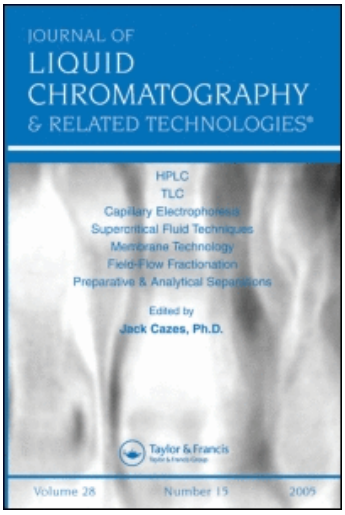

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A SENSITIVE ISOCRATIC LIQUID CHROMATOGRAPHY ASSAY FOR THE DETERMINATION OF DIPYRIDAMOLE IN PLASMA WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

For many years, dipyridamole (DP) has been used in the treatment of hypertension as a vasodilatator, but recently it has been recognized as an anti-platelet aggregation agent and to potentiate cytotoxic. A rapid and very sensitive (1 nM) procedure for the determination of free and protein-bound DP in plasma, using reversed-phase high-performance liquid chromatography on a μBondapark C18 (10μm) column (300 x 3.9 mm I.D.) with coulometric detection (+ 0.65 V), is reported. Free and bound DP were separated using ultrafiltration. The liquid-liquid extraction from plasma included solvent extraction using diethyl-ether and a preparative column to separate DP from constituents normally found in plasma. The particular columns used contained a specially modified form of diatomaceous earth which requires no-preconditioning washes. The overall recovery from plasma was 52 ± 12 % at the concentration of 0.5 ng/ml (1 nM). Concentrations of DP between 1 nM and 1 μM were measured in plasma with relative standard deviations under 7.6 % (n = 6). The subsequent determination of DP levels in patients orally administered from 107 to 500 mg/m^2/day showed that DP binding to plasma protein was higher than 90 % with a mean at 97.2 % (95.5 % to 92.3 %).

INTRODUCTION

Despite intensive efforts to discover new and effective anticancer drugs, the number of clinically useful cytotoxic agents has remained relatively stable. However, great progress has been made in understanding the mechanisms of cytotoxicity of currently available agents and in associating them with drugs which allow a biochemical modulation of cytotoxic agents like the anti-metabolite compounds. Dipyridamole (DP) is a structural analogue of the purine and pyrimidine nucleosides and is used clinically as a vasodilatator and recently, it was shown to potentiate anti-metabolite activity in a dose-dependent manner (1 - 3).

It therefore appears important to control the plasma level of this compound, which is mainly bound to plasma-protein. Several methods, e.g., spectrofluorimetry and high-performance liquid chromatography (HPLC) with spectrophotometric, fluorescence or amperometric detection were previously reported for the determination of DP in biological samples (4 - 6). In addition to being time consuming, spectrofluorimetric methods suffers from the low specificity inherent in non-chromatographic methods. The three reported HPLC methods suffered from a relative lack in sensitivity. Moreover, the HPLC assay using electrochemical detection was only applicable for the determination of DP in pharmaceuticals without developing any procedure for plasma preparation (6).

Yet, no method is available for the rapid determination of free and protein bound DP with a sensitivity lower than 5 ng/ml (10 nM).

The specificity of this method allowed the quantitation of free and total DP in plasma. It describes also a rapid, sensitive (1 nM) and selective HPLC method using a reversed-phase column and coulometric detection involving only a simple liquid-liquid extraction with a preparative column which requires no-preconditionning washes.

The assay was employed during a recent clinical pharmacology study to characterize the high protein binding rate of DP (> 90 %) after *per os* administration (7).

MATERIALS AND METHODS

Apparatus

The HPLC system was a computer-monitored GOLD PC apparatus (Model 126 pumps, Model 406 Interface), (Beckman, Gagny, France), an ESA Coulochem

5100A dual-electrode coulometric detector with an ESA guard cell (Cunow, Cergy Saint-Christophe, France), a μBondapak C18, 10 μm column (300 x 3.9 mm I.D.) and a WISP 512 autosamples (Waters, Millipore, Molsheim, France).

Reagents, Standard Solutions and Internal Standard

DP was purchased from Boehringer-Ingelheim (Reims, France) and indometacin (INDO) from Merck (Darmstadt, Germany). All other reagents were obtained from Prolabo (Paris, France) and were of the highest purity available.

A stock solution containing 5 mg/ml of dipyridamole (DP) was prepared in methanol. The internal standard stock solution indometacin (INDO) 5 mg/ml was also prepared in methanol. Plasma standard solutions of DP for the calibration curves were prepared by appropriate detection of the stock DP solutions with drug-free plasma so that concentrations from 1 to 200 nM were obtained. The plasma standards and the internal standard solution were freshly prepared each day of analysis.

HPLC Conditions

Isocratic elution conditions were adopted. The eluting solvent was methanol - 0.1 M sodium acetate buffer (pH 4.0) (55 : 45, $^{v}/_{v}$). Chem-Elut® columns (Analytichem International, Ann Harbor, Mi, USA) were used for plasma samples preparation. Throughout the study, deionized water (Milli Q water purification system, Millipore) was used.

Room temperature and a flow-rate of 1.0 ml/min were maintained throughout the analyses. A pressure of 100 bars (1 500 p.s.i.) was used. A volume of 20 μl of the samples in methanol was injected into the column.

Electrochemical Detection

The potentials corresponding to the limiting current wave of the DP and INDO were determined by generation of hydrodynamic voltammogram and the potential of the upstream electrode was set to + 0.05 V being lower than the rising portion of the voltammogram of the analyte.

This stabilized the baseline and allowed a better reproducibility of the chromatographic results. The second electrod was used to detect the analyte at its appropriate potential corresponding to the limiting current wave. Amounts of DP were quantified by comparison of peak height with that of a standard sample.

Sample Pretreatment

Blood samples were collected in EDTA tubes and immediately centrifuged at 3 000 g for 10 minutes. The separated plasma was rapidly frozen in polypropylene tubes at - 20°C until analysis. 500 µl of plasma was mixed for 10 s using a rotating mixing and then supplemented with 100 µl of INDO as internal standard.

Free and bound DP were separated using SM 13243 ultrafiltration units (Sartorius, Palaiseau, France) by centrifugation (4 000 g for 30 min).

The aqueous samples were added to the dry Chem Elut® column and left 3 to 5 minutes to absorbe and distribute as a thin film over the hydrophilic packing material. Two milliliters of diethyl-ether were used to eluate the first aliquot.

As the solvent was trickled through the column, it extracted DP from the aqueous layer. Four additioned milliliters of diethyl-ether were used to end the extraction. The extract was concentrated by evaporating the 6 ml of eluant under a gentle steam of nitrogen at ambiant temperature (10 min). The residue was reconstituted in 100 µl of methanol by placing the tube in an ultrasonic water bath for 10 min. The reconstituted residue was then vortexed and transferred to an autosampler microvial.

Plasma samples

A pharmacological clinical study was performed in patients who received oral administration of DP. Blood samples were collected at 8 a.m. and 5 p.m. every day from patients receiving three times daily (morning, midday and evening) DP doses ranging from 107 to 500 mg/m^2/day. Two daily blood samples were analysed by our HPLC method, one at 8 a.m. and the other one at 5 p.m. The blood samples collected at 8 a.m. were analysed before the morning administration of DP, therefore the results shown in table 4 took into account the total dose of the day before whereas the blood sample collected at 5 p.m. took into account only the morning and midday doses (Table 5). As the stability of freshly prepared standard solutions exposed to daylight could show considerable variations (5), all samples and standards were protected from light.

RESULTS

Electrochemical detection

The optimal potential between the two electrodes for the detection of DP and INDO was determined by making voltammograms. Figure 1 shows the

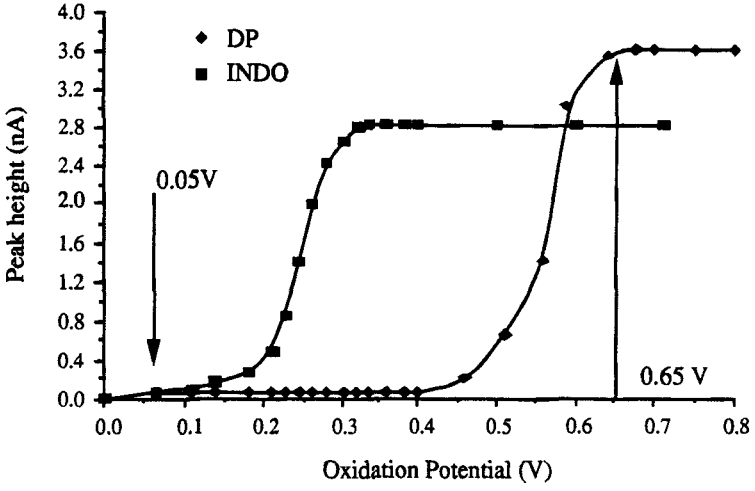

FIGURE 1
Voltammograms of dipyridamole (DP) and indometacin (INDO).

voltammograms for DP, INDO both at 200 ng/ml and the background current from mobile phase constituents. The optimal potential for the detection of DP and INDO was found to be + 0.65 V (Fig. 1).

The chromatogram from a blank plasma spiked with 100 ng of INDO (internal standard), is shown in Figure 2. At a signal-to-noise ratio of 3, the minimal detectable concentrations after the extraction procedure were 0.5 ng/ml (1nM) by injecting 20 µl of 100 µl of reconstituted plasma extract (Fig. 3).

Linearity and precision

In order to investigate the linearity of the extraction and ultrafiltration procedure, blank plasma samples were spiked with DP (1 to 200 nM). The calibration curve showed good linearity as expressed by the following equation :

$y = 0.026 (\pm 0.010) x - 0.053 (\pm 0.008)$

$r = 0.996 \pm 0.003$

The linearity of the extraction procedure without ultrafiltration. Blank total plasma samples were spiked with DP (0.2 to 10.0 µM). The calibration curve showed

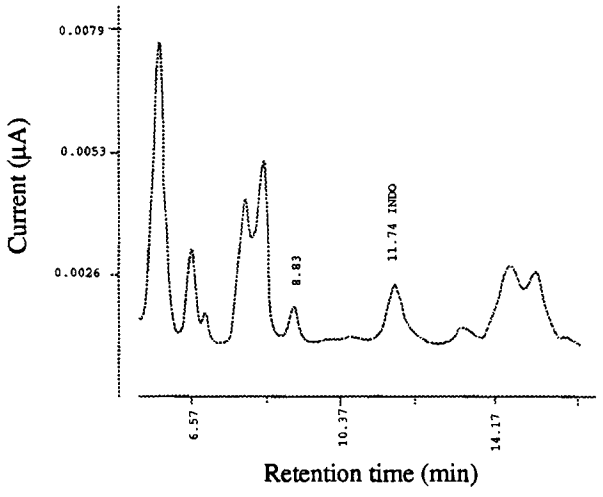

FIGURE 2

Representative chromatogram from 1 ml of plasma control extract, supplemented with 100 ng INDO as internal standard. The mobile phase was methanol - 0.1 M sodium acetate buffer (pH 4.0) (55 : 45, $^{v}/_{v}$). For chromatographic conditions, see text.

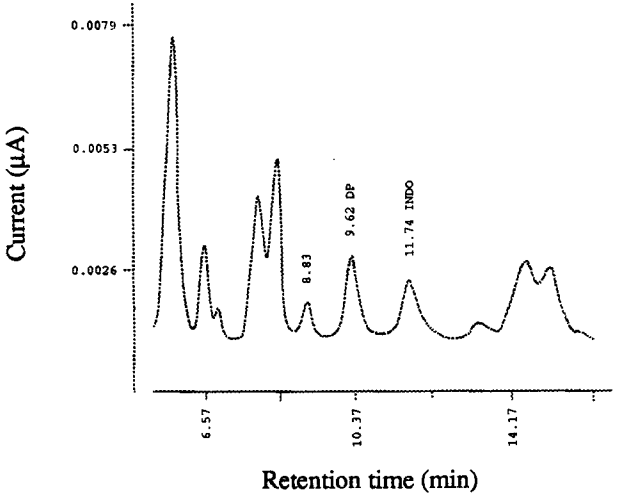

FIGURE 3

Representative chromatogram of a patient plasma sample supplemented with 100 ng INDO and containing DP at 14.6 nM. The mobile phase was as in Fig. 2. For chromatographic conditions, see text.

good linearity as can be seen from the following equation :

$y = 0.074 (\pm 0.006) x - 0.378 (\pm 0.005)$

$r = 0.998 \pm 0.001$

These calibrations curves were obtained from 3 different samples x and y are respectively the concentration of DP (μM) and the peak area, r is the correlation coefficient.

The within-day relative standard deviation (R.S.D.), based on six determinations, was less than 12.0 % for DP at concentrations ranging from 1 to 1 000 nM (Table 1).

The between-day R.S.D. was calculated by performing six analyses of plasma samples containing DP at five concentrations on different days that did not follow. A summary of these analyses is presented in table 2. The between-day R.S.D. were all under 16.0 % (4.3 to 15.3 %). The results indicate a relatively good precision of the assay.

DP stored at -20°C for up to 1 month showed no sign of decomposition and almost the same concentration values were measured (n = 6, table 2).These results suggest that in these storage conditions DP is stable for at least 1 month. As nearly the same concentration values were obtained after a storage at - 20°C for up to 6 months, we could consider that under these conditions DP is also stable for at least 6 months (data not shown).

TABLE 1

Within-day Variability of Total DP Plasma Assay (n = 6)

DP (nM)	Concentration measured (nM)	Coefficient of variation (%)	R.S.D. (%)
1	1.2	7.6	6.3
20	22.4	6.5	12.0
100	97.2	5.0	4.7
200	202.0	2.0	2.2
1 000	1 010.8	2.2	2.3

TABLE 2

Between-day Variability of Total DP Plasma Assay (n = 6)

DP (nM)	Concentration measured (nM)	Coefficient of variation (%)	R.S.D. (%)
1	0.9	9.6	12.0
20	23.0	8.5	15.3
100	96.8	7.0	6.1
200	204.4	4.3	4.7
1 000	1 008.0	4.8	4.3

TABLE 3

Mean Recovery Rates of Total DP at Five Different Concentrations (n = 3)

DP (nM)	Recoveries ± C.V. (%)
1	52 ± 12
20	74 ± 9
100	75 ± 8
200	86 ± 5
1 000	89 ± 5

Under this plasmatic extraction procedure, no major endogenous sources of interference was observed (Fig. 2 and 3) and the specificity between DP and the internal standard (INDO) was satisfactory. The retention times for DP and INDO were 9.6 ± 0.9 and 11.7 ± 1.2 minutes, respectively.

The mean recovery rates of DP from plasma over a concentration range from 1 to 1 000 nM (Table 3) were higher than 74 %, except at 1 nM (52 ± 12 %).

Absolute recovery of DP from plasma was assessed by comparing the peaks of the drug in plasma samples with those obtained by direct injection of DP standard.

Determination of DP in plasma

As DP is a base with a pKa of 6.4, accurate pH control is needed and should be ionised in the acidic sodium acetate buffer (pH : 4.0). Therefore, the mixture methanol - buffer (55 : 45, $^{v}/_{v}$) was chosen as it provides a good resolution between DP and the internal standard.

In order to evaluate the extraction procedure for the analysis of free and total DP, plasma samples of patients were investigated. Free and total DP levels in plasma were measured and the results (Tables 4 and 5) showed that DP is extensively bound to the plasma protein with a mean of free DP at 2.8 % (0.5 to 7.7 %). Statistically significant correlation coefficients were found between DP doses and plasmatic concentrations of free and total DP and also between free and total DP (7).

TABLE 4

Mean Plasma Free and Total DP Concentrations for Plasma Samples at 8 a.m.

DP dose (mg/m^2/day)	Number of patients	Total DP (μM)	Free DP (nM)	Percentage of free DP
107	1	0.146	-	
132	1	0.078	3.4	4.4
150	3	0.112	1.8	0.8
161	2	0.158	2.8	1.8
176	1	0.376	9.6	2.6
200	3	0.568	13.2	2.3
214	1	0.252	6.8	2.7
265	1	0.422	11.4	2.7
300	3	0.592	19.0	3.2
321	1	0.258	7.0	2.7
400	2	1.622	8.0	0.5
450	1	1.386	36.0	2.6
500	1	3.262	-	

- not enough plasma sample

TABLE 5

Mean Plasma Free and Total DP Concentrations for Plasma Samples at 5 p.m.

DP dose (morning + midday) (mg/m^2)	Number of patients	Total DP (μM)	Free DP (nM)	Percentage of free DP
88	1	0.590	12.8	2.2
100	3	0.728	55.4	7.7
107	2	0.794	12.0	1.5
176	1	3.990	54.4	1.4
200	3	2.858	58.4	2.0
214	1	1.298	35.2	2.7
250	2	2.332	43.2	1.8
300	2	6.222	215.4	3.5

DISCUSSION

Electrochemical detection was found to be 20 fold more sensitive than ultraviolet detection at 280 nm (4). No endogenous sources of interference from plasma was observed and the detection limit of the assay can be assigned at 1nM. Compared to other chromatographic procedures, this HPLC method has the advantage to be a very simple and rapid diethyl-ether extraction procedure. The sample preparation used is less complex than those mentioned in the literature (5) while being more sensitive. Overloading the column should be strictly avoided, since if more aqueous sample was added than the column was designed for, the sample could break through the bottom of the column into the collection tubes. The large surface area interfaced between the aqueous and organic layers which gave correct recoveries (Table 3) and eliminated emulsion problem.

These extraction columns contained a specially modified form of diatomaceous earth which requires no pre-conditioning washes however they are not reusable.

In conclusion, a very rapid and accurate method for the analysis of total and free DP has been described that can be employed for therapeutic monitoring or pharmacokinetic studies. Owing to the rapid extraction procedure by a highly

efficient liquid-liquid method and because of the sensitivity (0.5 ng/ml or 1 nM), the selectivity of the procedure and the rapidity, this HPLC assay is quite suitable for routine analysis in bioavailability studies.

As indometacin is also a drug prescribed, the ideal internal standard would be the methoxydipyridamole but this compound is not commercialized (8).

During the last decade, only few anticancer agents actually improved the therapeutic index. However, a better knowledge of the cytotoxicity mechanisms of commercialized drugs allowed to envisage the use of modulators. Such examples of modulation are found in trials associating various antineoplastic agents and DP. This vasodilating agent has recently been shown to potentiate antimetabolite activity in a dose-dependent manner (2, 3). It therefore appears very important to control the free plasma levels of this compound which is mainly bound to plasma protein (Table 4 and 5).

Acknowledgement

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