Journal of Chromatography, 225 (1981) 225—230
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 904

Note

High-performance liquid chromatographic assay for plasma dipyridamole monitoring

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(Received February 25th, 1981)

In recent years there has been an increasing number of reports on dipyridamole's role in the treatment of various thromboembolic diseases [1—4]. Until recently, however, there was no rapid and simple method for the analysis of the plasma concentration of dipyridamole. The available methods at that time involved spectrophotofluorometric procedures described by Beizenherz et al. [5], Zak et al. [6], and Mellinger and Bohorfoush [7]. Fluorescence assays have as a drawback interference from plasma components and other fluorophores; furthermore, the required extraction procedures are tedious and complicated. While our studies were in progress, three additional methods were reported for the analysis of dipyridamole [8—10]. Pedersen [8] described the detection of dipyridamole by high-performance liquid chromatography (HPLC) using either ethanol to precipitate protein from serum (Procedure A) or a 1.0 *M* Tris buffer at pH 8.6 (Procedure B). However, in both cases if an internal standard was used, it was added after the extraction of the drug from the serum. These procedures allow only for detection of injection volume error, column efficiency and the detector response, but don't detect any drug loss or problems during the most critical step, the extraction step. Procedure A requires a 15-min waiting period after adding the ethanol to allow for the protein to precipitate, while Procedure B requires 1 ml of serum to be extracted twice with 8 ml of diethyl ether [8]. This means 16 ml of diethyl ether must be evaporated to 2 ml and re-extracted with 0.1 *N* hydrochloric acid. Therefore Procedure B is actually a three-step extraction method and requires a large volume of the extraction solvent.

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0378-4347/81/0000—0000/$02.50

A newer technique [9] for the assay of dipyridamole in plasma added 100 ng of methoxy-dipyridamole as the internal standard in the extraction solvent. The extraction step, however, calls for 10 ml of dichloromethane for the separation of dipyridamole from plasma, and the amount of plasma extracted must be adjusted according to the concentration of dipyridamole in plasma. Furthermore, the internal standard used must be synthesized from dipyridamole, since it is not commercially available. The third report [10] used ion-pair chromatography with fluorescence detection. This method while extremely sensitive requires the availability of a fluorescence detector and the use of ion-pair chromatography which has the additional expense of buying PIC™ reagents.

The objectives of our study were to develop a method which: (1) employed a one-step extraction procedure; (2) used ultraviolet detection; and (3) did not involve paired-ion chromatography.

EXPERIMENTAL

Apparatus

The HPLC system uses a Waters Assoc. Model 6000A solvent delivery pump equipped with a U6K injector, a μBondapack C_{18} column (30 × 0.39 cm I.D.; particle size 10 μm) and a Model 440 absorbance detector. The signal from the detector was quantified using a Shimadzu Seisakusho Data Processor Chromatopac-E1A and Houston Instruments Omni-Scribe recorder.

Reagents

Dipyridamole, received from Rhodia (New York, NY, U.S.A.) was used as the standard throughout the study. Glass distilled acetonitrile and methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Any other reagents used were analytical grade. The mobile phase was a 50:50 mixture of acetonitrile and a 0.01 *M* solution of sodium phosphate in water adjusted to pH 7. Stock solutions of dipyridamole and lidocaine (the internal standard) in methanol (1 mg/ml, 10 μg/ml and 10 ng/ml) were stored at 4°C.

HPLC conditions

A flow-rate of 1.5 ml/min of the mobile phase produced a pressure of approximately 102 atm (1500 p.s.i.). The absorbance reading of dipyridamole in methanol was at 280 nm, since its maximum wavelength of absorption is at 285 nm [11].

Sample preparation

Standard plasma samples ranged from 0.01—2.0 μg/ml. The 1 and 2 μg/ml samples were prepared by first evaporating to dryness aliquots from a 10 μg/ml methanol stock solution of dipyridamole. The samples were then reconstituted with a 5-ml blank human plasma. The 0.5, 0.1, 0.05 and 0.01 μg/ml standards were prepared by dilution with human plasma of the 1.0 μg/ml standard.

Dipyridamole was extracted from the plasma standards and unknown samples by the following procedure. A 0.1-ml aliquot of 1.0 mg/ml methanol

solution of lidocaine (the internal standard) was added to a 15-ml culture tube. This solution was evaporated to dryness at 45°C by a stream of dry, filtered air. Then 1 ml of plasma and 0.5 ml of 0.1 *N* sodium hydroxide solution were added to the culture tube. This mixture was vigorously agitated for 15 sec on a vortex-type mixer. A 5-ml aliquot of ethyl acetate was added, and the plasma sample was agitated for 60 sec. Centrifugation at 1000 *g* for 6 min allowed the separation of the organic from the aqueous phase. A 4-ml aliquot of the organic phase was then transferred to a conical centrifuge tube. This aliquot was evaporated to dryness at 45°C as outlined above. The residue was reconstituted with 0.5 ml of the mobile phase. A 100-μl amount of each sample was then injected onto the column.

Linear regression analysis was performed on the results obtained from the standard plasma samples. The equation of the best fit for the standard curve was used to calculate the concentration of an unknown sample from the peak height ratio measured.

RESULTS AND DISCUSSION

Under the conditions described in the experimental section, dipyridamole and lidocaine have retention times of 5.5 ± 0.2 and 7.5 ± 0.2 min respectively. These conditions allow the detection in the range of 500 pg of dipyridamole injected onto the column. The extraction procedure permits the determination of plasma concentrations of dipyridamole as low as 5 ng/ml. Fig. 1 is a

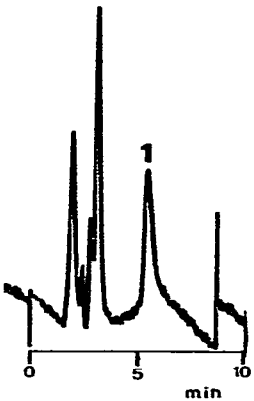

Fig. 1. Chromatogram obtained from a 50 μl injection of a 100 ng mobile phase solution of dipyridamole (1).

chromatogram of dipyridamole in the mobile phase at a concentration of 100 ng/ml; the amount of drug injected on the column was 5 ng. Fig. 2 illustrates the results obtained when plasma from a treated subject was assayed for dipyridamole. Fig. 2a is a plasma sample drawn before dosing of the subject with dipyridamole with the internal standard added. As may be seen from this figure, no interfering peaks occurred at the time corresponding to the retention time of dipyridamole or lidocaine. Fig. 2b is a chromatogram of a plasma sample obtained 1.33 h after the 50-mg dose of dipyridamole.

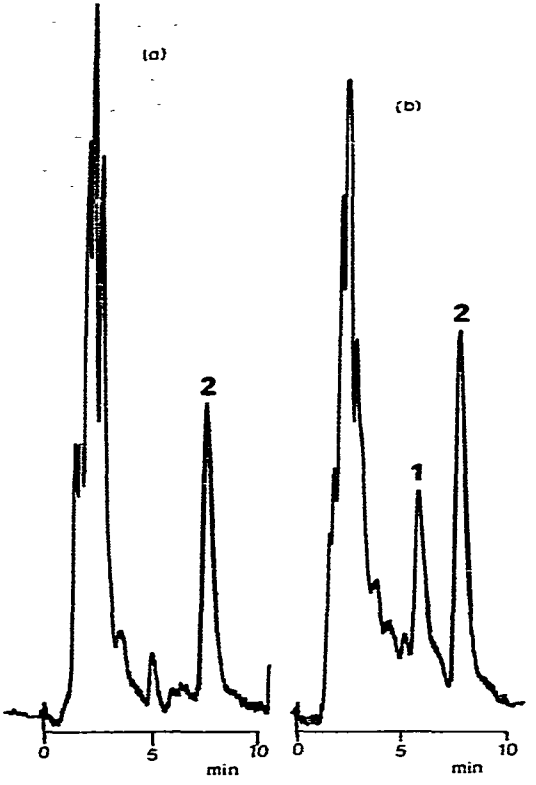

Fig. 2. Chromatograms obtained from human plasma samples. (a) Blank plasma sample obtained previous to dosing, with the internal standard, lidocaine (2), added. (b) Plasma sample drawn 1.33 h after a 50-mg oral dose of dipyridamole. The dipyridamole peak (1) corresponds to a plasma concentration of 101.7 ng/ml.

A standard curve for dipyridamole in plasma ranging from 0.01—2 μg/ml was prepared by plotting concentration against peak height ratio. A good linearity was obtained with a correlation coefficient of 0.9999 calculated by the least squares method. The slope of the line was calculated to be 0.0062, with a *y*-intercept of 0.1008.

The reproducibility of the assay was verified by extracting five plasma samples with a concentration of 0.1 μg/ml. The coefficient of variation of the peak height ratio of these samples was 4.84%. The percent recovery of dipyridamole from these standard plasma samples was 97.95 ± 2.36% with a coefficient of variation of 2.41%.

The preparation and extraction procedures of a sample require only 8 min, with 6 min of this being used in the centrifugation step. The longest time required in this assay, 15 min, was for the evaporation of the organic phase of the samples after the extraction step. A set of twelve samples, thus, can be prepared and is ready to inject onto the column within 30 min.

Dipyridamole is being considered as a possible antiplatelet agent in the treatment of thromboembolic diseases [8]. Dipyridamole plasma levels correlate well with the inhibition of platelet aggregation in patients after pros-

thetic heart valve replacement [12]. Thus it is desirable to monitor dipyridamole levels in a patient to attain proper dosing and maximum therapeutic efficacy.

The assay method was tested by monitoring the plasma concentration—time profile for dipyridamole after the administration of two intact 25-mg Persantine® tablets to two healthy male subjects. The volunteers fasted for 12 h prior to dosing and for the duration of the study. The range of the plasma levels observed over a 5-h period was 25—765 ng/ml (see Fig. 3). Subject 9A in Fig. 3 had a more erratic decay curve which may be indicative of enterohepatic recycling of the drug.

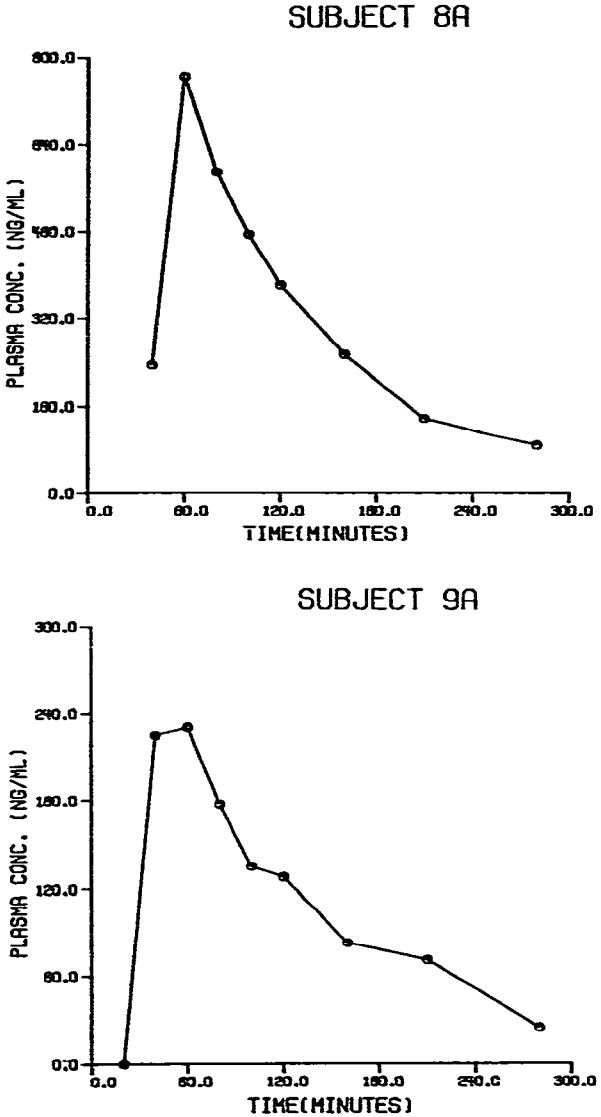

Fig. 3. Plasma concentration curves following the oral administration of two 25-mg tablets of dipyridamole to two healthy subjects.

The HPLC method discussed allows a specific, uncomplicated and rapid method of assaying for dipyridamole in plasma. This assay is currently in use in clinical pharmacokinetic studies designed to evaluate differences in dosage forms.

ACKNOWLEDGEMENTS

The authors would like to acknowledge support from Biomedical Research Support Grant No. 5S01RR05586-10 and the Graduate and Professional Opportunities Fellowship Program at Purdue University.

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