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Note

High-performance liquid chromatography with fluorescence detection of dazmegrel, a specific thromboxane A_2 synthetase inhibitor

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Specific thromboxane synthetase inhibitors have been shown to selectively inhibit thromboxane A_2 (TXA_2) synthesis while maintaining or increasing the formation of other eicosanoids [1–5]. The imidazole derivative, dazmegrel, selectively inhibits TXA_2 synthesis in experimental animals [1,2] and man [3–5] without reducing prostacyclin (PGI_2) synthesis. These effects suggest that specific TXA_2 inhibition may be beneficial in conditions where vasoconstriction and platelet thrombus formation are involved, and have led to studies with dazmegrel in hypertension [6], coronary artery occlusion [7], Raynaud's syndrome [8], small-caliber graft patency [9], sepsis [10] and suppression of platelet activity [1,2,11].

Measurement of experimental drug concentrations is often useful when studying the effects of the drug [3,4] and may be particularly important in cases where high variability in response is observed [9]. In this report we are describing a rapid and sensitive method for quantifying serum concentrations of dazmegrel using high-performance liquid chromatography (HPLC) with fluorescence detection. The method is suitable for relating serum concentrations of dazmegrel and the level of TXA_2 synthesis inhibition.

EXPERIMENTAL

Apparatus

A Varian Vista Model 5500 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) was used for solvent delivery. Samples were injected with a manual 100-μl loop injector (Valco, Houston, TX, U.S.A.). Separations were performed on a

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250 mm×4.6 mm LC-18-DB column (Supelco, Bellefonte, PA, U.S.A.) which contained a base-deactivated, 5-μm C_{18} reversed-phase packing. The effluent was continuously monitored with a McPherson Model 749 variable-wavelength spectrofluorometer (McPherson, Action, MA, U.S.A.).

Reagents

Acetonitrile, methanol and water were HPLC grade. The thromboxane synthetase inhibitor dazmegrel [UK-38485; 3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid] was obtained from Pfizer (Groton, CT, U.S.A.). The chemical structure of dazmegrel is shown in Fig. 1. A stock solution was prepared by suspending 3.0 g in 25 ml of water and adding sufficient 1.0 *M* sodium hydroxide until the dazmegrel was completely dissolved. The solution was then adjusted to pH 8.5 with 1.0 *M* hydrochloric acid and diluted to 100 ml with water. The stock solution was diluted further with saline as necessary. The internal standard, 3-indolebutyric acid (3IBA), was obtained from Aldrich (Milwaukee, WI, U.S.A.). A stock solution of 3IBA was prepared in 0.1 *M* sodium hydroxide at a final concentration of 10 μg/ml. Tritium-labeled thromboxane B_2 ([^{3}H]TXB_2, 113 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.) and TXB_2 standard was generously provided by Upjohn (Kalamazoo, MI, U.S.A.). All solvents and reagents were used without further purification.

Procedure

Blood was collected in a clean glass tube and incubated at 37°C for 1 h. After centrifugation at 1500 *g* for 15 min, 350 ng of 3IBA were added to a 400-μl aliquot of serum and vortex-mixed. To precipitate proteins, 600 μl of acetonitrile were added, vortexed and allowed to stand for 1 h. The sample was centrifuged at 12 000 *g* for 5 min. The supernatant was removed and evaporated to one fourth volume at 50°C under nitrogen. An aliquot of up to 100 μl of the remaining sample was injected directly on the HPLC instrument for analysis.

For HPLC, an isocratic solvent system consisting of 35:65 methanol–70 m*M* potassium phosphate buffer, pH 6.5, was used at a flow-rate of 1.5 ml/min. The effluent was monitored with a fluorescence detector with an excitation wavelength of 280 nm and an emission wavelength of 350 nm.

Serum levels of TXB_2, the stable hydrolysis product of TXA_2, were determined by radioimmunoassay (RIA) using a specific antiserum. Briefly, the RIA buffer contained 0.1 *M* potassium phosphate, 0.9% sodium chloride, 0.1% gelatin and 0.01% sodium azide, adjusted to pH 7.4. Working solutions of antiserum, tracer and standards were diluted with RIA buffer. For the assay, 0.1 ml each of anti-

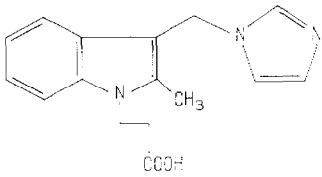

Fig. 1. Chemical structure of dazmegrel.

serum, tracer (5000 cpm) and sample were mixed and incubated at 4°C for 16–20 h. The free tracer was separated by addition of 1 ml of dextran-coated charcoal [0.4% Norit A charcoal (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and 0.015% 70 000 MW dextran in RIA buffer] and centrifugation at 1500 *g* for 15 min at 4°C. The supernatant containing the bound tracer was decanted directly into scintillation vials and mixed with 10 ml of Atomlight (New England Nuclear) for counting. The results were calculated from logit curves prepared using standard TXB_2. All samples were prediluted with RIA buffer as necessary and assayed as a three-step serial dilution with dilution factors of 1, 2 and 4.

RESULTS

The retention times for the internal standard (3IBA) and dazmegrel were 8.0 and 11.0 min, respectively, as shown in Fig. 2. The relative peak height, peak shape and retention times were identical in standard samples prepared in saline or rabbit serum. Calibration curves prepared by addition of standard dazmegrel to normal rabbit serum were linear from 50 ng/ml to 5.0 μg/ml, using calculations of peak-height ratios relative to 3IBA. Typically, the correlation coefficient (*r*)

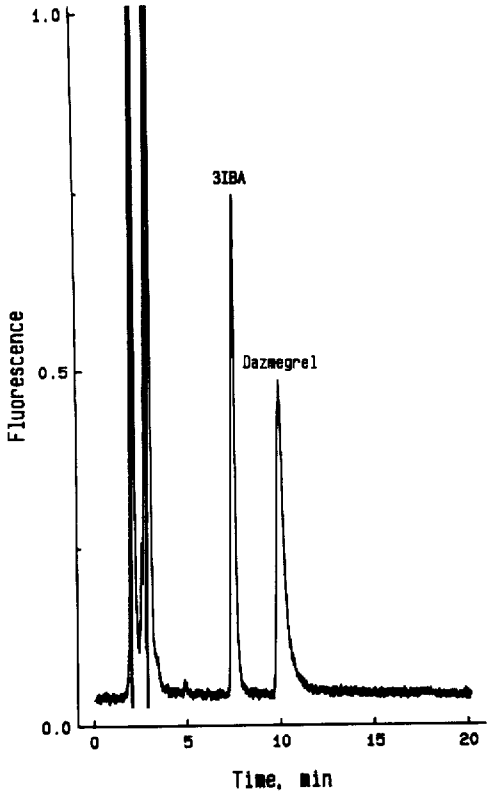

Fig. 2. Chromatogram of standard dazmegrel added to normal rabbit serum at a concentration of 1.25 μg/ml.

was > 0.99. The detection limit based on a signal-to-noise ratio of 3:1 was 25 ng/ml. The fluorescence intensity of dazmegrel with an excitation wavelength of 280 nm and emission wavelength of 350 nm increased with pH and reached a maximum at pH 8.5. The pH of 6.5, however, was selected for chromatography to optimize the fluorescence intensity and peak resolution.

The absolute recovery of 500 ng of dazmegrel added to 400 μl normal rabbit serum was 90.1 ± 4.5% (mean ± SD, $n=7$). The within-run coefficient of variation was 2.34% ($n=15$) at a concentration of 1.0 μg/ml in serum. The day-to-day coefficient of variation at the same concentration was 3.10% ($n=15$). A typical standard chromatogram with a dazmegrel concentration of 1.25 μg/ml in serum is shown in Fig. 2.

Eighteen male New Zealand white rabbits were treated with dazmegrel at a dosage of 30 mg/kg per day by mixing the inhibitor into the bottled water supply. Serum samples were collected before treatment and after four weeks. Chromatograms of rabbit serum before and after treatment with dazmegrel are shown in Fig. 3. An unknown substance, peak A, appears only in serum of treated rabbits and is presumably an unidentified metabolite of dazmegrel. It is absent in serum mixed with standard dazmegrel and serum from untreated rabbits. The serum

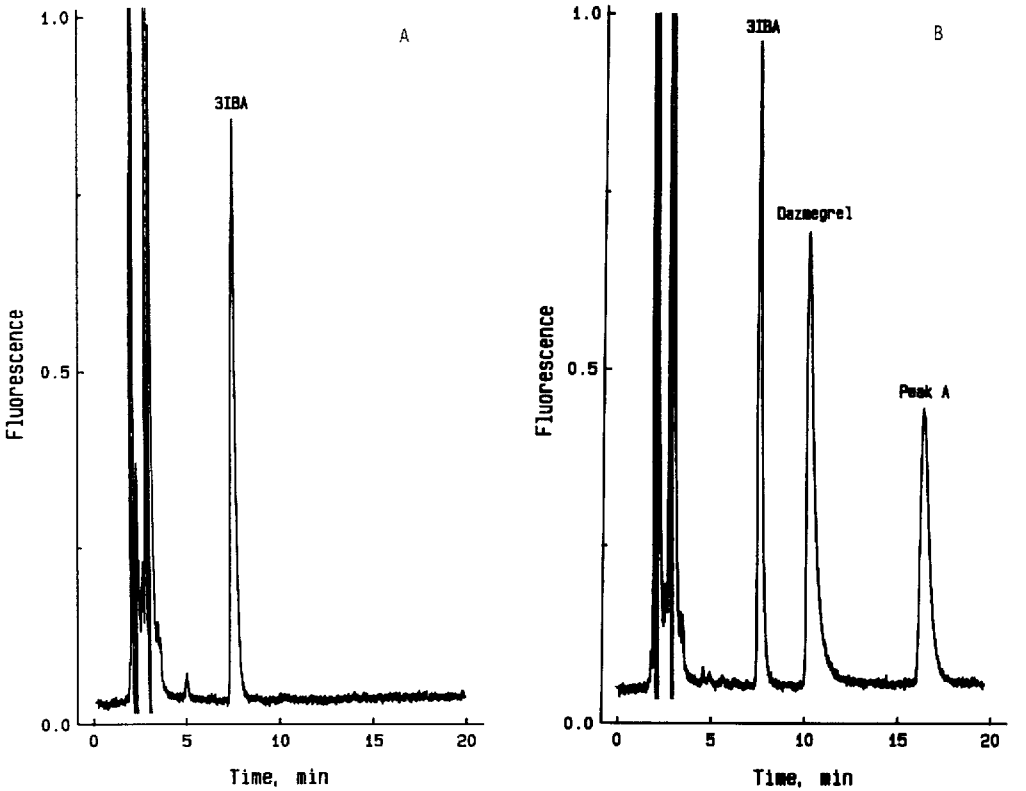

Fig. 3. (A) Chromatogram of normal rabbit serum prior to treatment with dazmegrel. (B) Chromatogram of a serum sample from a rabbit treated with dazmegrel. The dosage was 30 mg/kg per day added to the bottled water supply. Peak A is an unidentified peak, possibly a metabolite of dazmegrel.

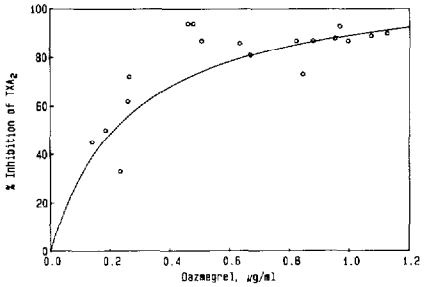

Fig. 4. Relationship of serum concentration of dazmegrel and the level of TXA_2 synthesis inhibition in rabbits treated with dazmegrel, 30 mg/kg per day. Each point represents an individual animal. Curve was determined by regression analysis of a double reciprocal plot ($r=0.77, p<0.0002$).

concentration of dazmegrel was 0.64 ± 0.33 μg/ml (mean ± S.D., $n=18$) and the inhibition of TXA_2 synthesis was $78 \pm 18\%$ (mean ± S.D., $n=18$). The relationship of dazmegrel concentration in serum and the percentage inhibition of TXA_2 synthesis, as determined by RIA of TXB_2, is shown in Fig. 4. It is apparent that high levels of inhibition ($>70\%$) of TXA_2 synthesis are maintained when the concentration of dazmegrel in vivo is above 0.4 μg/ml.

DISCUSSION

It is often advantageous to relate the concentration of experimental drugs to their pharmacological effects. In this report we have described a rapid and simple method for the determination of dazmegrel concentrations. The sensitivity of the method is within the range of pharmacological response in vivo. However, there are two precautions which affect the accuracy and reproducibility of the method. First, it is important that the supernatant obtained after addition of acetonitrile is evaporated to at least one fourth volume. If there is insufficient evaporation, the acetonitrile concentration remaining in the residual sample will be too high and cause peak broadening. Secondly, the retention times of 3IBA, dazmegrel and peak A are sensitive to pH. The fluorescence intensity of dazmegrel increases with pH, but dazmegrel and peak A also merge. The pH of 6.5, described in the procedure, was selected to optimize fluorescence and resolution of dazmegrel and peak A.

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