v) containing 180 m*M* hydrogen peroxide, was delivered at a flow-rate of 1.0 ml/min. Chromatography was performed at ambient temperature. The fluorescence was measured at excitation and emission wavelengths of 358 and 462 nm, respectively. The retention time of IDM was ca. 11 min.

Sample preparation

To 100 μl of serum were added 150 μl of acetonitrile in a polymer tube (1.5 ml), and the mixture was vortex-mixed. The mixture was centrifuged at 9600 *g* for 1 min, and a 10–100 μl aliquot of the supernatant was injected into the chromatograph.

Recovery study

The effective concentration of IDM is 0.3–3 μg/ml [21]. Consequently, for the recovery determination, concentrations of 0.1, 0.5, 1.0, 2.5 and 10.0 μg/ml

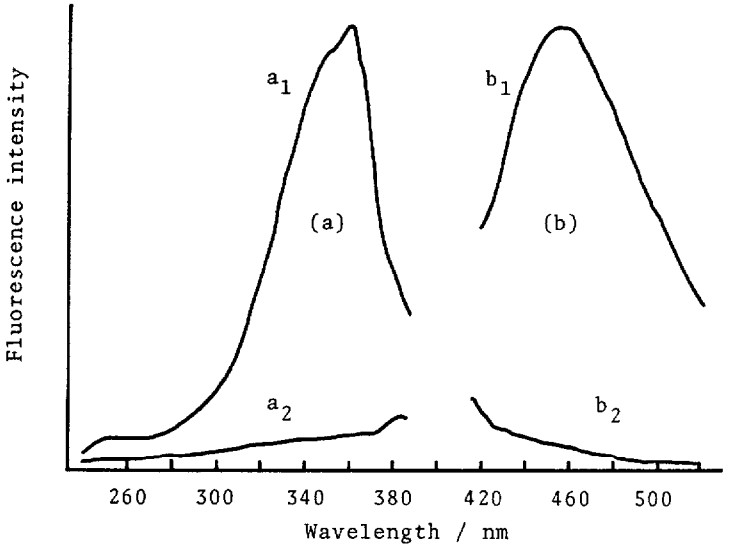

Fig. 2. Fluorescence excitation (a) and emission (b) spectra of indomethacin obtained by means of photochemical reaction with hydrogen peroxide. a_1 and b_1: 1.5 μg/ml indomethacin; a_2 and b_2: sample blanks for a_1 and b_1, respectively.

for IDM were spiked in blank serum (Consera), and run through the procedure as described for deproteinized serum. The peak-heights obtained for the deproteinized samples were compared with those of standards of IDM.

RESULTS AND DISCUSSION

Fig. 2 shows the excitation (a) and emission (b) spectra of IDM in the presence of hydrogen peroxide exposed to UV light. The excitation and the emis-

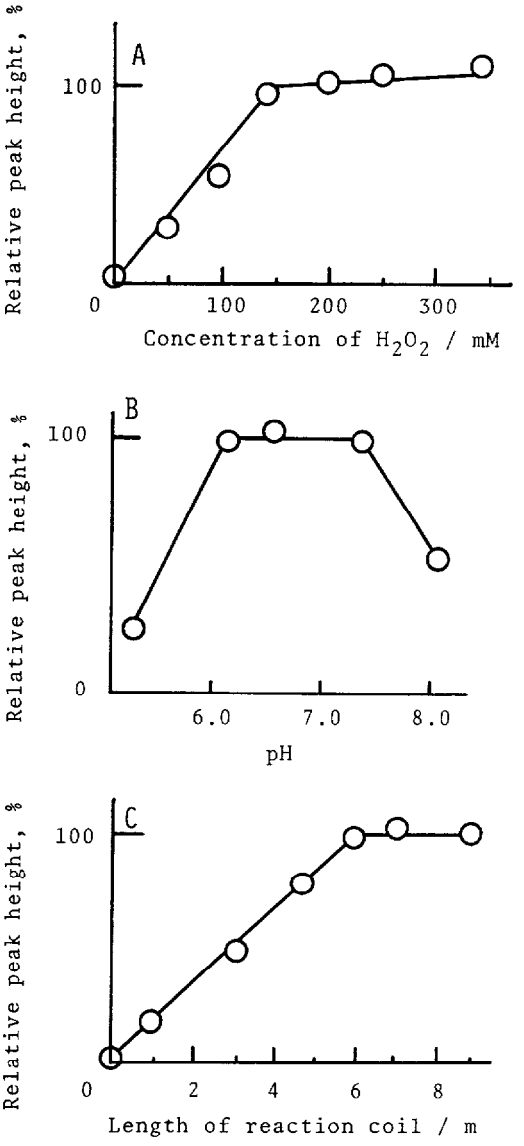

Fig. 3.

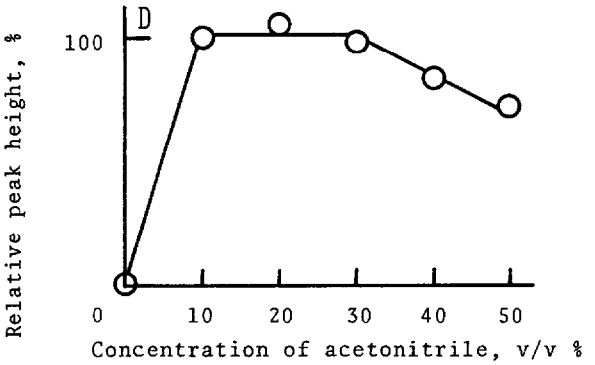

Fig. 3. (A) Effect of hydrogen peroxide concentration on the peak height. (B) Effect of pH on the peak height. (C) Effect of length of PTFE tube on the peak height. (D) Effect of acetonitrile concentration on the peak height. The photochemical reaction was carried out as described in Fig. 1. The amount of indomethacin was 50 ng.

sion maximum were at 358 and 462 nm, respectively. In order to confirm a fluorophore, the products were developed with a system of Søndergaard and Steiness [9] and two spots were confirmed. The R_F values were 0.76 (green–blue colour) and 0.17 (white–blue colour). The fluorescence colour and the R_F values of the fluorophores differed between the present method and the alkaline hydrolysis method.

Fig. 3A shows the effect of the hydrogen peroxide concentration on the fluorescence intensity, the maximum fluorescence being observed at a concentration of 180 m*M*. The addition of hydrogen peroxide to the mobile phase did not affect the separation of IDM. Fig. 3B shows the effect of the pH of mobile phase on the fluorescence intensity. The optimum pH range was 6.2–7.5, and pH 6.6 was adopted. Fig. 3C shows the effect of the length of the PTFE tube around the 15-W black light. The best results were obtained with a tube of length 7 m. The residence time of IDM in the photochemical reactor is 1.4 min.

Methanol, ethanol, 1-propanol, 2-propanol and acetonitrile were examined as organic solvents in the mobile phase. The mobile phase containing acetonitrile increased the fluorescence intensity of IDM. This finding indicated that acetonitrile is the important factor in the photochemical reaction of IDM. Fig. 3D shows the effect of the acetonitrile concentration on the fluorescence intensity. The optimum concentration of acetonitrile was 10–30% (v/v), but in order to use the same solvent for the chromatographic analysis and the on-line photochemical reaction, a concentration of 35% (v/v) was adopted.

The calibration graph was linear over the range 0.05–30 μg/ml by injecting 10 μl of IDM solution. If necessary, the sensitivity can be increased by enlarging the injection volume. The correlation coefficient was 0.9998, the slope 11.56 and the intercept −2.10. The relative standard deviation of standard IDM was 3.2% at 0.5 μg/ml ($n=8$) and 2.1% at 5.1 μg/ml ($n=11$) by injecting a volume

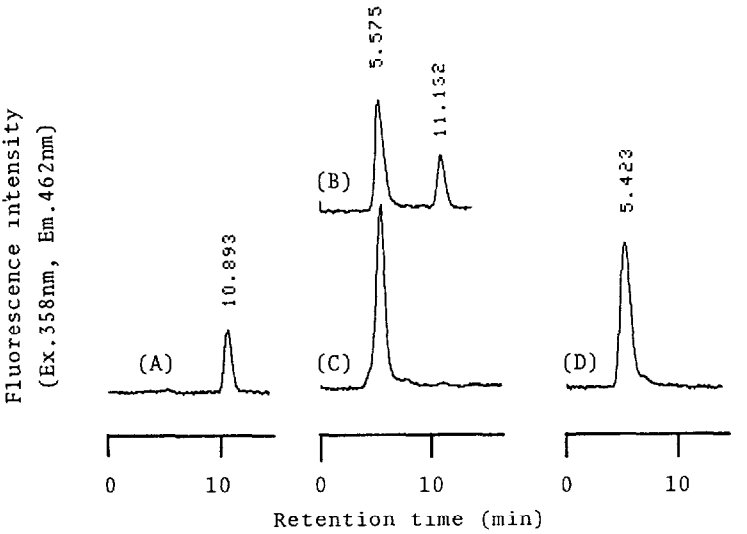

Fig. 4. Chromatograms of indomethacin and serum. (A) UV irradiation of standard indomethacin; (B) irradiation of indomethacin added to blank serum; (C) irradiation of blank serum; (D) same sample as in B, but without irradiation. HPLC conditions as in Fig. 1; amount of standard indomethacin, 10 ng.

TABLE I

RECOVERY OF INDOMETHACIN FROM SERUM

See Experimental for recovery study; HPLC conditions as in Fig. 1.

Concentration added (μg/ml)	Recovery (mean ± S.D.) (%)	n
0.1	93.6 ± 4.6	5
0.5	94.7 ± 3.2	8
1.0	92.6 ± 3.4	6
2.5	94.5 ± 3.8	6
10.0	96.2 ± 2.4	8
Mean recovery	94.3	

of 10 μl. The detection limit (signal-to-noise ratio = 5) was determined to be 10 ng/ml using a 100-μl aliquot of deproteinized serum.

Fig. 4 compares the chromatograms of (A) irradiated standard IDM, (B) irradiated IDM added to blank serum, (C) irradiated blank serum, and (D) non-irradiated IDM in blank serum. Chromatogram B shows a sharp peak with a retention time identical with that of the standard IDM, whereas the chromatogram of the non-irradiated sample (D) shows no peak at this position. Comparison of the chromatograms demonstrates the specificity of the present

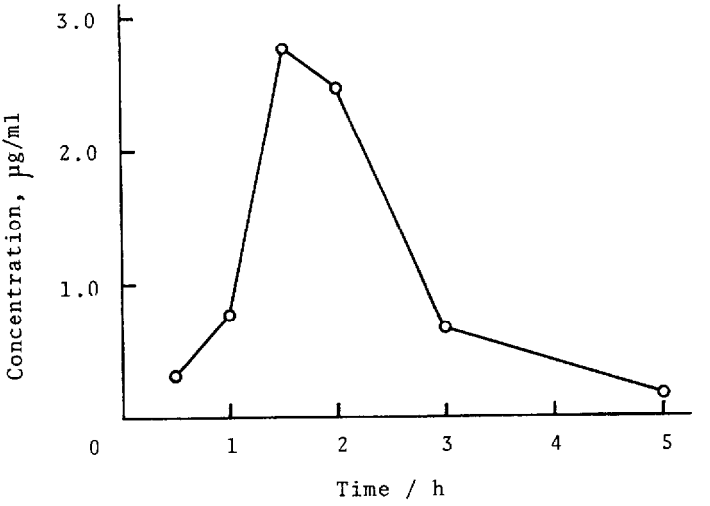

Fig. 5. Serum concentration curve following oral administration to a normal man of indomethacin (two 25-mg capsules) with water after fasting overnight. The determination was carried out as described in Experimental.

method. In addition, several compounds such as tryptophan, kynurenine, kynurenic acid, xanthurenic acid, melatonin, tyrosine, histidine, glucose, pyridoxine and ascorbic acid do not interfere with the analysis.

Table 1 summarizes the results of the recovery from serum. The mean recovery, all points on the spiked serum, was more than 92.6%. The mean recovery was 94.3%.

The serum concentration curve of IDM in a man is given in Fig. 5. The peak serum IDM concentration was obtained 1.5 h after oral administration. This observation is similar to that presented by Turakka and Airaksinen [22].

In conclusion, the present photochemical reactor is very simple and easily assembled. In addition, hydrogen peroxide and acetonitrile, the reagent for post-column derivatization, can be previously added in the mobile phase. The post-column reaction can be carried out only by UV irradiation, and the pump for the post-column derivatization could be omitted. This method is sensitive and specific enough to estimate IDM in human serum and is expected to be useful in therapeutic drug monitoring.

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