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Note

High-performance liquid chromatographic determination of dipyridamole

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Although there has been considerable interest in the antithrombotic potential of dipyridamole (DIP) [1—5], studies in different species and in some occasions in the same species, have produced variable results [4, 6—8]. Tyce et al. [9] have suggested that variability in response may be attributed to variability in plasma concentrations. Such intersubject variability in pharmacokinetics was reported by Mahoney et al. [10] who also suggested that the current clinical practice of empirical dosage schedules may be inappropriate. Rajah et al. [11] in man and Buchanan et al. [12] in rabbits demonstrated a positive correlation between plasma concentrations of DIP and effect on platelet behaviour. These data suggest that rational therapy with DIP requires maintenance of plasma concentrations above a certain threshold level. It is therefore necessary that simple and rapid methods be available for the determination of this drug in biological fluids.

Determinations of DIP concentrations have been carried out by spectrophotofluorometric methods [13—16] and by chromatographic [16—19] techniques.

The chromatographic procedures described to date are based either on fluorometric and ultraviolet (UV) detection. While fluorometry offers the advantage of sensitivity, it suffers from the disadvantage of a limited linear dynamic range. For instance, while both Schmid et al. [17] and Wolfram and Bjornsson [18] reported linear concentration—response relationships from 1—400 ng/ml and 1—500 ng/ml, respectively, variable amounts of plasma or blood must be used. It is necessary to calibrate the system for various sample volumes of blood or plasma particularly if whole blood is used [18]. Furthermore, DIP has endogenous fluorescence only at alkaline pH which can cause considerable difficulties with high-performance liquid chromatographic (HPLC)

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analysis due to instability of the support at high pH [17]. This problem can be overcome by the use of ion-pair chromatography wherein the fluorescence can be measured at acidic pH [18]. However, both fluorometric techniques doe not resolve potential problems of a relatively small linear dynamic range which complicates the methods by requiring varying amounts of plasma.

HPLC with UV detection has a wide linear dynamic range, does not require alkaline pH or ion-pair chromatography [19]. However, the reported method [19] does not take advantage of these characteristics. It requires both different volumes and different methods of extraction dependent upon concentrations and finally the method is externally calibrated. We describe an HPLC—UV detection method for DIP which uses uniform volumes of plasma, is sufficiently sensitive to follow the concentration of DIP in man for 48 h and is linear over the range of concentrations found in man at standard therapeutic regimen.

Experimental apparatus

The HPLC system used was a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 liquid chromatograph equipped with a U6K injector and a Model 440 dual-channel filter absorbance detector in conjunction with a Hewlett-Packard (Avondale, PA, U.S.A.) 3380A integrator. The column used was an Altex Ultrasphere 5-μm reversed-phase C18 column (25 cm × 0.46 mm I.D.) purchased from Beckmann (Canada).

Reagents

Dipyridamole was generously supplied by Boehringher Ingelheim (Burlington, Canada). Propranolol (P) was a kind gift from Ayerst Research Labs. (Montreal, Canada). Reagents for the preparation of buffers [tris(hydroxymethyl)aminomethane] (Tris) and both sodium and potassium phosphate salts were purchased from BDH (Toronto, Canada). N,N,N,N-Tetramethylethylene diamine (TMD) was purchased from Matheson Coleman and Bell (East Rutherford, NJ, U.S.A.). Diethyl ether used in the extractions was purchased from Canlab (Toronto, Canada) and was analytical grade. Acetonitrile for HPLC was purchased from Caledon Labs. (Mississauga, Canada). Water for the preparation of all buffers was glass-distilled in-house. Stock solutions of DIP and P, 5 mg/ml in ethanol, were prepared and stored at —20°C in darkness. Under these conditions the solutions were stable for at least nine months.

HPLC conditions

DIP and P were eluted with 33% acetonitrile in 0.02 *M* phosphate buffer containing 0.01 *M* TMD at pH 2.9. The flow-rate was 2.5 ml/min which generated a pressure of approximately 238 bar. The effluent was monitored at 280 nm [16]. Under these conditions both DIP and P could be detected and a baseline separation was readily achieved. The retention time for P was 6.5 min and for DIP it was 7.5 min. Quantitation was by determination of the ratio of the response for DIP to P. Both peak areas and peak heights were taken as a measure of the response of the two compounds.

Standard solutions

A series of serial dilutions from the stock solution of DIP (5 mg/ml) was prepared in ethanol. A 50-μl aliquot of each of these solutions was added to 5 ml of plasma. In this way, a series of concentrations ranging from 2 to 2000 ng/ml was prepared. The blank consisted of the addition of 50 μl of ethanol to 5 ml of plasma. The solutions so prepared were stored at −20°C and were found to be stable for a period of two years.

Sample preparation

To 1 ml of plasma were added 500 ng of P in 50 μl of ethanol. A spatula of sodium chloride was then added and the tube mixed to ensure saturation of the aqueous phase with the salt. One ml of 1 *M* Tris buffer at a pH of 10 was added to the plasma solution and this was further agitated to ensure maximum dissolution of the sodium chloride. Five ml of diethyl ether were added, the mixture was shaken for 5 min and centrifuted at 1500 *g* for 10 min. The clear organic phase was transferred to a second tube containing 3 ml of hexane. The DIP and P were then back-extracted into 50 μl of 0.1 *N* hydrochloric acid. After shaking and centrifugation, the ether layer was removed by aspiration. An aliquot of the aqueous phase or the total phase could be injected for analysis.

RESULTS

HPLC conditions

Factors controlling the elution of DIP from a 5-μm octadecyl silica gel column are the percent acetonitrile in the mobile phase, and the presence of TMD. The results are summarized in Table I.

TABLE I

THE EFFECT OF COMPOSITION OF THE MOBILE PHASE ON RETENTION TIME

Acetonitrile (%)	pH of the aqueous phase	Concentration of TMD (*M*)	Retention time (min)*
30	4	0	—**
30	4	0.01	13.75
35	4	0.01	8
33	2.9	0	10.32
33	2.9	0.01	7.49
33	2.9	0.02	6.82

*Flow-rate = 2.5 ml/min.
**No detectable peak within 15 min of injection.

Extraction procedure

In order to achieve high extraction efficiency from plasma into the relatively small volume of diethyl ether the plasma which was buffered to pH 10 was saturated with sodium chloride. Similarly, in order to facilitate the back-extraction of DIP from diethyl ether into 50 μl of acid, hexane was added to

the ether extract. The overall efficiency of extraction was 87%. This was determined by external calibration method with P acting as an external standard added to the final aqueous extract. In our hands omission of either sodium chloride or hexane resulted in an extraction of only trace amounts of DIP.

Linearity and reproducibility of the method

In the range from 2—2000 ng/ml the calibration curve was linear, following the equation $y = 0.14x + 0.009$ ($n = 20$; $r = 0.998$). At the 100 ng/ml level the relative standard deviation was 3% whereas at the 10 ng/ml level the relative standard deviation was 5% ($n = 5$).

DISCUSSION

The potential utility of monitoring plasma concentrations as an adjunct to therapy with DIP makes it necessary that methods for determining concentration of this drug in biological samples be as selective, simple and rapid as possible. In particular, if such therapeutic monitoring is to become generally used it is important that such techniques be readily established in different laboratories. HPLC using high-resolution columns and UV detection offers the best combination of selectivity and simplicity of instrumentation.

The percentage of acetonitrile in the mobile phase plays a role in determining the elution volume of DIP (Table I) with acidic conditions being a prerequisite [19]. This is expected for a basic drug. However, Table I also shows that TMD has a major effect on elution volume. The purpose of the diamine is to inactivate binding sites that may bind the highly polar nitrogenous base [20]. It is evident from the effect of TMD on elution volume that these binding sites, if not inactivated, can alter the elution volume of a basic analyte.

Recognizing the need for simplicity, conditions were developed which utilized single extraction steps as well as small volumes of solvents and extractants. In order to effect a high efficiency of extraction from buffered plasma we found it necessary to saturate the aqueous phase with sodium chloride. Salting out was also technically useful in that the protein separated out in a very narrow band at the interface between the aqueous solution and the diethyl ether. This permitted a facile transfer of organic phase into the second extraction tube. Similarly, in our hands, the addition of hexane to the diethyl ether prior to back-extraction was necessary in order to have a high extraction efficiency into 50 μl of 0.1 *N* hydrochloric acid. Again, there was a technical improvement in that the separation of the 50 μl of aqueous from organic phase was free of emulsion. When only diethyl ether was used there was emulsion between the organic phase and the 50 μl of aqueous phase.

The use of an internal standard simplified the entire procedure by eliminating the need for precise transfer of solvent and precise injection volume. Propranolol was used because the drug is readily available and since it is a tertiary amine its extraction characteristics are similar to DIP. Furthermore the differences in structure between analyte and standard permit a separation of these two compounds.

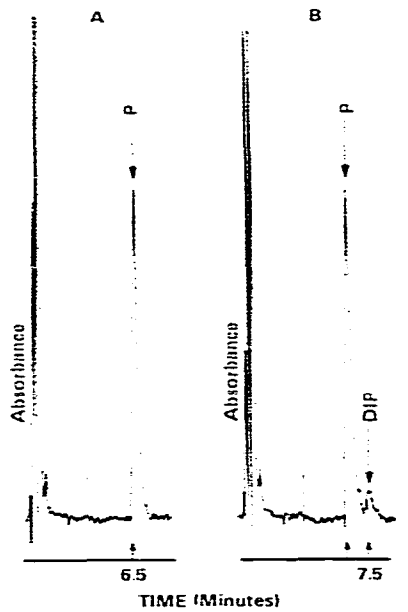

Fig. 1. Plasma prepared from a sample (A) prior to ingestion of DIP; and (B) 48 h after the last dose. The concentration was 10 ng/ml. These results were obtained by injecting a 25-μl aliquot of the 50-μl extract.

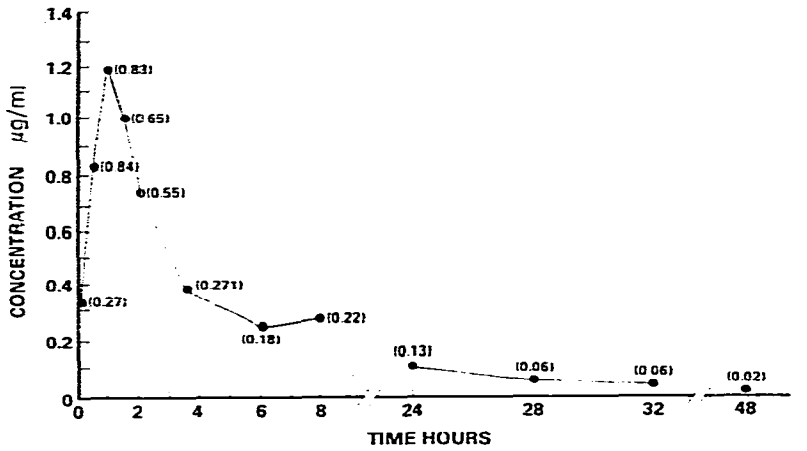

Fig. 2. Plasma concentration profile from ten volunteers receiving 75 mg DIP for 1 week t.i.d. Concentrations shown are averages with standard deviations in brackets. Sample for $T = 0$ was taken at 8:00 a.m. just prior to the last dose.

The extracts of blank plasma so prepared showed no interference to the detection of DIP at low concentrations of DIP (Fig. 1).

This technique is not quite as sensitive as a fluorometric method, but it is not markedly less sensitive. It was found to have sufficient sensitivity to permit a description of the plasma concentration profile over a 48-h period (Fig. 2).

The calibration is linear over the concentration range normally found in

man and uniform volumes can be used. Finally, the method is sufficiently simple and rapid to permit the extraction of 100 samples per day of plasma.

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