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Journal of Chromatography B, 805 (2004) 181–185
JOURNAL OF CHROMATOGRAPHY B
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Short communication

Determination of Tirofiban in human serum by liquid chromatography–tandem mass spectrometry

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Received 20 November 2003; received in revised form 2 February 2004; accepted 1 March 2004

Abstract

A liquid chromatography–tandem mass spectrometric (LC–MS–MS) method with a rapid and simple sample preparation was developed and validated for the determination of Tirofiban in biological fluids. Tirofiban in serum samples was extracted and cleaned up by using an automated solid phase extraction method. An external calibration was used. The mass spectrometer was operated in the multiple reaction monitoring mode (MRM). A good linear response over the range of 2–200 ng/ml was demonstrated. The accuracy for Tirofiban ranged from 94.8 to 110.8% within-day and from 103.0 to 104.7% between-day. The lower limit of quantification was 2 ng/ml. This method is suitable for pharmacokinetic studies.

Keyword: Tirofiban

1. Introduction

Tirofiban, *N*-(*n*-butanesulfonyl)-*O*-[4-(butane-4-piperidinyl)]-L-tyrosine hydrochloride (Fig. 1) belongs to a new class of drugs which is important for patients suffering from vaso-occlusive disorders such as myocardial infarction and unstable angina pectoris [1–4]. Tirofiban is a fibrinogen-receptor antagonist which inhibits platelet aggregation by competitively binding to membrane-bound glycoprotein complex GPIIb/IIIa on the surface of activated platelets, preventing the binding of fibrinogen [5–9]. Aggrastat (Tirofiban hydrochloride) was designed for intravenous administration [5,10,11]. Tirofiban has an elimination half-life of 2 h. Renal failure prolongs the half-life and continues inhibition of platelet aggregation refractory to transfusions of platelets. Extracorporeal elimination is the only option to prevent excessive haemorrhage in this condition.

A competitive radioimmunoassay (RIA) has already been reported for the determination of Tirofiban in plasma, however, it is not commercially available [12]. Another high performance liquid chromatographic–tandem mass spectrometry (LC–MS–MS) method with an insufficient sample preparation was developed for the analysis of Tirofiban in human plasma. After isolating the analyte by a three step liquid extraction, it was converted into its *N*-trifluoroacetyl derivative and analyzed by HPLC with atmospheric pressure negative ionization MS–MS-detection. The lower limit of quantification of the assay was 0.4 ng/ml [13].

This paper describes a LC–MS–MS method with a rapid and simple sample preparation using an automated solid phase extraction (SPE) to determine in serum the elimination of Tirofiban by hemofiltration, requiring smaller sample volumes and enabling a low limit of quantification.

2. Experimental

2.1. Chemicals

Reference substance of Tirofiban was not available. Instead, an Aggrastat® solution of infusion (PE container with 250 ml) Charge C518597 (MSD, Haar, Germany) was used. The solution contained 14.05 mg Tirofiban hydrochloride (=12.5 mg Tirofiban).

Acetonitrile LiChrosolv (for chromatography), methanol LiChrosolv (for chromatography), ammonium acetate (p.a.)

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doi:10.1016/j.jchromb.2004.03.003

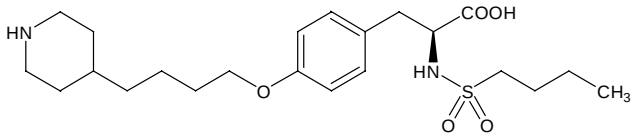

Fig. 1. Structure of Tirofiban.

and formic acid (p.a.) were purchased from Merck (Darmstadt, Germany). Pure water (18 MΩ) was obtained using an ion-exchange system RS 40E, SG Ionenaustauscher (Barsbüttel, Germany).

The SPE Cartridges NEXUS were obtained from Varian (Darmstadt, Germany).

2.2. LC–MS–MS analysis

2.2.1. Apparatus and chromatographic conditions

The LC–MS–MS system used was a Quattro micro (Micromass, Manchester, GB) equipped with an electrospray interface (ESI). Full scan mass spectrum was acquired by continual infusion of standard solution (concentration 100 ng/ml with 10 μl/min) and by scanning MS1 from *m/z* 200–500. The product ion mass spectrum was obtained by choosing the molecular ion as the precursor ion, scanning MS2 from *m/z* 100–500. The capillary voltage was 3500 V (positive ion mode) and the ion source temperature of 100 °C was applied. The desolvation gas flow (nitrogen) was 600 l/h at 300 °C.

The multiple reaction monitoring (MRM) was performed by monitoring the transitions between *m/z* 441.2 (parent ion) and *m/z* 140.1 (collision energy −32 eV, dwell time 200 ms) and between *m/z* 441.2 and 276.1 (collision energy −32 eV, dwell time 200 ms) with Argon as collision gas (pressure 6.5e–3 mbar). The sum of both transitions was used for the determination of analyte concentration.

The HPLC equipment consisted of a Dionex P580 HP-Gradient pump and an autosampler Dionex ASI 100 T (Idstein, Germany) with a Chromeleon Chromatography Data System (Dionex Softron, Idstein, Germany). The chromatographic separation was performed on a Purospher Star C18, endcapped, 55 mm × 2 mm (Merck) column with a Security Guard C18, 4 mm × 2 mm i.d. (Phenomenex, Aschaffenburg, Germany). The following mobile phase gradient was applied with solvent A (5/95/0.2, v/v/v) and solvent B (95/5/0.2, v/v/v) of a mixture of acetonitrile, ammonium acetate in water (0,002 mol/l) and formic acid. See Table 1.

The MassLynx Data System was applied for MS control and QuanLynx for peak area evaluation, regression analysis of standard curves and calculation of concentrations.

2.3. Sample preparation

2.3.1. Solid phase extraction

Tirofiban in serum samples was extracted and cleaned up by using an automated solid phase extraction method with NEXUS cartridges (Varian) in an ASPEC XL Sample Processor (Gilson).

Table 1
Mobile phase gradient—solvent A (5/95/0.2, v/v/v) and solvent B (95/5/0.2, v/v/v) of a mixture of acetonitrile, ammonium acetate in water (0.002 mol/l) and formic acid

Time (min)	A (%)	B (%)
0.0	100	0
0.1	100	0
2.5	20	80
3.0	20	80
3.2	100	0
7.0	100	0

The frozen serum samples (−30 °C) were thawed at room temperature, mixed and centrifuged. The stability of Tirofiban was investigated and no significant changes in the concentration could be found after freeze/thaw cycles, in samples after storage at −30 °C up to 3 months, at 4 °C for 2 days and at 22 °C for 1 day.

The sample volume of 0.1 ml serum was diluted with 0.1 ml of formic acid (9%) and extracted by pressing through the cartridges.

2.3.2. Extraction procedure

Condition: 1 ml methanol and 1 ml water
Load: 0.2 ml mixture of serum and formic acid
Wash: 1 ml water
Elute: 0.5 ml methanol

Eluates were evaporated to dryness at 72 °C in an air stream with a Techne DRI Block SC-3 (thermo-DUX, Wertheim, Germany), afterwards redissolved in 100 μl of mobile phase, and 10 μl were injected for LC–MS–MS.

3. Results

3.1. Mass spectrometry

The mass spectra of Tirofiban is shown in Fig. 2. The spectrum revealed a base peak at *m/z* 441 corresponding to the molecular ion $(M + H)^+$. The product ion mass spectra were obtained by choosing the molecular ion as the precursor ions. The fragment ion observed at *m/z* 140 correspond to the butane-4-piperidinyl group and the ion with *m/z* 276 is believed to result from a rearrangement of the butanesulfonyl group and the carboxyl group. Intensities of other fragments are lower than 30% (Fig. 3).

3.2. Chromatography

Because of the high specificity of the MS–MS method a complete chromatographic separation of analytes and matrix is not necessary. However, to achieve high-quality analytical data for samples with low levels of analytes originally in biological fluids, sufficient chromatographic retention of

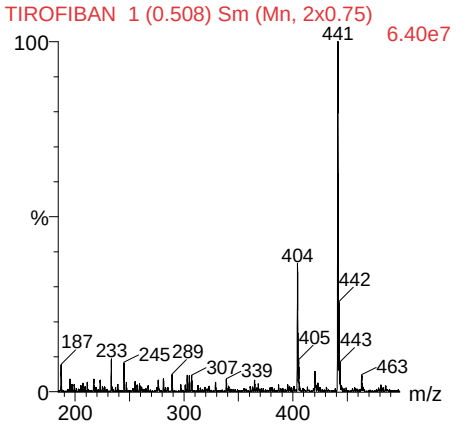

Fig. 2. Mass spectra of Tirofiban: the spectrum reveals a base peak at *m/z* 441 corresponding to the molecular ion (M + H)+.

the analyte is preferred to minimise signal suppression and other matrix effects. Therefore, we need to optimise the mobile phase to obtain high sensitivity and short analysis time. A mixture of acetonitrile, ammonium acetate in water (0.002 mol/l) and formic acid caused a high sensitivity of Tirofiban.

To exclude interferences from the biological matrix, chromatograms of the transitions between *m/z* 441.2 and 140.1 and between *m/z* 441.2 and 276.1 were controlled separately. No interferences and a low background noise were found (Fig. 4). The best sensitivity was achieved by monitoring the sum of both fragment ions.

MS–MS does not respond to ions originating from impurities of the biological matrices that differ in *m/z* from the selected ion. The retention time of Tirofiban was about 3.2 min.

3.3. Recovery and ion suppression

The recovery from serum was evaluated by comparing the peak areas of amounts mixed with assayed blanks and injected than directly with those of assayed samples. The ion

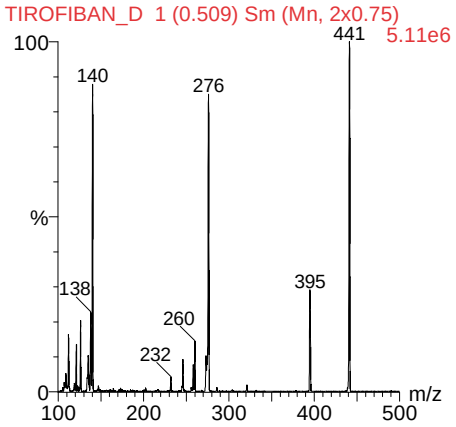

Fig. 3. MS–MS-spectra of Tirofiban.

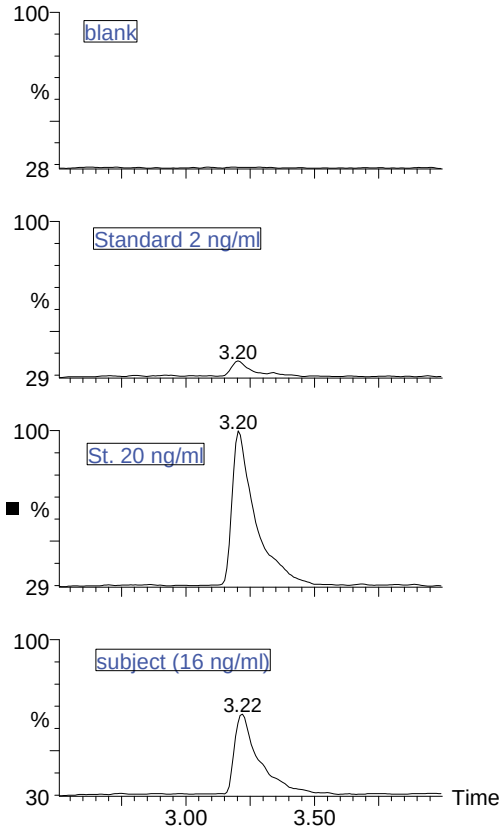

Fig. 4. Chromatograms of Tirofiban, retention time 3.2 min: blank sample, standard with 2 ng/ml, standard with 20 ng/ml and patient sample with 16 ng/ml.

suppression of Tirofiban in the mass spectrometer (effect of the matrix) were evaluated by comparing the peak areas of amounts injected directly with peak areas of amounts mixed with assayed blanks and injected than directly.

The slope of the linear regression line $y = mx + b$ of the response x of amounts mixed with assayed blanks (and than injected directly) and the response of the assayed samples y delivers the mean recovery of the concentration range investigated. The slope of the linear regression line $y = mx + b$ of the response x of directly injected amounts and the response of the amounts mixed with assayed blanks (and than injected directly) y delivers the mean ion suppression of the concentration range investigated. The peak areas of Tirofiban at seven concentrations in the range of 2 up to 200 ng/ml were used.

The graphs, evaluated with values in the concentration range from 2 to 200 ng/ml using the "Trend line function" of Excel, are shown in Figs. 5 and 6. The mean recovery in the ranges from LLOQ to 200 ng/ml was found to be about 100% and the ion suppression was about 30%.

3.4. Calibration graph

The calibration graph for Tirofiban was generated from MRM of increasing amounts of Tirofiban stan-

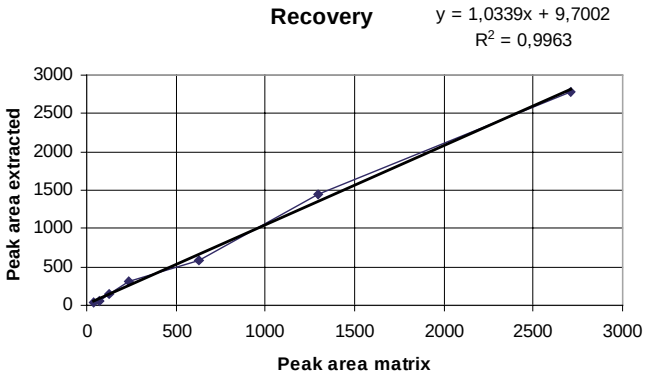

Fig. 5. Recovery of Tirofiban from serum in the concentration range from 2 to 200 ng/ml; Recovery = slope ∼ 100%.

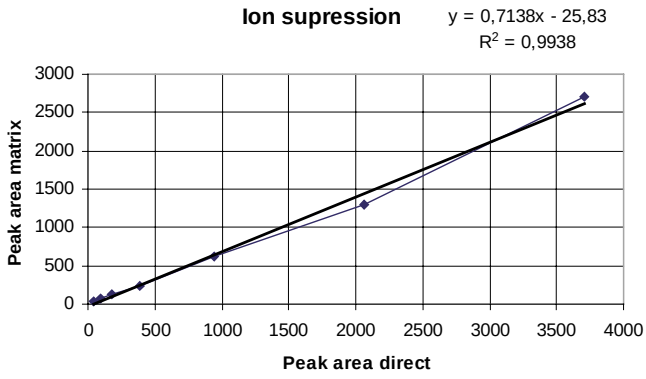

Fig. 6. Ion suppression of Tirofiban in serum matrix in the mass spectrometer in the concentration range from 2 to 200 ng/ml; Ion suppression = 1 − slope ∼ 30%.

dards. A quadratic calibration graph was constructed using least-squares regression of quantities versus peak area. A good response over the range of 2–200 ng/ml was demonstrated. Samples with a higher concentration of Tirofiban were diluted. The correlation coefficient of regression lines was 0.9986 or higher. See Table 2 and Fig. 4.

3.5. Reproducibility

The precision and accuracy of the method were assessed by determination of seven concentrations in six independent series of spiked serum samples as shown in Table 2. The accuracy was calculated to be from 94.3 to 108.5%. The coefficient of variation (CV) ranged from 0.4 to 9.3%. The lower limit of quantification (LLOQ) was obtained by comparing the true and measured values for the lowest standard data points and blanks of the six standard curves. The acceptance criterion was a deviation of less than ±20%. LLOQ of 2 ng Tirofiban/ml could be accepted.

Table 2
Determination of Tirofiban in serum: statistics of six independent standard curves (S.D., standard deviation; CV, coefficient of variation; IP, accuracy)

	Tirofiban added (ng/ml)							
	0	2	5	10	20	50	100	200
	Tirofiban measured (ng/ml)							
Mean		2.17	4.71	9.4	20.5	50.4	99.8	199.9
S.D.		0.20	0.27	0.54	0.85	2.21	2.39	0.86
CV (%)		9.3	5.7	5.8	4.1	4.4	2.4	0.4
IP (%)		108.5	94.1	94.3	102.4	100.9	99.8	100.0

4. Discussion

Liquid chromatography tandem mass spectrometry is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological matrices. There was a report [13] on the use of the highly sensitive and specific liquid chromatography–tandem spectrometry technique to measure Tirofiban in human plasma. But the three step liquid–liquid extraction and the subsequent derivatisation are very complicated. In this study, an analytical method of Tirofiban determination using LC–MS–MS after simple solid phase extraction and without derivatisation has been developed. Only the small sample volume of 0.1 ml serum was necessary to achieve a limit of quantification of 2 ng/ml.

Therapeutic concentrations range from 200 to 60 ng/ml while concentrations below 40 ng/ml only provide incomplete inhibition of platelet aggregation [14]. Our measured plasma concentration ranged from 266 ng/ml (before hemofiltration) to 9 ng/ml (after hemofiltration). The elimination of Tirofiban by hemofiltration could be shown.

The described method is suitable for routine measurements of Tirofiban in biological materials.

5. Conclusion

LC–MS–MS is the most sensitive method for a quantitation of Tirofiban in serum. Furthermore, the assay requires only an automatic simple sample preparation. To increase the sensitivity it is possible to increase the sample volume. This method is suitable for pharmacokinetic studies.

Acknowledgements

The authors thanks Merck Sharp and Dohme for financial support and Mrs. U. Mann for the technical assistance.

References

[1] B. Stein, V. Fuster, D.H. Israel, M. Cohen, L. Badimon, J.J. Badimon, J.H. Chesebro, J. Am. Coll. Cardiol. 14 (1986) 813.
[2] D.J. Fritzgerald, L. Roy, F. Catella, G.A. Fritzgerald, N. Engl. J. Med. 315 (1986) 983.
[3] D.J. Fritzgerald, F. Catella, L. Roy, G.A. Fritzgerald, Circulation 77 (1988) 142.
[4] C.W. Hamm, R.L. Lorenz, W. Bleifeld, W. Kupper, W. Wober, P.C. Weber, J. Am. Coll. Cardiol. 10 (1987) 998.

[5] G.D. Hartman, M.S. Egbertson, W. Halczenko, W.L. Laswell, M.E. Duggan, R.L. Smith, A.M. Naylor, P.D. Manno, R.J. Lynch, G. Zhang, J. Med. Chem. 35 (1992) 4640.
[6] G.A. Marquerie, E.F. Plow, T.S. Edginton, J. Biol. Chem. 254 (1979) 5357.
[7] J. Hawiger, S. Parkinson, S. Timmons, Nature 283 (1980) 195.
[8] E.I. Peerschke, M.B. Zucker, R.A. Grant, T.S. Egan, M.M. Johnson, Blood 55 (1980) 841.
[9] E. Rouslahti, M.D. Pierschbacher, Science 238 (1987) 491.
[10] M.S. Egbertson, C.T. Chang, M.E. Duggan, R.J. Gould, W. Halczenko, G.D. Hartman, W.L. Laswell, J.J. Lynch Jr., R.J. Lynch, P.D. Manno, J. Med. Chem. 37 (1994) 2537.
[11] J.J. Lynch Jr., J.J. Cook, G.R. Sitko, M.A. Holahan, D.R. Ramjit, M.J. Mellott, M.T. Stranieri, I.I. Stabilito, G. Zhang, R.J. Lynch, J. Pharmacol. Exp. Ther. 272 (1995) 20.
[12] E.L. Hand, J.D. Gilbert, A.S. Yuan, T.V. Olah, M. Hichens, J. Pharm. Biomed. Anal. 12 (1994) 1047.
[13] J.D. Ellis, E.L. Hand, J.D. Gilbert, J. Pharm. Biomed. Anal. 15 (1997) 561.
[14] D.J. Schneider, H.C. Herrmann, N. Lakkis, F. Aguirre, M.W. Lo, K.C. Yin, A. Aggarwal, S.S. Kabbani, P.M. DiBattiste, Am. J. Cardiol. 91 (2003) 334.