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Note

Specific determination of dipyridamole in serum by high-performance liquid chromatography

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During the last ten years a number of investigators have reported a beneficial effect of dipyridamole treatment of patients suffering from various thromboembolic diseases [1—4]. In some investigations the time lapse of serum concentrations of dipyridamole has been followed using spectrophotofluorimetric methods as described by Beizenhertz et al. [5], Zak et al. [6] and Mellinger et al. [7]. These methods are rather time-consuming, however, and, furthermore, a number of drugs with fluorescence potential may interfere with the analysis. The present paper describes a specific, sensitive and rapid method for the determination of dipyridamole in serum and other biological fluids by means of high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Apparatus

The HPLC system used was a Waters Model 600 liquid chromatograph equipped with a U6K injector, a μBondapak C_{18} column (30 × 0.39 cm I.D.; particle size 10 μm) and a Model 440 dual channel filter absorbance detector in conjunction with a Tarkan W + W 600 recorder.

Reagents

Dipyridamole was purchased from Böhringer-Ingelheim (Copenhagen, Denmark). All other reagents (analytical grade) were obtained from Merck (Darmstadt, G.F.R.). The eluting solvent was a mixture (75:25) of methanol and a 0.02 *M* solution of sodium acetate in water adjusted to pH 4 with acetic acid. Stock solutions of dipyridamole and indomethacin, 5 mg/ml in ethanol, could be stored at - 20° in darkness for up to six months. Two buffer solutions of 1 *M*

tris(hydroxymethyl)aminomethane, one adjusted to pH 8.6 with hydrochloric acid, were used in the extraction procedures. Ethanol (96%, w/v) was used for the protein precipitation of serum samples.

HPLC conditions

A solvent flow-rate of 2 ml/min producing a pressure of approximately 2000 p.s.i. was used in the experiments. The absorbance spectrum of a solution of dipyridamole in the solvent mixture showed a maximum at about 280 nm and consequently filters for this nominal wavelength in conjunction with instrumental absorbance settings of 0.05—0.005 a.u. were used for continuous detection of the compound. A volume of 25 μl of the samples in ethanol or buffered water was injected into the column.

Standard solutions

Dilute standard solutions in ethanol, which were made from the stock solutions, could be kept stable in darkness at 5° for several weeks. In order to estimate the stability in daylight of standards and samples prepared from serum, the decline in concentration of dipyridamole in freshly prepared solutions in 1 *M* Tris buffer (pH 8.6), in ethanol and in ethanol-precipitated serum was followed as a function of time in different types of reagent tubes (polyethylene, polypropylene and ordinary glass).

Sample preparation

Two preparation procedures were investigated.

Procedure A. To 100 μl serum were added 200 μl ethanol and after gentle mixing the sample was allowed to stand for 15 min for protein to precipitate. After further centrifugation at 600 *g* for 5 min, 25 μl of the clear deproteinized supernatant were injected directly onto the column.

Procedure B. To 1 ml of serum was added 1 ml of 1 *M* Tris buffer (pH 8.6) and the mixture was extracted twice with 8 ml diethyl ether. The combined ether extracts were then evaporated under nitrogen to about 2 ml and re-extracted with 230 μl of 0.1 *N* hydrochloric acid. The ether phase was removed and the acid phase was neutralized with 20 μl 1 *M* Tris. Samples of 25 μl were used for injection.

A few experiments on the isolation and determination of dipyridamole glucuronide as described by Zak et al. [6] were also performed. This procedure involves treatment of the serum residual from procedure B with β-glucuronidase and subsequent sample treatment according to procedure B.

In some experiments indomethacin was added to the ethanol phase (A) or to the 0.1 *N* hydrochloric acid phase (B) and used as an internal standard for control of the injection volume, column efficiency and detector response.

Quantitation

Determination of the serum concentration of dipyridamole was carried out as follows. The peak height of the serum sample (P_{se}) was compared to the peak height of a standard (P_{st}) of 1 μg/ml. The calculations related to the sample preparation procedures, A and B, respectively, were as follows:

$$\frac{P_{se} \times 3 \times 0.936}{P_{st}} = \text{serum dipyridamole } (\mu g/ml)$$

and

$$\frac{P_{se}}{4 \times P_{st}} = \text{serum dipyridamole } (\mu g/ml)$$

RESULTS AND DISCUSSION

Sample preparation procedures

One of the main advantages of HPLC in comparison to other chromatographic procedures is the possibility of a simple and quick sample-preparation procedure. Precipitation of serum proteins with ethanol or trichloroacetic acid has previously been found to be an adequate sample preparation procedure, which allows direct injection of the deproteinized serum into the chromatograph without any damaging effect on the column [8]. The ethanol precipitation procedure affords complete recovery of the dipyridamole in serum, contrary to the inconsistent recoveries found by the use of trichloroacetic acid precipitation. However, the precipitation technique involves sample dilution and this method only allows determination of serum concentrations above approximately 0.1 μg/ml. The quantitative extraction procedure used by other investigators [6, 9] was consequently slightly modified by adapting the extraction volumes in a way that allowed determination of much smaller concentrations. The experiments with the isolated dipyridamole glucuronide metabolite showed that the present method can easily be adapted to specific determination of this metabolite. This is of interest in pharmacokinetic investigations.

Chromatographic conditions

Reversed-phase packing compounds have been used in the HPLC determination of a number of different drugs [10], and the μBondapak C_{18} packing compound was chosen for this analysis. The pH and composition of the eluting fluid are important factors in the determination of retention volume and magnitude of detector signal. In the present analysis, the optimum detector signal was obtained with a pH between 4 and 7, and based on its excellent column compatibility a 0.02 *M* acetate buffer adjusted to pH 4 was chosen. Satisfactory retention volumes of 5—15 ml were obtained using 65—75% methanol in the buffer as eluent, and the retention volumes were not influenced by changes in pH. When indomethacin was used as internal standard it was necessary to lower the methanol content of the eluent to obtain complete analytical separation. Fig. 1 shows a chromatogram, developed with 65% methanol in acetate buffer at pH 4, using a flow-rate of 2 ml/min.

Linearity and sensitivity

A standard curve of dipyridamole in the concentration range 0.1—10 μg/ml showed a linear relationship between concentration and peak height (absorbance units) with a slope of 3.319×10^{-3} a.u. per μg, y intercept = 2.4×10^{-5} a.u., and $r = 0.9999$. The coefficient of variation in duplicate serum analyses

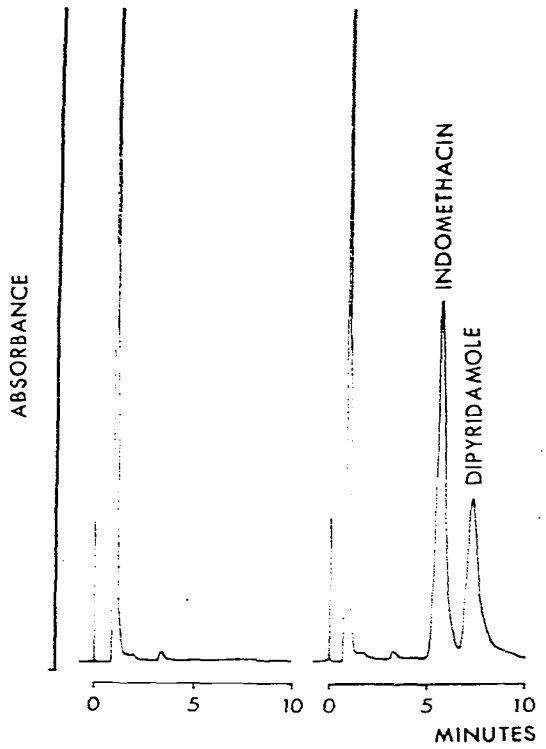

Fig. 1. Left: chromatogram from a normal serum sample treated as described in text (procedure A). Right: chromatogram from a person who had ingested dipyridamole. Indomethacin was added as internal standard (procedure A). Chromatographic conditions as described in the text.

was always below 2%. The detection limit in serum, defined on the basis of the amount of compound injected that caused an absorption of two times the standard deviation of baseline noise, was found to be 0.8 ng of injected compound, corresponding to about 100 ng/ml in procedure A and about 8 ng/ml in procedure B. The analytical sensitivity of the photometric method, expressed as the amount of drug giving a detector response of 0.008 a.u., was calculated to be about 0.016 μg.

Interference and recovery

Interference from naturally occurring substances or other drugs was not found in analyses of serum samples from a number of patients under treatment with dipyridamole. In particular, no interference was found from drugs used in anti-thrombotic therapy, including aspirin, sulfinpyrazone and coumarin anticoagulants. No interference from the dipyridamole glucuronide was found in either of the sample preparation procedures.

Experiments with the addition of known amounts of dipyridamole to serum with subsequent sample preparation according to procedure A or B resulted in complete recovery of the drug. However, the concentration of dipyridamole in the supernatant of deproteinized serum or plasma was constantly found to be 6.8% ± 1.1 S.E. higher than the expected value; this is probably due to dis-

placement of dipyridamole from the denaturated serum protein particles. The recovery of dipyridamole from extracted serum samples was 100.4% ± 0.4 S.E.

Stability

The stability of freshly prepared standard solutions exposed to daylight showed considerable variation. The rate of degradation of dipyridamole seemed to follow a first-order function, and the rate constant was obviously dependent on the light intensity in the laboratory. The maximum degradation rate was found in the solutions with Tris-buffered water, in which the half-time of dipyridamole varied from about 3 to 30 h. The corresponding half-time in ethanol was 12—240 h. Samples prepared with deproteinized serum were stable for several days. No difference between the reagent tubes was found, and there was no evidence of adsorption on the glassware or plastic tubes. These findings strongly indicate that all samples and standards should be protected from light.

Applications

In a number of clinical and experimental studies it has been shown that dipyridamole either alone or in combination with other drugs can be used as an anti-platelet agent in the prevention of thrombo-embolic disease [1—4, 11]. Although dipyridamole is an inhibitor of platelet phosphodiesterase, inhibition of platelet aggregation in vitro has only been demonstrated in concentrations about 10 times the maximum concentration obtained during continuous therapy. This is in contradiction to the strikingly beneficial effect of dipyridamole in the treatment of thrombo-embolic disorders characterized by increased platelet turnover. Recently, Summers et al. [12] have studied the effect of

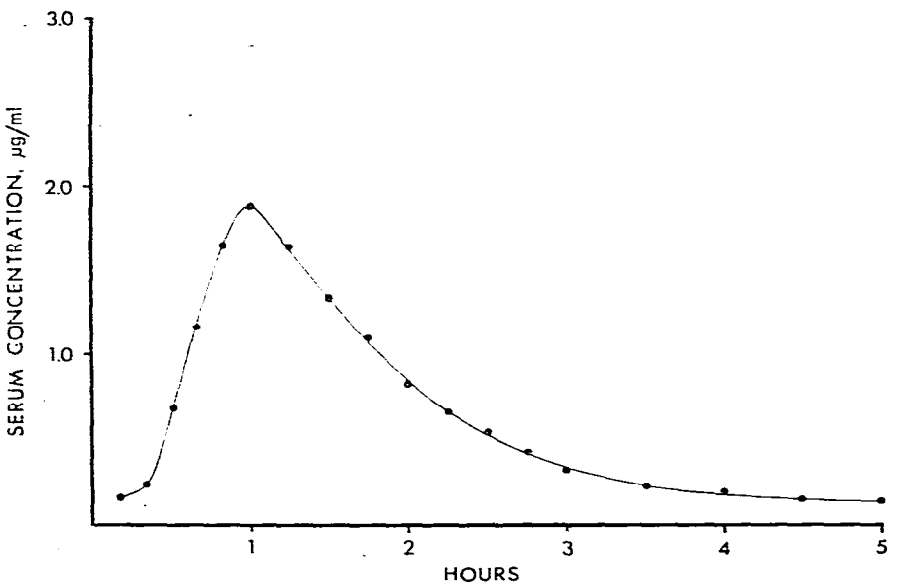

Fig. 2. Serum concentration curve following oral administration of dipyridamole (1.25 mg/kg) to a normal man. Determinations were carried out as described in the text (procedure A).

dipyridamole on [^{14}C]adenosine uptake in human platelets ex vivo, and the results showed a significant inhibition of adenosine uptake which was strongly correlated to the plasma concentration of dipyridamole. Another report [9] has stated a correlation between the dipyridamole plasma level and inhibition of platelet aggregation in patients after isolated mitral valve replacement. But these results seem to be based on an insufficiently described technique and remain to be verified. However, these investigations call for a more detailed description of dipyridamole pharmacokinetics in man. Until now, only inadequate spectrofluorimetric assay methods have been available for the study of dipyridamole plasma concentrations.

The present HPLC method was developed in order to reduce the time required for sample treatment and in order to increase precision and specificity. Fig. 2 shows the serum concentration curve after oral administration of 1.25 mg/kg to a human volunteer. Pharmacokinetic analyses of a number of such experiments [13] have revealed that the drug exhibits first-order kinetics with regard to absorption, distribution and elimination and fits the model of an open two-compartment system.

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