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DETERMINATION OF THE α,β-ADRENOCEPTOR BLOCKER YM-09538 IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic method for the determination of the α,β-adrenoceptor blocker 5-{1-hydroxy-2-[2-(*o*-methoxyphenoxy)ethylamino]ethyl}-2-methylbenzenesulphonamide hydrochloride (YM-09538) in plasma, using 5-di-*n*-butylaminonaphthalene-1-sulphonyl chloride as a reagent for fluorescence labelling, is described. The detection limit is 20 ng/ml, which is sensitive enough to determine YM-09538 plasma levels after the oral administration of effective doses to dogs and humans.

INTRODUCTION

Imai et al. [1] have introduced various phenylethylamine derivatives as combined α- and β-adrenoceptor antagonists. Among these derivatives, 5-{1-hydroxy-2-[2-(*o*-methoxyphenoxy)ethylamino]ethyl}-2-methylbenzenesulphonamide hydrochloride (YM-09538) showed dose-dependent antihypertensive effects in rats and dogs without inducing tachycardia [2] which, as a homeostatic reflex mechanism [3], generally occurs after the administration of vasodilating drugs. For pharmacological and biopharmaceutical studies on YM-09538, a simple and sensitive method for assaying its concentration in plasma is required.

In order to increase the sensitivity of high-performance liquid chromatography (HPLC), derivatization to introduce a suitable fluorophore is necessary. Of various reagents for fluorescence labelling, 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dans-Cl) has been widely used and applied in HPLC [4, 5]. A structurally related reagent, 5-di-*n*-butylaminonaphthalene-1-sulphonyl chloride (Bans-Cl) [6] has been used in combination with thin-layer chromato-

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graphy (TLC) or mass spectrometry to determine amino acids or some other amines [7—9]. In the present paper an HPLC method for determining YM-09538 concentrations in plasma using Bans-Cl is described.

EXPERIMENTAL

Chemicals

YM-09538 (Fig. 1, I) and the structurally related compound (II), which was used as the internal standard (I.S.), were synthesized in our laboratory by the method of Imai et al. [1].

Bans-Cl (Fig. 1, III) and Dans-Cl were purchased from Tokyo Kasei (Tokyo, Japan). All other reagents and solvents used were of analytical-reagent grade.

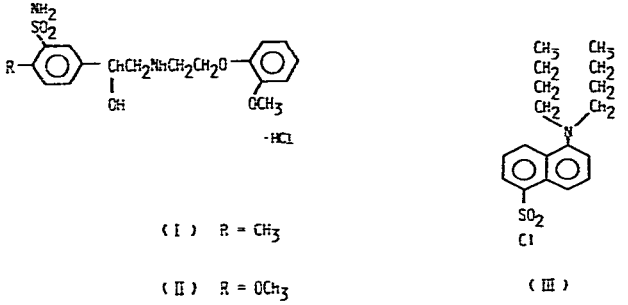


Fig. 1. Chemical structure of YM-09538 (I), I.S. (II) and Bans-Cl (III).

HPLC

The chromatograph used for HPLC consisted of a TWINCLE high-pressure pump (Jasco, Tokyo, Japan), a Model U6K injector (Waters Assoc., Milford, MA, U.S.A.) and a stainless-steel column (15 cm × 4 mm I.D.) packed with LiChrosorb SI-60 (particle size 5 μm) (Merck Japan, Tokyo, Japan). Fluorescence was measured at 500 nm with the 365-nm line of a medium-pressure mercury lamp, using a Model FP-110 spectrofluorimeter (Jasco). The mobile phase was benzene—methanol (50:1); the flow-rate was 2 ml/min. When many samples were analysed, a WISP 710A automatic sampler (Waters Assoc.) was employed for sample injections.

Assay procedure

To 1 ml of plasma were added 2 ml of an aqueous solution containing 1 μg of I.S. and then about 0.5 g solid $NaHCO_3$. The mixture was extracted with 4 ml of ethyl acetate, centrifuged and the ethyl acetate layer was evaporated to dryness under reduced pressure. To the residue were added 0.1 ml of an aqueous solution containing 300 μg sodium hydrogen carbonate and 0.2 ml of acetone containing 100 μg of Bans-Cl. After 90 min at 45°C, 4 ml diethyl ether were added, the mixture was washed with 3 ml of distilled water for 10 sec and the diethyl ether layer was evaporated to dryness at 40—50°C. The residue was dissolved in 0.1 ml of benzene and 5—10 μl of the solution were injected into the HPLC system.

Calibration was effected by applying the procedure to prepared plasma stan-

dards containing various concentrations of YM-09538 (20—1000 ng).

Physical properties and reaction conditions for the YM-09538 Bans derivative

YM-09538 (2 mg) was subjected to reaction with Bans-Cl as described above and the derivative was purified on a 100-mesh silica gel column (10 cm × 1 cm I.D.). Elution was performed with benzene—methanol (20:1).

Excitation and emission spectra of the derivative, dissolved in the HPLC mobile phase, were obtained on a Hitachi MPF-4 fluorescence spectrophotometer equipped with a xenon lamp. The slits were adjusted to 5.5 nm for both excitation and emission; the scanning speed was 20 nm/min. To confirm the structure of the fluorescent compound, we used a Hitachi RMU-6M mass spectrometer at an emission current of 40 μA, an electron energy of 20 eV, an ion source temperature of 170°C and an accelerating voltage of 1.3 kV.

The optimal derivatization time and temperature were evaluated as follows. Plasma extracts, each containing 500 ng of YM-09538, were dissolved in the reaction medium described under Assay procedure and incubated at 60°C, 45°C or room temperature (about 15°C). After appropriate intervals, each sample was admixed with 4 ml of diethyl ether, 0.5 ml of ethyl acetate containing 500 ng of the I.S. Bans derivative and 3 ml of distilled water, and then centrifuged. The organic layer was analysed by HPLC as described above and the peak-height ratios were plotted against incubation time.

Animal and human studies

After an overnight fast, four male beagles, weighing 11.5—12.5 kg, received 3 mg/kg of YM-09538 as a 0.3 mg/ml aqueous solution via a stomach tube. In the human study, three male volunteers, 24—35 years of age, received 50-mg YM-09538 tablets orally after overnight fasting. After appropriate intervals, blood samples were collected by venipuncture, using heparinized syringes, centrifuged and the plasma was stored frozen until taken for assay.

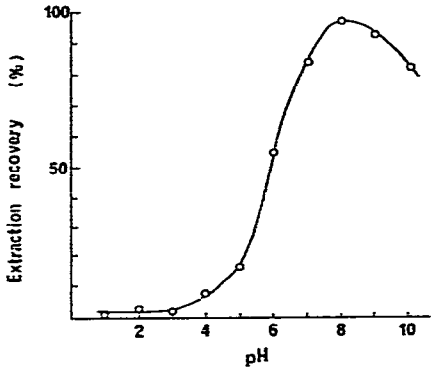
RESULTS AND DISCUSSION

Extraction of YM-09538 from plasma

The extraction of YM-09538 from plasma, using ethyl acetate, was pH dependent. At pH 7.5—8.5 the extraction was efficient, but there was a marked decrease in recovery at pH values below 7 (Fig. 2). Similar extraction curves were obtained with the other organic solvents examined. Maximal recoveries obtained at pH 8.0 with diethyl ether, chloroform and *n*-hexane were 55, 70 and 40%, respectively; almost 100% of the administered drug was extracted at pH 8.0 with ethyl acetate. To simplify the present assay procedure, we added solid sodium hydrogen carbonate to obtain an aqueous alkaline solution and achieved good results. Upon saturating a mixture consisting of 1 ml of plasma and 2 ml of aqueous I.S. solution with sodium hydrogen carbonate, the pH was about 8.5.

Studies on YM-09538 Bans derivative

Mass spectrometric analysis demonstrated that the fluorescence derivative was the mono-Bans derivative of YM-09538. It showed molecular ions at *m/z*



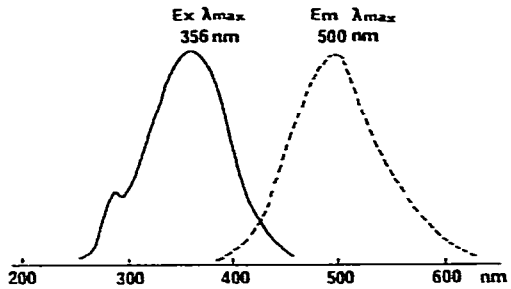


Fig. 2. Effect of pH on the extraction of YM-09538 from dog plasma, the pH of which was adjusted by adding 0.1—1.0 *N* HCl or 0.1—1.0 *N* NaOH solution. The YM-09538 extracted was quantitated by HPLC as described in the text.

Fig. 3. Excitation and emission spectra of the YM-09538 Bans derivative.

697, and the characteristic fragment ions of $(M-C_3H_7)^+$ at *m/z* 654, which had facilitated the discovery of the molecular ions in the spectra of Bans derivative mixtures of amino acids [6]. Mass fragmentation analysis (*m/z* = 213, 255, 373 and 497) confirmed that Bans was attached at the secondary amino group of YM-09538.

The excitation and emission spectra of the YM-09538 Bans derivative in the mobile phase are shown in Fig. 3. As the maximal excitation wavelength was 356 nm, the most intense emission lines of a mercury lamp at 365 nm could be used. When the maximal excitation wavelength of a compound of interest is close to one of the intense emission lines of a mercury lamp, for HPLC we advise the use of a fluorescence detector equipped with a mercury lamp rather than a xenon lamp, based on considerations related to the stability of the line and the size and cost of the instrument. In this study, fluorescence was measured at the maximal emission wavelength (500 nm).

At 45°C, the reaction of Bans-Cl with YM-09538 was quantitative within 60 min, whereas at room temperature, it was incomplete after incubation for 80 min (Fig. 4). When the mixture was incubated at 60°C, the reaction rate was higher than at 45°C, but, within 10 min the peak-height ratio reached a plateau, which was about 40% lower than at 45°C, and no further increase in the peak-height ratio was observed even on prolonging the incubation to 2 h. It appears that the reagent is decomposed rapidly at 60°C. Concomitantly, the yellow colour of the reagent disappeared on incubating the reaction medium for 10 min at 60°C. Therefore, in the analysis of plasma samples, the reaction mixtures were incubated at 45°C for 90 min to ensure complete derivatization.

HPLC

During derivative formation, Bans-Cl gives as many fluorescent side-products as Dans-Cl. With YM-09538, most of these side-products remained in the aqueous layer when the reaction mixture was shaken with diethyl ether and

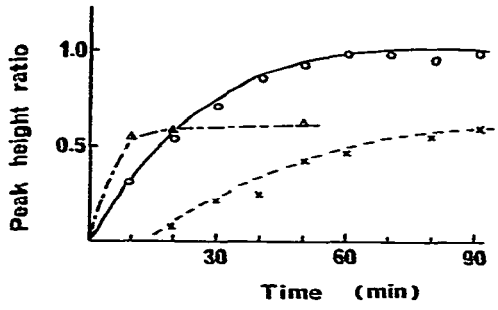


Fig. 4. Reaction of YM-09538 with Bans-Cl at 60°C (△ — · — △), at 45°C (○——○) and at room temperature (× — — — ×).

water. However, some fluorescent side-products were extracted into the organic layer and had to be separated from the YM-09538 and I.S. derivatives. HPLC proved very useful for this purpose.

The YM-09538 Bans derivative could be analysed by either normal-phase or reversed-phase chromatography. When the latter method was employed, the peak of interest was separated from interfering peaks on an RP-18 column (Merck Japan), using acetonitrile—water (1 : 1) as the eluent. However, the time required for separation exceeded 15 min and the sensitivity of this method was relatively low (limit of detection ca. 100 ng/ml).

The application of normal-phase chromatography was more favourable. The chromatograms obtained from human plasma samples are shown in Fig. 5. The Bans derivatives of YM-09538 and I.S. gave sharp peaks at retention times of 2.9 and 4.0 min, respectively; the drug-free control plasma gave no interfering peaks. When the peak-height ratios of YM-09538 and I.S. were plotted against the amount of YM-09538 added to the standard samples, a linear response was obtained over the concentration range 20—1000 ng/ml. Regression analysis of

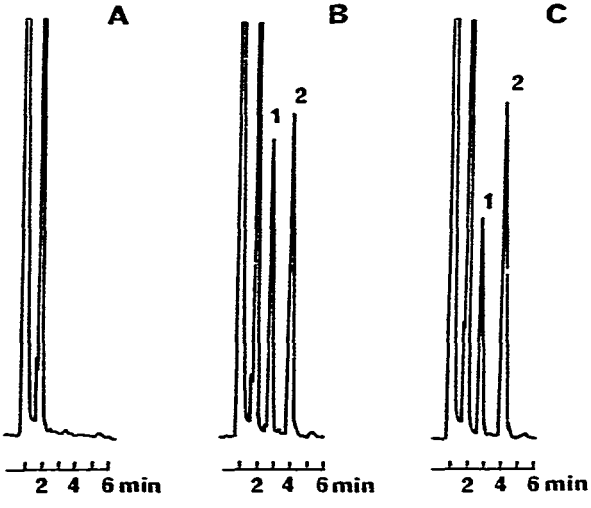


Fig. 5. Chromatograms of (A) control human plasma, (B) control plasma spiked with 0.5 μg of YM-09538 and 1 μg of I.S. and (C) plasma obtained from a volunteer 3 h after oral administration of 50 mg of YM-09538. Peaks: 1 = YM-09538; 2 = I.S.

these data gave slope 0.0018, intercept 0.023 and correlation coefficient 0.9987 (n = 18). The intra-assay coefficient of variation over the range 20—1000 ng/ml was less than 2.6% and the inter-assay coefficient of variation at 1000 ng/ml was 1.8% (n = 6).

Comparison of YM-09538 Bans and Dans derivatives

YM-09538 was subjected to reaction with Dans-Cl in a manner similar to that of YM-09538 with Bans-Cl and the natures of the two compounds were compared. The maximal excitation wavelength of the Dans derivative was 350 nm; that of the Bans derivative, at 356 nm, was closer to the line of the mercury lamp used (365 nm). This observation accords with the results of Seiler et al. [6], who pointed out the advantages of using Bans-Cl rather than Dans-Cl in the determination of various amines. The maximal emission wavelength of the Dans derivative was 505 nm.

The Dans derivative was more polar than the Bans derivative. Both compounds were subjected to HPLC and we found the Dans derivative to be eluted with a retention time of 4.1 min and the Bans derivative was eluted at 2.9 min.

Application of the method

The application of the method to determinations of plasma levels in dogs and humans is demonstrated in Fig. 6. After the oral administration of YM-09538, the plasma concentration in dogs and humans reached its maximum at 0.5 and 2 h, respectively; elimination half-lives were about 2.1 and 3.5 h, respectively. When the areas under the plasma concentration—time curves per dose were compared, the values in humans were 20—30 times higher than those in dogs.

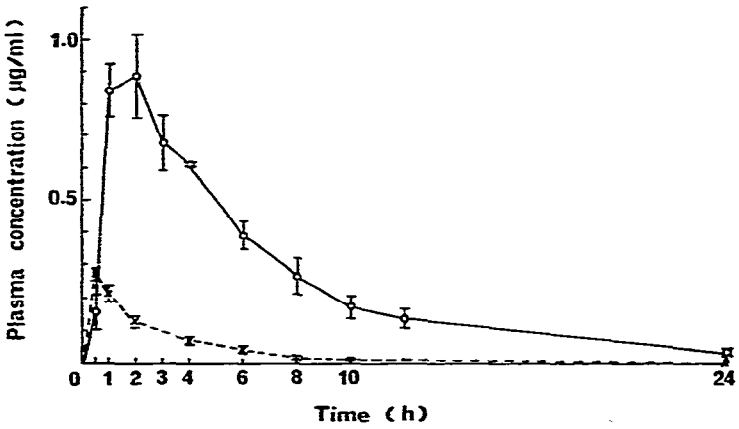


Fig. 6. Plasma concentration of YM-09538 in dogs after oral administration of 3 mg/kg (x – – – x) and in humans after oral administration of 50 mg (o———o). Each point represents the mean ± S.E.M. of 3—4 experiments.

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