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Short Communication

Determination of picotamide in human plasma and urine by high-performance liquid chromatography

Tiziano Fossati, Simona Parisi, Gianalfredo Abbiati* and Carlo Castiglioni

LPB Research Institute, Via dei Lavoratori 54, 20092 Cinisello B., Milan (Italy)

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ABSTRACT

A high-performance liquid chromatographic method for the determination of picotamide in human plasma and urine is described. After addition of an internal standard (bamifylline), the plasma and urine samples were subjected to liquid–liquid extraction and clean-up procedures. The final extracts were evaporated to dryness and the resulting residues were reconstituted in 100 μl of methanol–water (50:50, v/v) and chromatographed on a LiChrosorb RP-SELECT B reversed-phase column coupled to an ultraviolet detector monitored at 230 nm. Chromatographic analysis takes about 10 min per sample. The assay was linear over a wide range and has a limit of detection of 0.005 and 0.1 μg/ml in plasma and urine, respectively. It was selective for picotamide, accurate and robust and thus suitable for routine assays after therapeutic doses of picotamide.

INTRODUCTION

Picotamide (Plactidil) [N,N′-bis(3-picolyl)-4-methoxy-isophthalamide] (CAS 80503-63-8) (see Fig. 1 for chemical structure) is a new antiplatelet drug which has been shown in *in vitro* and *ex vivo* experiments to have platelet inhibitory effects in animals and man [1,2]. It inhibits thromboxane A_2, without interfering with the production of vasodilatory and antiaggregatory prostaglandins [3], and it simultaneously blocks the platelet receptors for thromboxane A_2 synthase and cyclic endoperoxides [4].

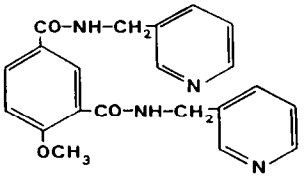

Fig. 1. Structure of picotamide [N,N′,-bis(3-picolyl)-4-methoxy-isophthalamide].

The present report describes a relatively simple yet highly specific liquid chromatographic procedure for the detection of picotamide in human body fluids, which is sensitive and precise enough to measure the plasma and urine concentrations of unchanged drug that may be met in pharmacokinetic studies and therapeutic drug monitoring. Preliminary pharmacokinetic data of patients receiving picotamide orally are presented.

EXPERIMENTAL

Materials

Stock solutions of picotamide, pharmaceutical grade (Istituto Farmaceutico PB, Cinisello Balsa-

0378-4347/92/$05.00

mo, Italy), were prepared in methanol at concentrations of 1 mg/ml. They were stable for at least one month if stored at 0–4°C. Standard solutions were prepared from stock solutions by dilution with methanol.

Bamifylline (internal standard) [8-benzyl-7-(N-ethyl-N-(β-hydroxyethyl)aminoethyl)theophylline] was obtained from Chiesi Farmaceutici (Parma, Italy). Analytical-grade acetonitrile, chloroform, methanol, 2-propanol, ethyl acetate, hydrochloric acid (37%), sodium hydroxide and sodium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany).

Apparatus and chromatographic conditions

The analyses were conducted on a Waters system equipped with a Model 712 intelligent sample processor and a Model 600 E solvent delivery system (Waters Assoc., Milford, MA, USA), a reversed-phase column (LiChrosorb RP-SELECT B, C_8, 25 cm × 4 mm I.D., particle size 5 μm) (Merck) maintained at room temperature and a Model 490 E UV detector monitored at 230 nm. The mobile phase was acetonitrile–0.05 *M* sodium dihydrogenphosphate (pH 5.5) (28:72, v/v) delivered isocratically at a flow-rate of 1 ml/min. The mobile phase was filtered through a 0.45-μm filter and degassed with helium during use.

The detector was coupled to a computer terminal (Maxima 820 Data System, Waters Assoc.) for data acquisition and evaluation.

Plasma sample collection

Human blood samples were collected by direct venipuncture and put in heparinized tubes. Plasma was separated by centrifugation at 670 *g* and stored frozen until analysed.

Sample preparation

To heparin-treated plasma (1 ml) and urine (0.1–1 ml), 0.02 ml of a methanolic solution of bamifylline (0.05 and 2.5 mg/ml) was added. Then the samples were alkalinized using 25% aqueous ammonia (60 and 100 μl for plasma and urine, respectively) and extracted with 5 ml of chloroform–isopropanol (95:5, v/v) by shaking for 20 min. The layers were separated by centrifugation at 2000 *g* for 15 min and the organic extract was transferred to new test tubes and evaporated to dryness. The residues were dissolved in 100 μl of 0.6 *M* hydrochloric acid (1.2 *M* for urine), 0.6 ml of ethyl acetate were added and the samples were then vortex-mixed for 15 s. After centrifugation (2000 *g* for 3 min), the organic phase was discarded and the acidic aqueous phase was evaporated to dryness under vacuum. The residues were dissolved in 0.1 ml of methanol–water (50:50, v/v) and 25 μl were injected into the HPLC system.

Validation procedure

Drug-free plasma with known amounts of picotamide added was analysed concurrently with each set of unknown samples. At least five different concentrations across the working range were measured in duplicate. Because of the wide range of picotamide concentrations encountered in different body fluids and experimental conditions, it was necessary to use different calibration graphs, in the range 0.005–2 μg/ml for plasma and 1–100 μg/ml for urine. Calibration curves were calculated by the least-squares method. Peak-area ratios between the compound and the internal standard were used to generate the linear least-squares regression lines. Concentrations of picotamide in the unknown samples were obtained by interpolation from these calibration curves using peak-area ratios from unknown samples. Plasma blanks were used to monitor for interference.

The intra- and inter-assay precision were checked by preparing quality control (QC) samples at the start of the validation study. Intra-assay (within-day) precision was obtained by replicate analysis of plasma and urine samples on the same day. Inter-assay (day-to-day) precision was established by analysing the QC samples on various days over four weeks.

The recovery of picotamide was checked by comparing the peak-area ratios measured in plasma extracts spiked with the compound with the peak-area ratios of equal amounts of directly injected picotamide solution. The internal standard was added just before injection into the chromatograph.

RESULTS AND DISCUSSION

Optimal chromatographic separation of picotamide and bamifylline was achieved using a mobile phase of 0.05 *M* sodium dihydrogenphosphate (pH 5.5)–acetonitrile (72:28, v/v) at a flow-rate of 1 ml/min. Examples of chromatograms from 1-h post-dose plasma and 0–4 h urine samples of a volunteer treated orally with picotamide (300 mg) are shown in Fig. 2A and C, respectively. The response for picotamide corresponded to 1.2 μg/ml in plasma and 223 μg/ml in urine. The chromatographic peaks had baseline separation and the extract of drug-free plasma (Fig. 2B) and urine (Fig. 2D) showed no peaks that could interfere with analysis of the unchanged compound. Approximate retention times were 7 and 10 min for bamifylline and picotamide, respectively.

Of the various organic solvents tested to isolate picotamide from biological fluids, a chloroform–2-propanol mixture (95:5) gave quantitative recoveries for both the drug and the internal standard, but interfering endogenous components were often present in the chromatograms.

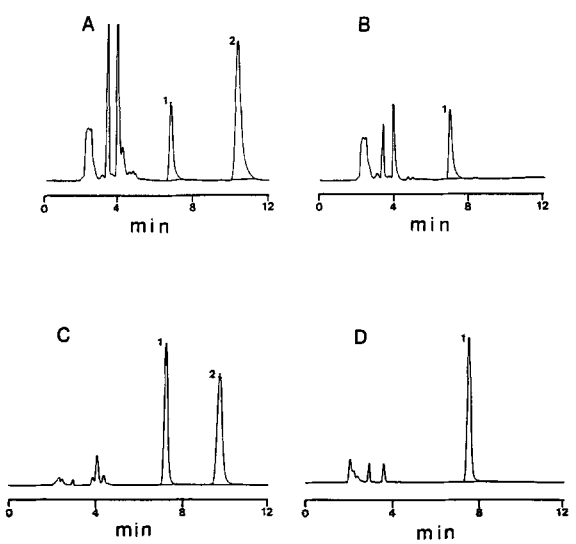

Fig. 2. Chromatograms of extracts from a 1-h post-dose plasma sample (A) and 0–4 h urine sample (C) of a healthy volunteer after oral picotamide (300 mg). (B) and (D) are the predose plasma and urine, respectively. Peaks: 1 = bamifylline (internal standard); 2 = picotamide.

The extraction procedure with the chloroform–2-propanol mixture was thus followed by a clean-up step before instrumental analysis and this reduced the problem. Under these conditions overall mean recovery, summarized in Table I, was 86% (S.D. = 3%) and 95% (1%) for picotamide and 83% (5%) and 89% (4%) for the internal standard (1 and 50 μg/ml, $n = 4$) in plasma and urine, respectively [coefficients of variation (C.V.) were less than 5% at each concentration tested]. The recovery of picotamide did not show dependence on concentration over the range investigated in plasma (0.005–2 μg/ml) and urine (1–100 μg/ml), hence regression analysis over these concentration ranges gave high linearity in both body fluids ($y = 0.380x + 0.0015$, $r > 0.99$, in plasma; $y = 9.7x + 1.5$, $r > 0.99$, in urine).

Blank samples did not give a response for picotamide, and the intercept of the linear regression line was not significantly different from zero in plasma or urine. The minimum detectable amount was assumed to be the lowest validated point of the plasma and urine standard curves, *i.e.* 0.005 and 1 μg/ml in plasma and urine, respectively, using 1 ml of body fluid.

Human plasma and urine QC samples spiked with different amounts of picotamide were assayed with each of the HPLC chromatographic

TABLE I

RECOVERY OF PICOTAMIDE FROM SUPPLEMENTED DRUG-FREE AND URINE SAMPLES AND COEFFICIENTS OF VARIATION FOR REPLICATE ASSAYS (n = 3)

Concentration added (μg/ml)	Recovery (%)	C.V. (%)
Plasma		
0.025	90	3.8
0.1	86	3.1
0.5	85	5.3
1	85	0.7
Urine		
1	95	4.9
2	96	4.0
100	94	0.8

TABLE II

INTER- AND INTRA-ASSAY PRECISION AND REPRODUCIBILITY OF THE METHOD (n = 5)

Plasma			Urine		
Concentration added (μg/ml)	Concentration found (μg/ml)	C.V. (%)	Concentration added (μg/ml)	Concentration found (μg/ml)	C.V. (%)
Intra-assay					
0.005	0.0047	9.8	1	0.97	7.5
0.010	0.0097	2.1	10	9.95	5.2
0.025	0.022	9.6	40	41.9	4.9
0.1	0.099	3.5	100	100.2	2.1
0.5	0.516	2.5			
Inter-assay					
0.025	0.026	9.1	1	0.96	8.8
0.1	0.102	7.1	10	10.1	3.1
0.4	0.400	4.4	40	42.8	8.3
0.8	0.816	2.9	100	97.0	4.4
1.6	1.632	2.2			

runs in support of this study. Intra-assay C.V. at the low levels were 9.8 and 7.5%, respectively, in plasma (0.005 μg/ml) and urine (1 μg/ml), and for all higher concentrations it was less than 10% in both body fluids. The results, summarized in Table II, indicate that the method has acceptable reproducibility too, the inter-assay C.V. being $\leq 10\%$ in all cases.

The relative error [R.E. = $(F - A)/A \times 100$] of these QC samples, calculated from the deviation of the mean concentration found (F) from the nominal value (A), indicated inter-assay variation from 2 to 4% in plasma and from 1 to 7% in urine.

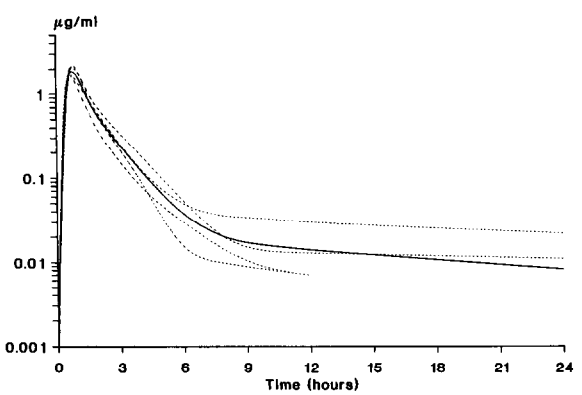

Fig. 3. Individual (broken lines) and mean (solid line) plasma concentration–time curves of picotamide after an oral dose (300 mg) to four healthy male volunteers.

The utility of the analytical method was established by analysing plasma and urine samples from healthy male volunteers given oral doses of picotamide in a pilot kinetic study. The individual plasma concentration–time curves of the drug for four subjects after 300-mg picotamide tablets are depicted in Fig. 3. The plasma specimens were obtained from 15 min to 24 h after drug administration. The observed time to maximum concentrations was in the range of 0.5–0.75 h with a mean (±S.D.) of 0.69 (±0.13), indicating rapid absorption of the tablet formulation from the gastrointestinal tract. The peak plasma concentration was on average 2.02 ± 0.37 μg/ml, with five-fold individual variability. The plasma concentration then fell to levels close to the limits of the analytical procedue at 8–24 h after dosing.

About 28% of the orally administered dose was recovered as unchanged drug in the 0–24 h urine of these subjects, mostly (about 24%) during the first 4 h after dosing. Therefore the method reported here has sufficient sensitivity to mon-

itor the plasma and urine levels of unchanged drug that might be met in pharmacokinetic and monitoring studies.

Further, more detailed clinical investigations, including a dose proportionality study and characterization of the multiple-dose kinetics of picotamide, are now in progress in our laboratory.

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