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Note

Rapid, sensitive determination of dipyridamole in human plasma by high-performance liquid chromatography

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Dipyridamole is widely used as coronary vasodilator. Further, in the last years the combination of acetylsalicylic acid with dipyridamole is increasingly being used as an antithromboticum. The study of the bioavailability of dipyridamole in various pharmaceutical formulations as well as in combination-drugs, as mentioned above, requires specific and sensitive plasma level evaluations.

Quantitative analysis of dipyridamole in biological fluids has been performed by fluorescence measurements [1, 2]. These direct determinations without chromatographic separation do not distinguish between other fluorescent substances (metabolites and other drugs). A newer technique [3] allows the separation of glucuronides. An especial advantage of a chromatographic technique is that it permits the use of an internal standard. This improves the precision of the determination and facilitates the handling of small sample volumes. A recent publication [4] describes the detection of dipyridamole by means of high-performance liquid chromatography. The sample preparation described there is more complicated and the UV detection is less specific and sensitive.

The aim of this study was to develop a rapid, simple and specific method for determining dipyridamole in small plasma samples.

EXPERIMENTAL

Reagents and chemicals

Dipyrimidamole (R-A 8 BS) and the internal standard methoxy-dipyridamole (R-A 764 BS) were of analytical grade (Dr. Karl Thomae, Biberach, G.F.R.) (see Fig. 1). Dichloromethane, sodium hydroxide, methanol and tris(hydroxymethyl)aminomethane (Tris) were of p.a. quality (E. Merck, Darm-

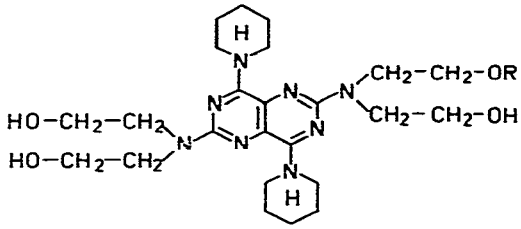

Fig. 1. Structural formulae of dipyridamole (R-A 8 BS), R = H, and the internal standard methoxy-dipyridamole (R-A 764 BS), R = OCH_3.

stadt, G.F.R.). The Tris—HCl buffer was 0.2 *N* (in water), pH 8.6. ^{14}C-labelled dipyridamole (specific radioactivity 3.75 mCi/mmole ($2.75 \cdot 10^5$ $sec^{-1} \cdot mg^{-1}$) was synthesized in the isotope laboratory of the Biochemical Department of Dr. Karl Thomae.

Apparatus

Chromatograph: Perkin Elmer 601. Detector: Spectrofluorimeter 801 Farrand Optical, excitation 415 nm, emission 478 nm. Integrator: Hewlett-Packard 3370B. Recorder: Perkin Elmer/Hitachi 56. Column: 125 × 4.6 mm I.D., filled with LiChrosorb RP-18, 5 μm (E. Merck). Pressure 1100 p.s.i. temperature 20°. Mobile phase: methanol—0.2 *M* Tris · HCl buffer (80:20), at a flow-rate of 1 ml/min.

Collection of samples

Blood samples were withdrawn with heparinised Sarstedt Monovetten^R (Nümbrecht, G.F.R.) and centrifuged to obtain plasma.

Analytical procedure

A 0.2-ml aliquot of plasma (range 0.1—0.5 ml) was pipetted into a 15-ml glass-stoppered test-tube, and 1 ml of 1 *N* sodium hydroxide and 10 ml dichloromethane, containing 100 ng internal standard, were added. After mixing for 10 min on a shaking machine the upper phase was withdrawn and discarded. The organic phase was filtered through a piece of filter-paper into another test-tube and evaporated to dryness under a gentle nitrogen stream in a water-bath at 30°. The residue was reconstituted with 50 μl of the solvent mixture and injected into the chromatograph. The amount of plasma taken for analysis should be adjusted according to the dosage. For dosings of 50 mg dipyridamole, 200 μl plasma are appropriate.

Calibration curve

Standards corresponding to 0, 10, 20, 40, 80, 100, 200, 400 ng of dipyridamole dissolved in 20 μl methanol were added to 0.5 ml plasma. After incubation for 1 h at 37° the analysis was performed as described above. The volume of 0.5 ml plasma was taken to show the absence of interfering peaks even for large plasma volumes.

Experiments demonstrating high specificity

To make sure that the dipyridamole peak was not overlapped by a metabolite with the same chromatographic behaviour, the eluate from the column

was re-chromatographed on silica gel G thin-layer plates (No. 5271, E. Merck) in the solvent systems toluene—isopropyl alcohol—ethanol—ammonia (70:15:15:1) and *n*-butanol—methyl ethyl ketone (80:20). The R_F values of dipyridamole are 0.60 and 0.80, respectively. The plates were dried in a stream of cool air and then inspected under UV light (254 nm).

Recovery

The recovery was established by liquid scintillation counting of ^{14}C-labelled dipyridamole. The labelled compound was added to plasma as described above. Following phase-separation the amount of radioactivity of the two phases was measured by liquid scintillation counting (Packard Tri-Carb, Model 3380).

RESULTS AND DISCUSSION

Sensitivity

The fluorescence detector enabled us to detect less than 200 pg of dipyridamole, as shown in Fig. 2. The detection limit in plasma is lower than 5 ng/ml as shown in Fig. 3.

Fig. 2. Chromatograms showing (a) 500 pg and (b) 100 pg of pure dipyridamole.

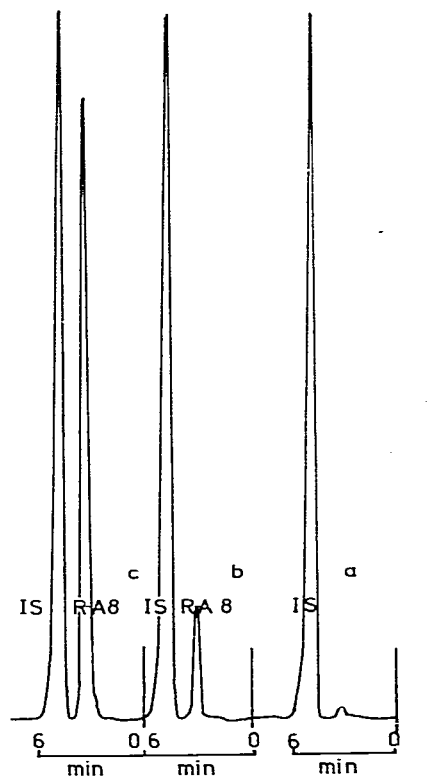

Fig. 3. Chromatograms of dipyridamole showing human plasma blank with (a) internal standard; (b) 20 ng/ml R-A 8; (c) 160 ng/ml R-A 8.

Selectivity

The thin-layer re-chromatography of the high-performance liquid chromatography (HPLC) eluate demonstrated the absence of metabolites with the same chromatographic behaviour on the reversed-phase column. Ten different human plasma samples obtained from heparinized, citrate-treated and EDTA-treated blood showed practically no interference in the chromatogram.

Calibration curve

The calibration curve is linear between 10 and 400 ng dipyridamole. It is reasonable to adjust the plasma volume corresponding to this concentration range.

Precision and accuracy

Within-day precision was established on a drug-free plasma. Dipyridamole was added at a concentration of 100 ng/ml, 200 ng/ml and 400 ng/ml (Table I).

Day-to-day precision was established by analysing drug-containing plasma samples of a pharmacokinetic experiment and analysing them on different days (Table I).

TABLE I

PRECISION OF DIPYRIDAMOLE DETERMINATION ON ONE DAY (A) AND BETWEEN DAYS (B)

C.V. = coefficient of variation.

	Plasma level (ng/ml)	Mean peak ratio	C.V. (%)	No. of determinations
A	100	0.426	3.5	4
	200	0.861	0.8	4
	400	1.707	1.7	4
B	580	2.466	1.3	6

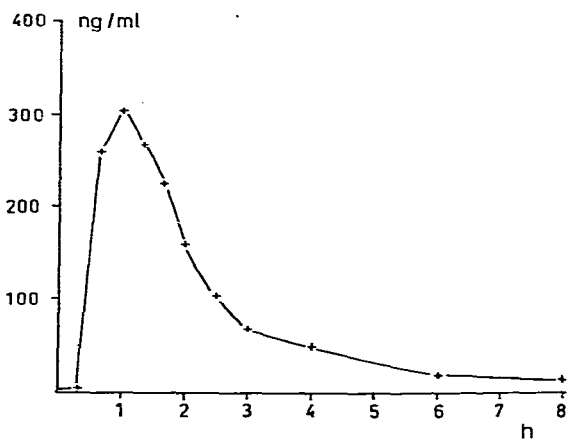

Fig. 4. Plasma concentration of dipyridamole in a human subject following peroral administration of 25 mg.

Using liquid scintillation counting, the recovery of ^{14}C-labelled dipyridamole was 88 ± 3.8% (n = 5) at a level of 100 ng/ml. For a single-step extraction, this result is excellent.

Application

Fig. 4 shows the plasma concentration of dipyridamole in a human subject following peroral administration of 25 mg dipyridamole as a tablet. This plasma concentration level agrees with the total fluorescence measurement.

This method is very suitable for routine analysis of bioavailability studies, as it is simple (due to the selective extraction), rapid (because of the short retention times), precise (on using an internal standard), specific (only dipyridamole is detected) and sensitive (with the fluorescence detector). One person is able to perform 40 analyses a day. The results demonstrate that HPLC with fluorescence detection has a high sensitivity and high selectivity, they further give evidence that HPLC is an important tool for pharmacokinetic studies.

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