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Short communication

Determination of ticlopidine in human plasma by high-performance liquid chromatography and ultraviolet absorbance detection

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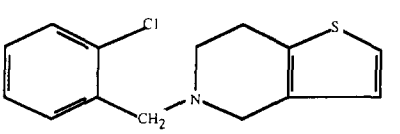
Abstract

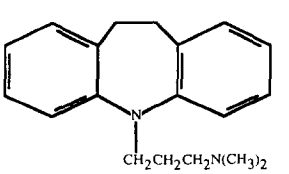
A simple HPLC method has been developed for the determination of ticlopidine in human plasma. Plasma samples were buffered at pH 9 and extracted with *n*-heptane–isoamyl alcohol (98.5:1.5, v/v). Imipramine was used as internal standard. Chromatography was performed isocratically with acetonitrile–methanol–0.05 *M* KH_2PO_4 (20:25:55, v/v) at pH 3.0 containing 3% triethylamine at a flow-rate of 1 ml/min. A reversed-phase column, Supelcosil LC-8-DB, 15 cm × 4.6 mm I.D., 5 μm particle size, was used. The effluent was monitored by UV absorbance detection at 235 nm. The method showed good accuracy, precision and linearity in the concentration range 5–1200 ng/ml. The limit of quantitation was 5 ng/ml, with a precision (C.V.) of 8.91%, which is the same as that achieved by other authors with a previously published GC–MS method. The procedure described in this paper is simple and allows the routine assessment of ticlopidine plasma concentration in pharmacokinetic studies following therapeutic doses in human subjects.

1. Introduction

Ticlopidine [5-(2-chlororbenzyl)-4,5,6,7-tetrahydrothieno-(3,2-C)pyridine hydrochloride, Fig. 1], an inhibitor of platelet aggregation, proved to be endowed with a potent antithrombotic activity in several animal models, and is therapeutically used in the prevention of stroke and myocardial infarct in high risk patients [1–3]. Ticlopidine hydrochloride is marketed as Tiklid® in Europe



Ticlopidine



Imipramine (internal standard)

Fig. 1. Chemical structures of ticlopidine and imipramine (internal standard).

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and as Panaldine® in Japan, whereas it is in clinical trials in USA and Canada [4].

Pharmacokinetic studies have demonstrated that ticlopidine is well absorbed through the gastrointestinal tract; about 98% of circulating drug is reversibly bound to plasma proteins. Its terminal half-life proved to be less than 8 h after single administration, whereas it increased to about 96 h in a repeated-dose regimen, this being likely attributable to the appearance of an additional kinetic phase after the multiple dosing, not detectable after a single dose. The drug is cleared from the body through biotransformation processes and only scarcely through renal excretion of non-modified parent drug [1,5].

From previous publications, ticlopidine can be assayed in plasma by HPLC with spectrophotometric detection with a limit of quantitation (LOQ) of 50 ng/ml or by GC with thermoionic detection or by GC–MS with LOQs of 20 and 5 ng/ml, respectively [4,6,7].

The aim of the present investigation was to set up and validate a simple HPLC assay for ticlopidine using UV absorbance detection which would be applicable to pharmacokinetic studies with the same LOQ (5 ng/ml) as that of the GC–MS assay previously published [7].

This goal was achieved optimising the selectivity and reproducibility. Extraction was carefully investigated in order to recover 100% of the analyte and nil of its more polar metabolites, which led to a very good selectivity. The choice of the Supelcosil LC-8-DB column resulted from a specific study on the complete separation of internal standard and ticlopidine. The high sensitivity was reached as the analytical conditions selected allowed 43% of the ticlopidine contained in 1 ml of plasma to be injected.

2. Experimental

2.1. Chemicals

All solvents and reagents were of analytical or HPLC grade and were purchased from Carlo Erba (Milan, Italy), Merck (Darmstadt, Germany), Pierce (Rockford, IL, USA). HPLC grade water was produced by the Milli-Q-G system (Millipore, Bedford, MA, USA). Ticlopidine hydrochloride and imipramine hydrochloride (used as an internal standard, I.S.) were purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus and chromatographic conditions

The HPLC was consisted of two pumps (Model 880-PU, Jasco, Tokyo, Japan), an UV absorbance detector (Model 841 LC spectrophotometer from Waters-Millipore, Milford, MA, USA), an autosampler (Model 851-AS from Jasco), a computerised integrator (Data System Model 450 MT 2 from Kontron, Zürich, Switzerland) and the analytical column (Supelcosil LC-8-DB, 15 cm × 4.6 mm I.D., 5 μm particle size; Supelco, Bellefonte, PA, USA) equipped with a guard column (Supelguard LC-8-DB, 2 cm × 4.6 mm I.D., 5 μm particle size; Supelco).

Analysis was carried out isocratically, using a mobile phase of acetonitrile–methanol–0.05 *M* KH_2PO_4 pH 3.0 containing 0.2% of triethylamine (20:25:55, v/v) filtered through a 0.45-μm membrane (type HATV, Millipore). The flow-rate was 1.0 ml/min and the effluent was monitored at 235 nm. Under these conditions, the retention times for ticlopidine and the I.S. were 7.6 and 11.6 min, respectively (see Fig. 2).

2.3. Preparation of standard and quality control (QC) samples

A stock solution of ticlopidine 1 mg/ml (as free base) in methanol was further diluted to yield appropriate working solutions for the preparation of the calibration plasma standards; an aliquot of 20 μl of each working solution was then added to 1 ml of blank human control plasma to give 5, 10, 20, 50, 120, 400 and 1200 ng/ml. QC samples were obtained by spiking 1 ml of blank control plasma with 20 μl of another set of working solutions prepared in order to obtain final concentrations of 25, 60 and 600 ng/ml of plasma. A stock I.S. solution was prepared at a concentration of 1 mg/ml in methanol and diluted to 20 μg/ml with methanol to obtain the working solution. All stock and

working solutions were stored at 0–4°C and proved to be stable for at least one month.

2.4. Sample preparation

Frozen samples were allowed to thaw at room temperature. In 100 × 16 mm glass tubes with PTFE-lined screw caps, 1-ml aliquots of plasma were transferred and 20 μl of I.S. solution and 1 ml of 0.5 *M* phosphate buffer pH 9 were added and vortex-mixed briefly. The analytes were extracted with 7 ml *n*-heptane–isoamyl alcohol mixture (98.5:1.5, v/v) on a rotating shaker at 32 rpm for 15 min. After centrifugation at 1500 *g* for 5 min, the organic layer (6 ml) was transferred to a centrifuge conical glass tube and evaporated to dryness at 60°C under a gentle stream of nitrogen. The solution was reconstituted with 200 μl of mobile phase, vortex-mixed for 10 s and centrifuged at 1500 *g* for 3 min. This solution was transferred to autosampler vials and 100-μl aliquots were injected for assay.

2.5. Calibration and calculations

Seven calibration samples covering the expected concentration range (5–1200 ng/ml) were daily extracted and processed together with unknown and QC samples. The calibration graph was obtained plotting the weighted ($1/\text{concentration}^2$) plasma concentration of ticlopidine vs. ticlopidine/I.S. peak-height ratios by the linear regression method. The ticlopidine concentration in QC and unknown samples was calculated using the above regression equation.

3. Results and discussion

3.1. Linearity and reproducibility

The linearity of the assay was ascertained from a seven point calibration graph with 5 replicates carried out in the range 5–1200 ng/ml. A mean coefficient of correlation (r^2) of 0.99934, an accuracy (percentage difference between nominal and actual values) from back calculated concentration not exceeding +1.40 and −2.70% and a precision (C.V.) <7.19% were obtained. Data are shown in Table 1.

3.2. Intra-day and inter-day precision and accuracy

Intra-day precision and accuracy were achieved from the values of QCs and that of the lowest concentration (5 ng/ml) assayed with 5 replicates with reference to a calibration curve in the range 5–1200 ng/ml. Accuracy ranged from −9.00 to +5.78% and precision was <9.08%.

Inter-day precision and accuracy were calculated from 17 replicates of the QCs, analysed on different days. Accuracy ranged from −0.30 to

Table 1
Linearity and reproducibility of ticlopidine assay evaluated from the back-calculated concentrations of five calibration curves

Concentration added (ng/ml)	Mean concentration found (ng/ml)	Accuracy (%)	Precision (%)
1200	1200.60	+0.05	4.62
400	389.19	−2.70	4.08
120	120.75	+0.63	4.35
50	50.70	+1.40	4.32
20	19.84	−0.78	4.14
10	9.99	−0.06	7.19
5	5.01	+0.12	4.12

Linear regression led to the following relationship: ticlopidine found (y) = $-1.62 \times 385.25x$, $r^2 = 0.99934$, where x is the ticlopidine/I.S. peak-height ratio.

Table 2
Intra-day and inter-day precision and accuracy of ticlopidine assay

Concentration added (ng/ml)	*n*	Mean concentration found (ng/ml)	Accuracy (%)	Precision (%)
Intra-day				
600	5	630.53	+ 5.09	9.08
60	5	60.60	+ 1.00	8.19
25	5	26.44	+ 5.78	7.51
5	5	4.55	− 9.00	8.51
Inter-day				
600	17	603.14	− 0.52	7.50
60	17	59.82	− 0.30	9.04
25	17	24.51	− 1.96	8.90

−1.96%, precision was <9.04%. Data are shown in Table 2.

3.3. Limit of quantification (LOQ)

The LOQ proved to be as low as 5 ng/ml with values of precision and accuracy of 8.51% and −9.00%, respectively (Table 2).

3.4. Selectivity

No significantly interfering peaks from the matrix were found at the retention times of both ticlopidine and internal standard (Fig. 2), as judged from the analysis of more than 50 blank plasma samples. As described in the literature [8,9], ticlopidine is cleared from the body through biotransformation processes giving various metabolites, which however are more polar than the parent drug and thus are not extracted and do not interfere in the assay.

3.5. Recovery

The extraction yield in the investigated concentration range proved to be on average 100.56% with a mean C.V. of 4.69%, evaluated from three concentrations assayed in triplicate.

3.6. Stability

No significant change in ticolpidine concentration was detected in plasma samples stored at room temperature over 24 h nor after three freeze–thaw cycles. The analytes proved to be stable also in samples reconstituted with mobile phase, stored into autosampler vials at room temperature for at least 24 h.

3.7. Pharmacokinetic application

The method was used to assay the ticlopidine plasma concentration in 24 healthy subjects treated in fasting status with the drug, i.e. one tablet of Tiklid containing 250 mg of active ingredient. Fourteen seriated blood samples were drawn over a period of 24 h after dosing.

Fig. 3 shows the plasma concentration–time profiles for ticlopidine observed in two of the above subjects, one laying in the higher and the other in the lower concentration range. Fig. 3 shows a high variability in the ticlopidine plasma concentrations, in agreement with data previously obtained by other authors [5]. This behaviour usually occurs with drugs that meet a relevant hepatic first pass effect, and justified the size of the test group of 24 healthy volunteers in the bioequivalence study that was carried out using this analytical method. The test-group size is, in fact, a crucial problem in bioequivalence trials

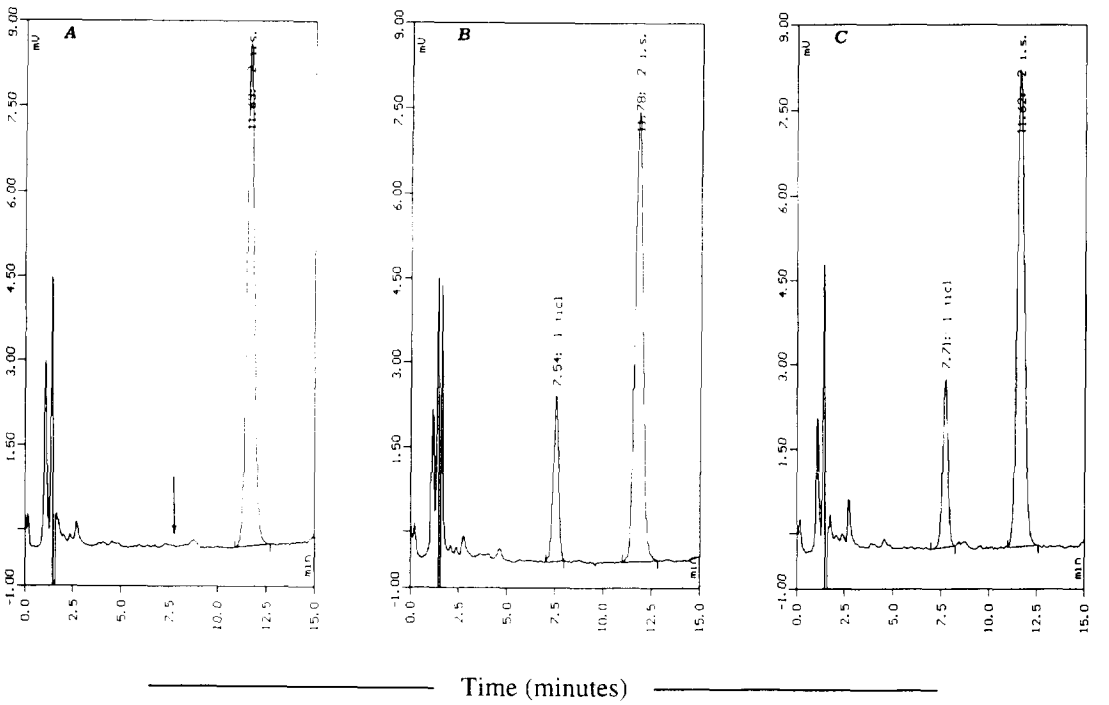

Fig. 2. Representative chromatograms of plasma extracts. (A) Drug free human plasma spiked with imipramine (internal standard). The arrow indicates the retention time of ticlopidine. (B) Blank plasma spiked with 120 ng/ml of ticlopidine. (C) Plasma sample of a subject 3 h after a 250-mg oral dose of ticlopidine, 124.4 ng/ml.

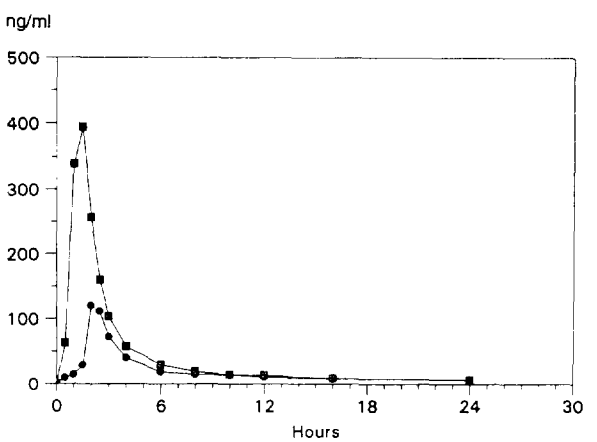

Fig. 3. Plasma concentration–time profile of ticlopidine in 2 healthy volunteers treated with the drug (Tiklid, 250 mg).

largely depending on the data dispersion encountered.

4. Conclusions

In conclusion this method proved to be suitable for pharmacokinetic and bioavailability studies.

The instrumentation involved is very common in pharmacokinetic laboratories, the I.S. as well as the column and chemicals all are easily available on the market. Working with an autosampler injector, a skilful operator can process within 24 h (one working day) an analytical run consisting of 40 unknown samples, plus 7 calibration samples and 6 QCs. The method thus is simple, of low cost and has a limit of quantitation comparable with that previously described by Arnoux et al. [7] for a GC–MS method.

This method was carefully validated for pharmacokinetic and bioavailability investigation in humans, its robustness was demonstrated in a pre-study validation and in the study specific validation carried out on more than 1000 unknown samples assayed in 30 analytical runs.

Possible application of this assay in pharmacokinetic and toxicokinetic studies in animals may require an extensive redevelopment prior to the appropriate validation in other species and matrices.

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