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Liquid chromatographic method for the determination of ticlopidine in human plasma

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Abstract

A simple high-performance liquid chromatographic method for determination of ticlopidine in human plasma using ultra violet detection was developed. The separation of the investigated compound and internal standard was achieved on a C_{18} BD column with a 0.01 *M* potassium dihydrogen phosphate buffer (pH 4)–acetonitrile–methanol (20:40:40, v/v) mobile phase. The detection was performed at 215 nm. The compounds were isolated from plasma by Bond Elut C_{18} solid-phase extraction, the mean absolute recovery was 84.9%. The limit of quantitation was 10 ng ml^{-1}, the limit of detection was 5 ng ml^{-1}. The bioanalytical method was validated with respect to linearity, within- and between-day accuracy and precision, system suitability and stability. All validated parameters were found to be within the internationally required limits. The developed analytical method for ticlopidine was found to be suitable for application in pharmacokinetic studies and human drug monitoring.

Keywords: Ticlopidine

1. Introduction

Ticlopidine (TIC) a tieno-[3,2-c]-pyridine derivative is an inhibitor of platelet aggregation in human plasma. TIC has been used in the treatment of thromboembolism, cerebral infarction and transient ischemic attack [9,10].

Several HPLC methods have been developed for quantitative determination of TIC in human plasma [1–4]. Until now these methods have limits of quantitation which are not sufficiently sensitive for bioequivalence studies. For sample processing, ethylacetate was used as extracting solvent, however, in contrast to the liquid–liquid extraction procedure described in the literature [2,4], we preferred solid-phase extraction (SPE). The method of Arnoux et al. [1] reported to ensure 88–99% recovery using Extrelut column for sample processing was also tried, then abandoned due to relatively low recovery (55–60%). Fujimaki et al. [3] eluted the sample with hexane–chloroform from a Sep-Pack C_{18} cartridge and chromatographed the eluate on a Nucleosil 7 C_{18} analytical column with phosphate buffer (pH 7)–acetonitrile as mobile phase. The reversed-phase chromatography with the mobile phase of phosphate buffer–acetonitrile as reported by several investigators [1,3,4], as well as the mobile phase of methanol–water–ammonia (80:20:1, v/v) [2] were found to be appropriate for the chromatography of TIC and the internal standard in human plasma.

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Nevertheless, for the purposes of the present analytical work, we have selected a phosphate buffer (pH 4)–methanol–acetonitrile mixture as mobile phase since it produced less noisy chromatograms than the other eluents.

The aim of our study was to develop of an HPLC method for the bioequivalence investigation of TIC containing drugs.

The method was required to be suitable for monitoring TIC plasma levels in the concentration range of 10–1000 ng ml^{-1}. It should be noted that the mean values of TIC concentration after oral administration of 250 mg twice daily to healthy volunteers is about 0.2–0.9 μg ml^{-1} [10].

Ticlopidine dichloro derivative, which we used as internal standard for quantitative determinations has been described in a number of reports [1–4].

2. Experimental

2.1. Materials

Ticlopidine (TIC) {5-(*o*-chlorobenzyl)-4,5,6,7-tetrahydrotieno-[3,2-c] pyridine hydrochloride} and the internal standard (I.S.) {5-[2,4-(*o*-dichlorobenzyl)] - 4,5,6,7 - tetrahydrotieno - [3,2 - c] pyridine} were provided by Egis Pharmaceuticals (Budapest, Hungary). The structural formulae of the compounds are depicted in Fig. 1.

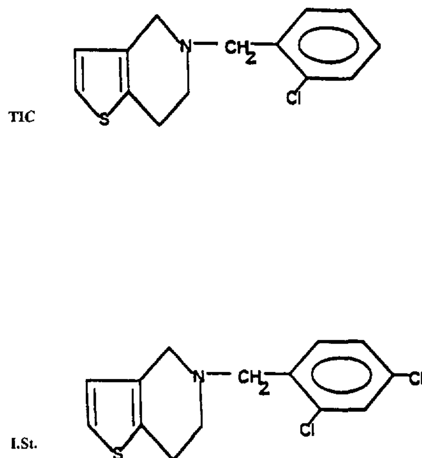

Fig. 1. Structural formulae of ticlopidine (TIC) and the internal standard (I.S.).

Methanol, acetonitrile, ethylacetate were all LiChrosolv grade and purchased from Merck (Darmstadt, Germany). Bond Elut C_{18} cartridge of 1 ml was the product of Varian (Harbor City, CA, USA). Sodium hydroxide, disodium hydrogen phosphate, potassium dihydrogen phosphate and EDTA disodium salt were obtained from Reanal (Budapest, Hungary). All the chemicals used were of analytical grade.

2.2. Chromatographic conditions

For liquid chromatography an LC-6A pump, an SPD-6A detector and a C-R6A integrator were used (Shimadzu, Kyoto, Japan). For sample injection a SIL-6B (equipped with SCL-6B system controller) (both Shimadzu, Kyoto, Japan) autosampler fitted with a 50-μl loop was applied.

The separation was accomplished at ambient temperature (air-conditioned room with temperature of 22±2°C), on a BST Rutin 10, C_{18} BD (250×4 mm I.D.) analytical column equipped with a BST Rutin 10, C_{18} (30×4 mm I.D.) guard column (Bio-Separation Technologies, Budapest, Hungary). (BST Rutin C_{18} is equivalent to Hypersyl BDS C_{18} (Shandon) stationary phase). The flow-rate was 1.0 ml min^{-1}. The detection wavelength was 215 nm. The mobile phase consisted of 0.01 *M* potassium dihydrogen phosphate buffer (pH 4), acetonitrile and methanol (20:40:40 v/v). The eluent was filtered through a 0.45-μm nylon 66 membrane (Supelco, Bellefonte, PA, USA) and degassed by purging it with high purity nitrogen.

2.3. Solutions

Stock solutions (1 mg ml^{-1}) of both TIC and the I.S. were prepared with methanol. When stored at −20°C, both stock solutions were stable for at least 8 weeks.

Working standard solutions of 10 and 1 μg ml^{-1} concentrations were obtained by diluting the stock solutions with methanol. The working standard solutions were prepared freshly every week and stored in a refrigerator at 4°C.

Blank human plasma was prepared from blood obtained by venipuncture from the cubital vein. As anticoagulant, disodium-EDTA was used at a con-

centration of 1 mg ml^{-1} of whole blood. The anticoagulant was dissolved in distilled water (100 mg ml^{-1}) and 0.1 ml of the solution was added to 10 ml of blood. Whole blood supplemented with the anticoagulant was centrifuged at 1500 *g* for 10 min and the resulting plasma was stored at −20°C until processing.

2.4. Sample processing

To 1 ml of sample 500 ng of the I.S. (50 μl of a 10 μg ml^{-1} concentration methanolic solution) and 1 ml of 1.0 *M* sodium hydroxide solution were added. After homogenising it by short vortexing, the sample was transferred to a Bond Elut C_{18} solid-phase extraction (SPE) cartridge (1 ml) previously activated with 1 ml of methanol and with 1 ml of 0.01 *M* sodium hydroxide. After sample application, the cartridge was washed with 4 ml of methanol–0.01 *M* sodium hydroxide 1:1 mixture then it was dried by sucking air through the cartridge. The solute was eluted with 0.5 ml of ethylacetate. The eluate was evaporated to dryness under a stream of nitrogen at 37°C. The dry residue was dissolved by vortexing in 200 μl of the mobile phase.

2.5. Method validation

The present method was validated according to internationally accepted criteria [5–7].

2.5.1. Quality control (QC) samples

For method validation, quality control samples were prepared from pooled human plasma, in advance at 50, 250 and 1000 ng ml^{-1} TIC levels. QC samples were stored deep-frozen at −20°C in 1.2-ml aliquots. Each QC sample was fortified with the I.S. (500 ng to 1 ml of plasma) just prior to sample processing.

2.5.2. System suitability test

Working standard solutions (10 μg ml^{-1}) of both TIC and the I.S. were chromatographed with 5–5 parallel injections.

2.5.3. Summary of calibration curve parameters

Calibration samples were prepared by adding 0, 10, 50, 100, 250, 500 and 1000 ng of TIC to 1 ml of blank pooled human plasma. Each sample was fortified with 500 ng of the I.S. The samples were processed and chromatographed as described above. Six parallel determinations were made at each concentration level.

For constructing calibration curve, peak area ratios of the TIC and internal standard were plotted against nominal concentration values using the KALIB computer program of the Department of Pharmacokinetics, Egis Pharmaceuticals. The calibration curve was fitted using combinatory method [8]. The *F* test for linearity and linear regression analysis were chosen for testing linearity.

2.5.4. Within-day precision and accuracy

The within-day precision and accuracy of the present method were determined by the analysis of quality control (QC) samples at three different concentration levels. Five parallel determinations were made at each level.

2.5.5. Between-day precision and accuracy

The between-day precision and accuracy were determined by the analysis of QC samples at 3 different concentration levels on 5 different days covering a 4-week study period.

2.5.6. Determination of absolute recovery

Blank plasma samples (1 ml) spiked with 50, 250, 500 and 1000 ng of TIC were processed without the addition of I.S. by the solid-phase extraction procedure. The internal standard (500 ng) was added only to the evaporation residue of the eluate dissolved in the mobile phase. Three parallel determinations were made at each concentration level. The peak area ratios obtained with the extracted samples were compared to those of the corresponding aqueous solutions of TIC and the I.S..

2.5.7. Stability test

The stability of TIC in human plasma was studied at 50, 250 and 1000 ng ml^{-1} levels after 4 and 6 weeks of storage at −20°C. The samples were fortified with the internal standard right prior to processing.

Three parallel determinations were made at each concentration level and for both storage periods.

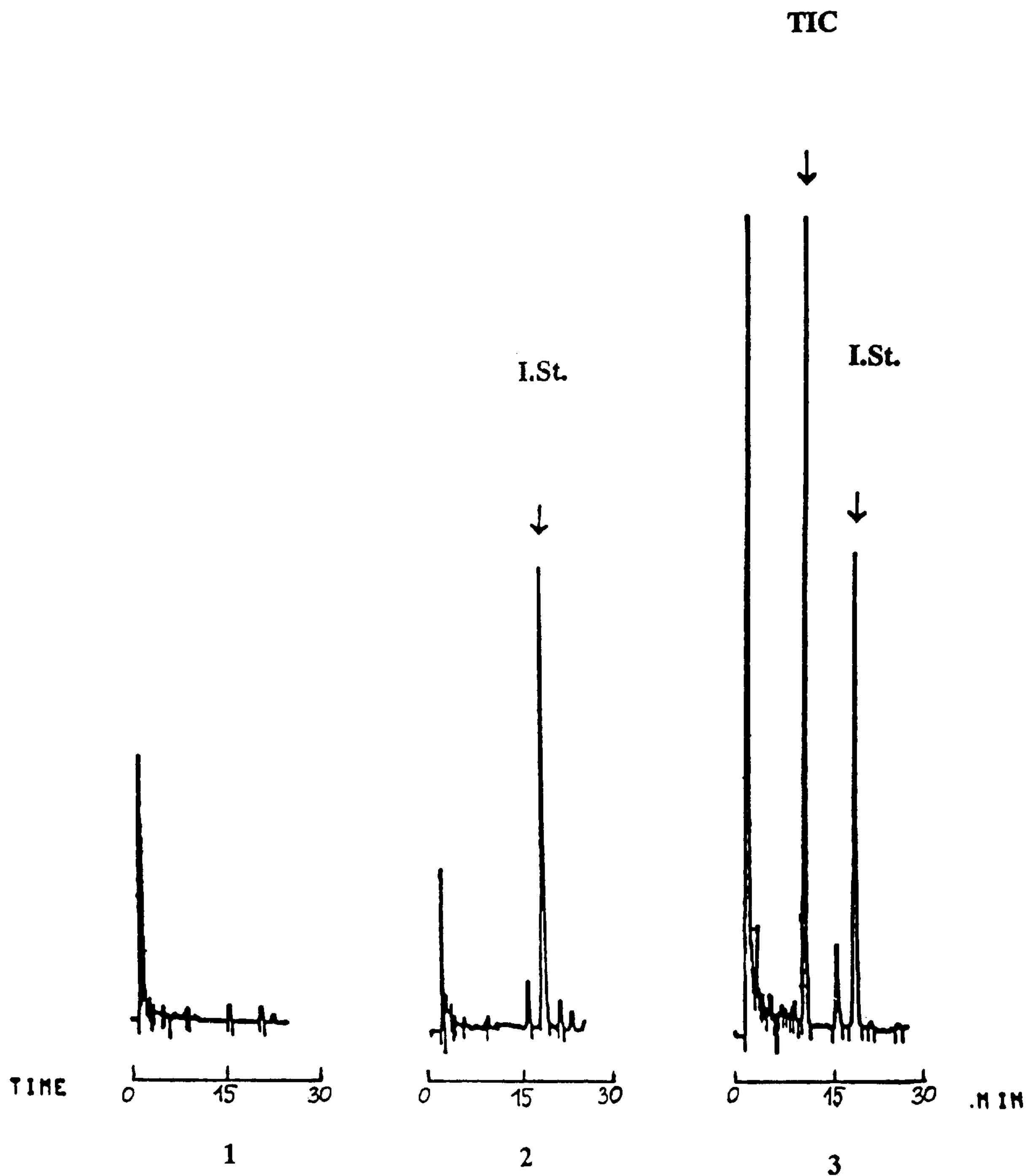

Fig. 2. Chromatograms of a characteristic blank plasma extract (1), the extract of a plasma sample spiked with 500 ng of I.S. only (2) and the extract of a plasma sample spiked with 1000 ng TIC and 500 ng I.S. (3). Chromatographic conditions are described in the text.

3. Results and discussion

The stationary phase (BST Rutin 10, C_{18} BD) and the ternary mobile phase [0.01 *M* potassium dihydrogen phosphate buffer (pH 4)–acetonitrile–methanol (20:40:40, v/v)] provided symmetrical peak shape for both TIC and the internal standard.

Fig. 2 shows the chromatogram of a blank plasma extract and that of a spiked (1000 ng of TIC and 500 ng of the I.S.) plasma extract obtained after processing the samples with Bond Elut C_{18} (1 ml) solid-phase extraction cartridge. Fig. 3 shows a typical chromatogram of a volunteer's plasma sample after oral administration of 250 mg TIC. As is seen in the figures, no endogenous peak interferes with the chromatographic peak of either TIC or the I.S.

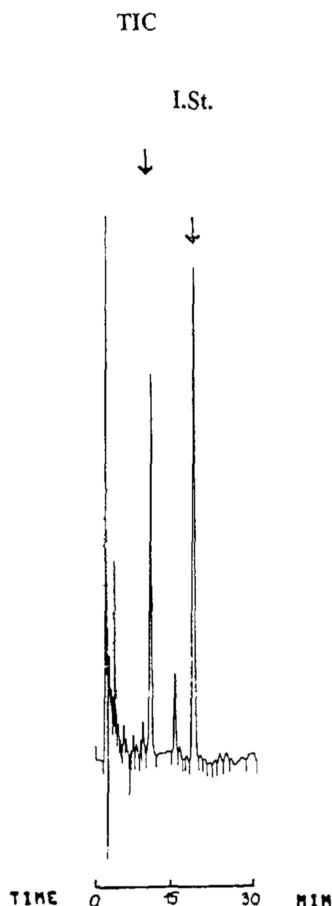

Fig. 3. Representative chromatogram of human plasma extract of a volunteer 2 h after oral administration of 250 mg TIC. Chromatographic conditions are described in the text.

Table 1
Summary of the data of ticlopidine calibration curve

Nominal concentration (ng ml^{-1})	Ticlopidine/I.S. peak area ratio (mean±S.D., n=6)	R.S.D. (%)
10	0.013±0.003	19.97
50	0.096±0.014	14.31
100	0.185±0.015	8.37
250	0.486±0.012	2.44
500	1.026±0.072	7.04
1000	2.014±0.049	2.44

Equation of the calibration curve: $y=-0.007615+0.002021x$, R=0.998.

Based on the system suitability test the mean retention time for TIC was 9.78 (0.013) min, while the same parameter for the I.S. was 18.26 (0.034) min. On the basis of five parallel determinations, the reproducibility (R.S.D. %) of the retention time was 0.13% for TIC and 0.18% for the I.S., whereas the reproducibility of the peak area values was 1.15% and 1.69% for TIC and the I.S., respectively.

The results of method validation are presented in details in Tables 1 and 2.

The reproducibility of the method (R.S.D. %) never exceeded the 20% limit accepted for the analysis of biological samples. On the basis of the validation parameters, the limit of quantitation (LOQ) achieved with present method was 10

Table 2
Precision

Nominal concentration (ng ml^{-1})	Measured concentration (mean±S.D.) (ng ml^{-1})	Accuracy (%)	R.S.D. (%)
Within-day (n=5)			
50	51.64±4.07	103.3	7.89
250	247.94±8.86	99.2	3.57
1000	1064.0±37.45	106.4	3.52
Between-day (n=5)			
50	51.22±4.75	102.4	9.29
250	253.12±10.57	101.2	4.17
1000	1076.22±85.35	107.6	7.93

ng ml^{-1} of plasma, while the limit of detection (LOD) amounted to 5 ng ml^{-1}.

The linearity of the calibration curve obtained from the calibration points by combinatoric weighting [8] was good (Table 1). The equation of the calibration curve covering the 10 to 1000 ng ml^{-1} of TIC plasma concentrations was $y=-0.007615+0.002021x$ ($R=0.998$), where y stands for TIC/I.S. ratio and x denotes TIC plasma concentration.

The R.S.D. % values of within- and between-day precision determined by the analysis of QC samples were always lower than 10% and the corresponding accuracy values never exceeded 8% (Table 2).

The absolute recovery of TIC was 84.9%, on the average, and the concentration dependence of recovery was negligible.

The stability tests indicated that in stock solutions both TIC and the I.S. were stable up to 6 weeks. The mean accuracy was 96.2% for TIC and 101.2% for I.S.

As regards the stability of TIC in human plasma, no significant decomposition was observed after either 4 or 6 weeks of storage at −20°C. The accuracy of TIC in the plasma after storage was between 94.2% and 107.7%.

Experiments carried out for investigating the robustness of the method indicated that sample processing using Bakerbond C_{18} (1 ml) SPE cartridge and chromatography using Nucleosil 5 C_{18} stationary phase and methanol–water–25% ammonia (80:20:1, v/v) were also feasible.

The results of validation show that the method is suitable for the determination of TIC in human plasma. Moreover the present method is appropriate for pharmacokinetic studies and drug level monitoring in humans.

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