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Short Communication

Sensitive determination of free and plasma protein-bound dipyridamole by high-performance liquid chromatography

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ABSTRACT

For many years dipyridamole (DP) has been used in the treatment of hypertension as a vasodilator, but recently it has been recognised as an anti-platelet aggregation agent and to potentiate anti-metabolite activity. A rapid and sensitive (20 n*M*) procedure for the determination of free and protein-bound DP in plasma, using reversed-phase high-performance liquid chromatography on an Ultrasphere XL ODS (3 μm) column (70 mm × 4.6 mm I.D.) with ultraviolet detection (280 nm), is reported. Free and bound DP were separated using ultrafiltration. Concentrations of DP between 0.1 and 10 μM were measured in plasma with a relative standard deviation of $<9.6\%$. The subsequent determination of DP levels in patients orally administered 450 mg per day showed that DP binding to plasma protein is higher than 90%.

INTRODUCTION

Dipyridamole (DP) is a commonly used vasodilating agent which has recently been shown to potentiate anti-metabolite activity in a dose-dependent manner [1,2]. It therefore appears important to control the plasma level of this compound, which is mainly bound to plasma protein. Several method, *e.g.*, spectrofluorimetry and high-performance liquid chromatography (HPLC) with fluorescence or amperometric detection, have previously been reported for the determination of dipyridamole in biological samples [3–8]. In addition to being time-consuming, spectrofluorimetric methods suffer from the low specificity inherent in non-chromatographic methods. Three of the reported HPLC methods [3–5] used fluorimetric or amperometric detection. To our knowledge, the only HPLC method using spectrophotometric detection [6] was based on two different procedures for plasma preparation. Using this procedure for sample preparation makes the method inconvenient and time-consuming, and additionally this method suffers from a relative lack of sensitivity.

This paper describes a rapid (<60 min, including ultrafiltration and work-up), sensitive (20 n*M*) and selective HPLC method for the determination of DP, using

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a reversed-phase column and spectrophotometric detection, that is applicable to both free and plasma protein-bound DP. Further, the method involves only a simple extraction with diethyl ether of an alkalinized sample followed by evaporation of the organic solvent.

EXPERIMENTAL

Apparatus

The HPLC system was a computer-monitored GOLD PC apparatus (Model 126 pumps, Model 166 detector), an Ultrasphere XL ODS 3-μm column (70 mm × 4.6 mm I.D.) (Beckman, Gagny, France) and a WISP 512 autosampler (Waters Millipore, Molsheim, France).

Reagents

DP was purchased from Boehringer-Ingelheim (Reims, France) and indomethacin (INDO) from Merck (Darmstadt, Germany). All other reagents were obtained from Prolabo (Paris, France) and were of the highest purity available. The eluting solvent was methanol–0.02 *M* ammonium acetate buffer (pH 5.0) (65:35, v/v). Tris buffer solutions (1 *M* Tris, adjusted to pH 10.0) were used in the extraction procedures.

HPLC conditions

Room temperature and a flow-rate of 1.5 ml/min were maintained throughout the analyses. The absorbance spectrum of a solution of DP in methanol showed a maximum at 280 nm, and this wavelength was selected for UV detection. A pressure of 67 bar (1000 p.s.i.) was used. A volume of 20 μl of the samples in methanol was injected into the column.

Plasma samples

Samples were collected from three subjects, two females and one male, receiving 450 mg of dipyridamole orally (75 mg, six times daily). Blood samples were collected in EDTA tubes 4 h after the last DP dose and immediately centrifuged at 3000 *g*. As the stability of freshly prepared standard solutions exposed to daylight could show considerable variations [3], all samples and standards were protected from light. The separated plasma was frozen in polyethylene tubes at −20°C until analysis.

Sample treatment

Thawed plasma samples of 1 ml were diluted with an equal volume of Tris buffer (pH 10.0) and INDO (internal standard; 150 ng/ml) and mixed with 8 ml of diethyl ether. As DP is a base with a pK_a of 6.4, accurate pH control is needed; the use of alkalinized aqueous phase in the buffer prevents ionization of DP. The samples were immediately extracted using a vortex mixer at high speed for 3 min.

followed by freezing for 1 h to separate the aqueous and organic phases. The diethyl ether phase was collected in another tube, then evaporated to dryness at room temperature. Using diethyl ether as the extraction solvent is technically advantageous: because of its lower specific gravity and freezing point than water, it can be easily separated from the aqueous phase by freezing. The residue was dissolved in 200 μl of methanol, and 20-μl samples were injected. Free and bound DP were separated using SM13243 ultrafiltration units (Sartorius, Palaiseau, France) by centrifugation (4000 *g* for 30 min). After ultrafiltration, the free DP was extracted with diethyl ether, the organic phase was evaporated and the residue was dissolved in 100 μl of methanol.

RESULTS

Sensitivity

The minimum detectable concentration of DP was 10 ng/ml (20 n*M*) on injecting 20 μl of 200 μl of reconstituted plasma extract at a sensitivity setting of 0.0001 a.u.f.s. DP could be determined with acceptable precision ($\leqslant$9.6%) (Table I) at a concentration of 0.1 μM (Fig. 1).

Linearity and precision

The calibration graphs of concentration *versus* peak area for both DP and INDO in plasma were linear over the concentration range studied of 0.1–10 μM ($r > 0.990$). The within-day relative standard deviation (R.S.D.), based on triplicate determinations, was less than 3.4% for DP at all concentrations, except at 0.1 μM (9.6%), and was less than 7.6% for INDO at all concentrations (Table I). The between-day R.S.D. was calculated by performing triplicate analyses of plasma samples containing DP at five concentrations on three different days. A summary of the analyses is presented in Table II. The between-day R.S.D.s were all less than 7% (2.3–6.2%), except at a 0.1 μM concentration of DP, for which we have no explanation. The calibration graph obtained by adding DP to control plasma before the ultrafiltration was linear from 20 to 60 n*M* ($r > 0.990$).

TABLE I

WITHIN-DAY VARIABILITY OF PLASMA ASSAY ($n = 3$)

DP (μM)	R.S.D. (%)	INDO (μM)	R.S.D. (%)
0.1	9.6	0.1	7.6
0.2	2.2	0.2	5.2
1.0	2.1	1.0	2.0
2.0	3.1	2.0	4.1
10.0	3.4	10.0	2.6

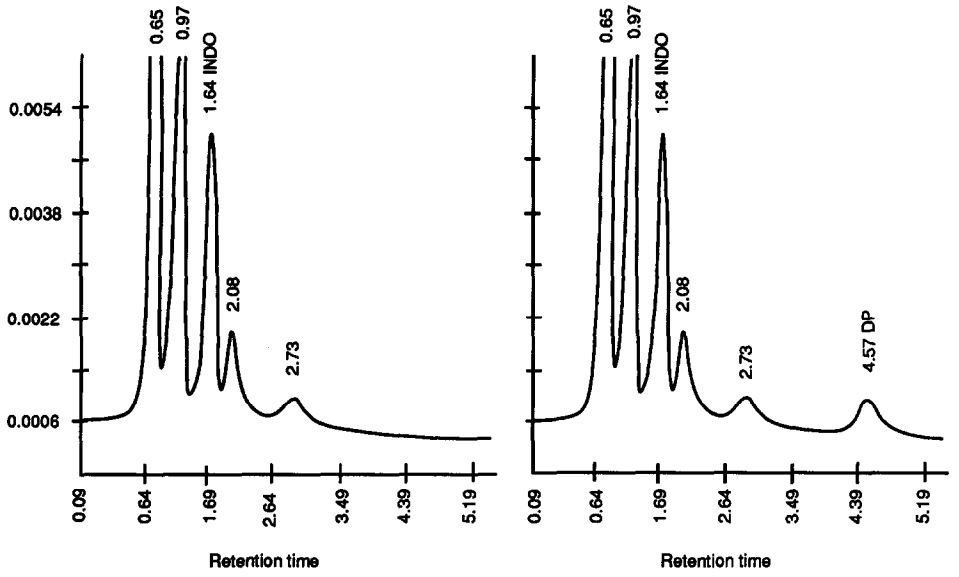

Fig. 1. Left: chromatogram of a control DP-free plasma sample. Right: chromatogram of a plasma sample containing 50 ng/ml DP. INDO (150 ng/ml) was added as an internal standard. For chromatographic conditions, see text. Retention time in minutes.

TABLE II

BETWEEN-DAY VARIABILITY OF PLASMA ASSAY (n = 9)

DP (μM)	Concentration measured (mean ± S.D.) (μM)	R.S.D. (%)	Recovery (%)
0.1	0.074 ± 0.009	12.4	74
0.2	0.158 ± 0.004	2.3	79
1.0	0.830 ± 0.024	2.9	83
2.0	1.781 ± 0.082	4.6	89
10.0	9.105 ± 0.561	6.2	91

TABLE III

PLASMA DP CONCENTRATION AFTER 75 mg ORALLY SIX TIMES DAILY (4 h AFTER THE LAST DP DOSE)

Patient No.	Total DP (μM)	Free DP (μM)	Free DP (%)
1	1.078	0.080	7.4
2	1.374	0.126	9.1
3	1.358	0.122	8.9

Determination of DP in plasma

DP levels in plasma were measured and the results (Table III) showed that DP is extensively bound to the plasma proteins (> 90%).

DISCUSSION

One of the main advantages of HPLC over other chromatographic procedures is the possibility of a very simple diethyl ether extraction and a rapid sample preparation procedure. While giving the same sensitivity, the extraction procedure used is less complex than those mentioned in the literature [3].

This method is very suitable for routine analysis in bioavailability studies, as it is simple (owing to the selective extraction), rapid (because of the short retention time), selective (only DP is detected) and sensitive (the minimum detectable concentration is 20 n*M*).

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REFERENCES

1 J. L. Grem and P. H. Fischer, *Cancer Res.*, 45 (1985) 2967.
2 M. Barberi, J. L. Merlin and B. Weber, *J. Cancer Res. Clin. Oncol.*, 116 (1990) S620.
3 K. M. Wolfram and T. D. Bjornsson, *J. Chromatogr.*, 183 (1980) 57.
4 F. Fontani, G. P. Finardi and G. Targa, *J. Chromatogr.*, 280 (1983) 181.
5 C. Deballon and M. Guernet, *J. Pharm. Biomed. Anal.*, 6 (1988) 1045.
6 A. Kirstein Pedersen, *J. Chromatogr.*, 162 (1979) 98.
7 G. Beisenherz, F. W. Koss, A. Schule, I. Gebauer, R. Bärisch and R. Fröde, *Arzneim.-Forsch.*, 10 (1960) 307.
8 S. B. Zak, H. H. Tallan, G. P. Quinn, I. Fratta and P. Greengard, *J. Pharmacol. Exp. Ther.*, 141 (1963) 392.