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Use of LC–MS/MS to cross-validate a radioimmunoassay for the fibrinogen receptor antagonist, Aggrastat (tirofiban hydrochloride) in human plasma

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Abstract

A method based on LC–MS/MS was developed for the determination of the fibrinogen-receptor antagonist Aggrastat in human plasma. The drug is isolated from plasma by liquid extraction and converted into its *N*-trifluoroacetyl derivative prior to analysis by HPLC with atmospheric pressure negative chemical ionization MS/MS detection. A structural analog is used as the internal standard and the lower quantifiable limit of the assay is 0.4 ng ml^{-1} with a relative standard deviation of 7%. This assay was used to cross-validate the existing immunoassay by analysis of plasma from patients receiving the drug. The specificity of the immunoassay was thereby confirmed.

Keywords: Aggrastat; Tirofiban hydrochloride; Plasma; Radioimmunoassay; Liquid chromatography–tandem mass spectrometry; Cross-validation

1. Introduction

Fibrinogen-receptor antagonists are an important new class of drugs for the prevention of thrombosis in patients suffering from vaso-occlusive disorders such as myocardial infarction and unstable angina pectoris [1–4]. These substances inhibit platelet aggregation by competitively binding to the membrane-bound glycoprotein complex GPIIb/IIIa [5–9] on the surface of activated platelets thus, preventing the binding of fibrinogen.

Aggrastat (tirofiban hydrochloride; MK-0383) is a fibrinogen-receptor antagonist designed for intravenous administration [9–11]. A competitive radioimmunoassay (RIA) has already been reported for the determination of this drug in plasma [12] and has been used to study its pharmacokinetics in healthy male volunteers [13]. The RIA used 5 μl of plasma and has a lower quantifiable limit of 1 ng ml^{-1} with satisfactory accuracy and precision. The assay had been tested using parallelism, accuracy by standard addition and plasma fractionation experiments and met all our acceptance criteria for specificity and there

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was no evidence for the presence of cross-reacting metabolites in either human plasma or urine.

The development of an alternative and highly specific method based on LC–MS/MS has now allowed a definitive demonstration of the RIAs specificity. The characteristics of the mass spectrometric method and its cross-validation with the immunoassay are described.

2. Experimental

2.1 Materials

Aggrastat, *N*-(*n*-butanesulfonyl)-*O*-[4-(butane-4-piperidinyl)]-L-tyrosine hydrochloride, and its phenylsulphonyl analog, L-702,128 (Fig. 1), were synthesized by the Medicinal Chemistry Department, Merck Research Laboratories, West Point, PA.

Acetonitrile, *n*-butyl chloride and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ), ethanol (anhydrous) from Kodak-IBI (New Haven, CT), toluene from EM Science (Gibbstown, NJ), ammonium acetate, dimethylaminopyridine and trifluoroacetic anhydride from Sigma (St. Louis, MO), perchloric acid (70%) from Frederick Smith (Columbus, OH) and air (hydrocarbon-free), nitrogen and argon (both 99.999%) from West Point Supply (West Point, PA).

2.2 Preparation of standard solutions

Stock solutions of Aggrastat and its internal standard were prepared as 1 mg ml^{-1} solutions of the free base in water–acetonitrile (1:1 v/v). Working standard solutions were prepared by dilution of the stock solutions and 50 μl aliquots of the working standard solutions were added to 0.5 ml aliquots of control plasma to provide plasma concentrations of Aggrastat equivalent to 100, 40, 20, 10, 4, 2, 1, 0.4 and 0.2 ng ml^{-1}.

2.3 Extraction procedure

A plasma sample (0.5 ml) was placed in a 13×100 mm borosilicate screw-capped culture tube. After the addition of internal standard solution (50 μl, 5 ng) and 0.5 ml of 0.1 M perchloric acid, the tubes were briefly vortex mixed and extracted with 5 ml of *n*-butyl chloride by shaking for 10 min. After centrifugation, the upper organic phase was aspirated to waste and the aqueous residue re-extracted with ethyl acetate (2×5 ml) with shaking and centrifugation as above. The combined extracts were transfered into a fresh borosilicate tube and the solvent was removed under a stream of nitrogen using a TurboVap LV Evaporator (Zymark, Hopkinton, MA) at 40°C. Traces of water were removed by reconstituting the extract in an azeotropic mixture of 250 μl of toluene–ethanol (1:1, v/v) and re-evaporation as above. The *N*-trifluoroacetyl derivatives were prepared by addition to the dried residue of 400 μl of a solution of dimethylaminopyridine (10 mM) in acetonitrile and trifluoroacetic anhydride (40 μl). After incubation for 30 min at 40°C, acylation was essentially complete. Excess reagents were removed in a stream of nitrogen and the derivatized extracts reconstituted in 150 μl of mobile phase prior to analysis by LC–MS/MS. Quality control samples were prepared by addition of the drug (in duplicate) to plasma at concentrations of 0.4, 4 and 40 ng ml^{-1}.

The absolute recoveries of Aggrastat and its internal standard were determined in the following manner. Aliquots (0.5 ml) of control plasma were spiked with analyte to yield concentrations of 0.4, 4 and 40 ng ml^{-1}. After extraction, the internal standard (5 ng, equivalent to 10 ng ml^{-1}) was added to the extracts and the derivatized extracts were assayed by LC–MS/MS. The area ratios obtained were compared with those of the appropriate mixtures of unextracted reference solutions. The recovery of the internal standard (at 10 ng ml^{-1}) of plasma was determined by reversing the role with the analyte. The recoveries of the drug at 0.4, 4 and 40 ng ml^{-1} were 92, 92 and 95%, respectively. The measured recovery of the internal standard was 103%.

2.4 LC–MS/MS

LC–MS/MS was performed on a Sciex (Thornhill, Ontario) Model API III (plus) triple-quadru-

pole mass spectrometer interfaced via a Sciex heated nebulizer probe to a liquid chromatograph consisting of a Hewlett-Packard (Wilmington, DE) model 1050 solvent-delivery system and model 1050 autoinjector equipped with a 100 μl loop. Separations were effected using a 250 × 4.6 mm i.d. Zorbax RX-C_{18} column (5 μm) from DuPont (Wilmington, DE). The mobile phase was acetonitrile–1 mM ammonium acetate (41.5:58.5, v/v) at pH 6.0 with a flow rate of 1 ml min^{-1}. The volume of extract injected was 25 μl and samples were chromatographed in batches of 80–120 under the control of a Macintosh IIFX computer running Sciex's RAD (routine acquisition and display) software. The nebulizer probe temperature setting was 500°C. The nebulizing gas (air) pressure and auxiliary (make-up gas) settings were 80 psi and 1 l min^{-1}, respectively.

Negative atmospheric pressure chemical ionization was effected by a corona discharge needle (+6.2 μA) and negative ions were sampled into the triple-quadrupole mass analyzer via a 0.0045 in pinhole aperture. The curtain gas was nitrogen at 0.7 l min^{-1}. The selected reaction monitoring (SRM) mode was used. The mass spectrometer was programmed to admit the pseudo-molecular ions $[M-H]^-$ at m/z 535 and 555 for the *N*-trifluoroacetyl derivatives of the drug and internal standard, respectively, via the first quadrupole mass filter (Q_1) with collision-induced fragmentation in Q_2 (collision gas argon at density of 250×10^{12} atoms cm^{-2}) and monitoring via Q_3 the product ions at m/z 415 (Aggrastat) and 141 (internal standard). The orifice potential, electron multiplier and interface heater settings were −60 V, +4.4 kV and 50°C, respectively. The dwell time was 200 ms. Peak-area ratios obtained from the SRM chromatograms of the derivatives of the analyte (m/z 535→415)/internal standard (m/z555→141) were computed using Sciex's MacQuan software. The ion at m/z 415 in the product ion mass spectrum of derivatized Aggrastat was selected in preference to that of the lower mass (and greater potential for non-specificity) at m/z 121. Calibration curves were constructed using a weighted (reciprocal of concentration) linear least-squares regression and concentrations of the drug in test samples were calculated by interpolation from the calibration curve.

2.5 Radioimmunoassay

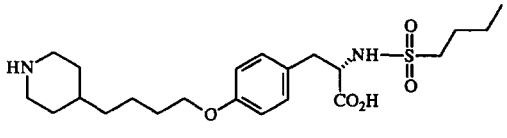
Radioimmunoassay was conducted according to the procedures reported previously [12].

2.6 Statistical analysis of data

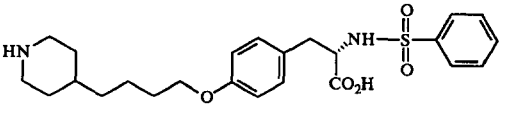
Statistical comparisons of data resulting from cross-validation experiments were performed using the paired *t*-test and regression analysis programs supplied in the statistical analysis package of Excel, Version 4.0 (Microsoft, Redmond, WA). Fixed and proportional errors were determined from the 95% confidence limits around the slope and intercept functions of the linear regression. Random error was assessed from the relative standard deviation (RSD) of the individual test data/reference data ratios.

3. Results

The objective of this research was to develop a definitively specific assay against which the RIA for Aggrastat could be definitively cross-validated. LC–MS/MS was selected because, in addition to its specificity, its sensitivity is comparable to that



Aggrastat™



L-702,128

Fig. 1. Structures of Aggrastat and its internal standard, L-702,128 as the free bases.

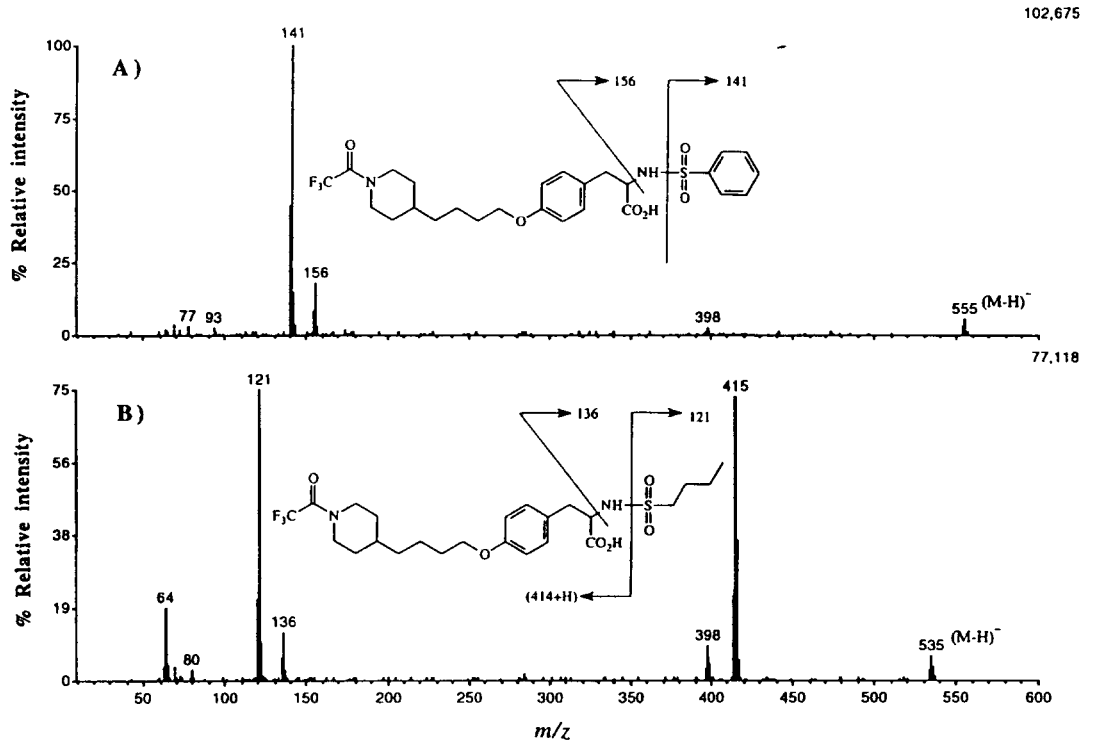


Fig. 2. Negative product ion mass spectra (background subtracted) of the pseudo-molecular ions of the *N*-trifluoroacetyl derivatives of (A) the internal standard L-702,128 (*m/z* 555) and (B) Aggrastat, (*m/z* 535).

of RIA and method development and validation are simple and rapid.

3.1 LC–MS/MS

Attempts to chromatograph Aggrastat underivatized using conventional reversed phase chromatography without ion pairing agents were not entirely satisfactory. Based on our previous experiences with the orally active substance L-734, 217 [14], we chose to analyze Aggrastat after conversion to its *N*-trifluoroacetyl derivative, which considerably reduced its polarity and allowed the use of chromatographic conditions compatible with the mass spectrometer. In contrast to L-734,217, however, the derivative of Aggrastat showed approximately tenfold greater sensitivity in the negative rather than positive ion mode. The negative product ion mass spectra of the *N*-trifluoroacetyl derivatives of Aggrastat and its internal standard are shown in Fig. 2.

3.2 Chromatographic system suitability

The precision of the chromatographic system was determined prior to each analysis by injection of a test solution containing Aggrastat and its internal standard (both at 200 pg on column) five times. Typically the RSD of the peak-area ratio was 3.5%.

3.3 Calibration

The calibration curves showed good linearity using a weighted (reciprocal of concentration) linear regression.

3.4 Precision and accuracy

The intra-assay precision was determined by the analysis of five sets of control plasma containing known quantities of the drug. The inter-assay precision and accuracy were determined by analy-

Table 1
Intra- and inter-assay accuracy and precision for the determination of Aggrastat in plasma by LC–MS/MS

	Aggrastat concentration (ng ml^{-1})	Mean recovery (%)	RSD (%)
Intra-assay ($n = 5$)	0.4	93.0	6.4
	1	92.1	7.6
	2	99.6	2.3
	4	102.8	3.1
	10	100.5	3.2
	20	105.2	2.8
	40	104.3	4.8
	100	101.6	2.0
Inter-assay ($n = 5$)[a]	0.40	106.2	5.7
	4.0	101.5	3.3
	40.0	103.6	3.2

[a] Determined in duplicate.

sis of quality control samples at 0.4, 4 and 40 ng ml^{-1} in duplicate on five analytical occasions. The results are shown in Table 1. Acceptable accuracy, defined as mean % (found/actual), was observed over the range 0.2–100 ng ml^{-1}. The intra-assay precision at 0.2 ng ml^{-1} was 11.6%, which exceeds the value (10%) required for defining the limit of quantitation (LOQ) in our laboratories. Accordingly, the LOQ was set at 0.4 ng ml^{-1}. The intra-assay precision profile of the LC–MS/MS-based assay is shown in Fig. 3. That for the RIA is included for comparison.

3.5 Cross-validation of the RIA and LC–MS/MS

The assays were cross-validated by the analysis of plasma obtained from subjects participating in a clinical study. Fifty-two samples were collected from four patients who had received Aggrastat intravenously at a dose of 6 μg kg^{-1}. SRM chromatograms of derivatized extracts of plasma from human subjects dosed intravenously with the drug are shown in Fig. 4. Extracts of plasma containing no drug showed peak-area ratios that were essentially zero and no interferences from endogenous or drug-related plasma components were observed with the measurement of either the patient drug or its internal standard.

The samples were assayed by both RIA and LC–MS/MS and, after detection and exclusion of outliers (Dixon's test [15]), data were compared by ratio analysis, paired t-test and regression analysis [16]. In these comparisons, the intrinsically specific LC–MS/MS-based method was designated the "reference" procedure and the RIA was the "test" method. Aggrastat concentrations below the LOQs of either one or both methods were excluded from the statistical comparison. The mean plasma concentration–time profiles obtained by both methods are shown in Fig. 5. The mean areas under the 0–5 h plasma concentration–times curves determined by RIA and LC–MS/MS were 29.49 ± 4.0 and 28.48 ± 3.1 ng h ml^{-1}, respectively.

The results of statistical analyses are shown in Table 2. The mean ratio (RIA/LC–MS) was 1.01 with an RSD of 12.5% and the total assay bias was +1%. The paired t-test showed no significant differences between the analytical methods. Regression analysis showed confidence intervals around slopes and intercepts consistent with the absence of fixed or proportional biases. The regression curve is shown in Fig. 6.

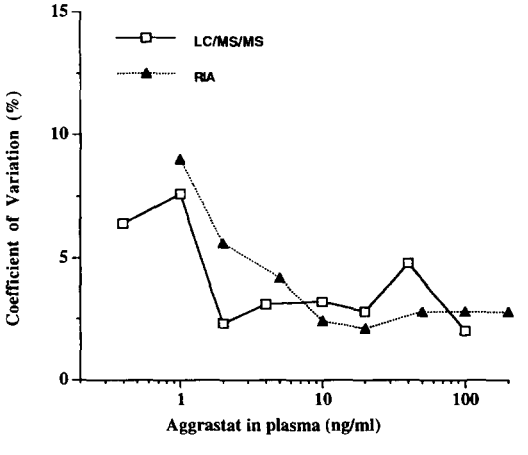


Fig. 3. Comparison of the intra-assay precision profiles for the determination of Aggrastat in plasma by LC–MS/MS and RIA.

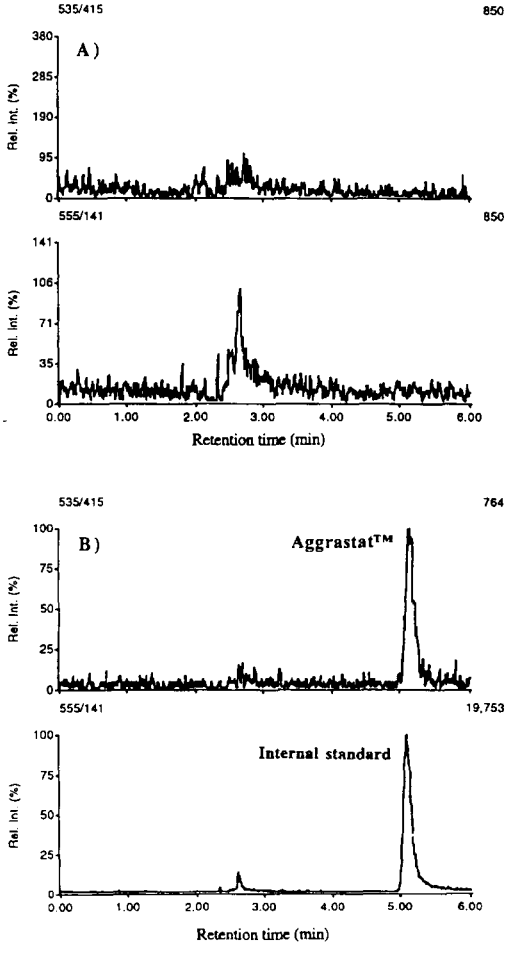


Fig. 4. Selected reaction monitoring (SRM) chromatograms of derivatized extracts of a patient's plasma collected (A) pre-dose and (B) 6 h after intravenous administration of Aggrastat. The drug concentration in the latter is ca. 0.8 ng ml^{-1}.

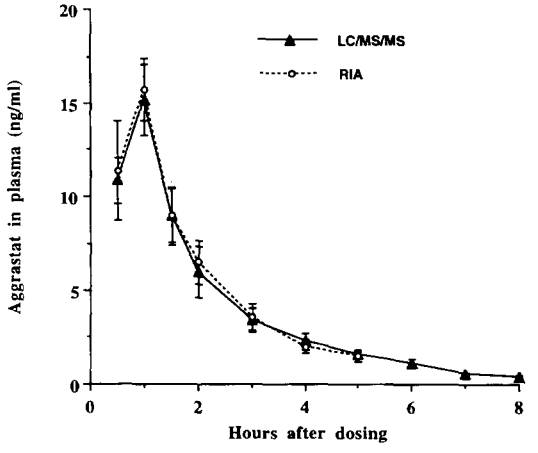


Fig. 5. Mean concentration–time curves ($n = 4$) of Aggrastat in the plasma of patients receiving intravenous doses (0.6 $\mu g\ kg^{-1}$) of Aggrastat. The drug concentrations were measured by both LC–MS/MS and RIA.

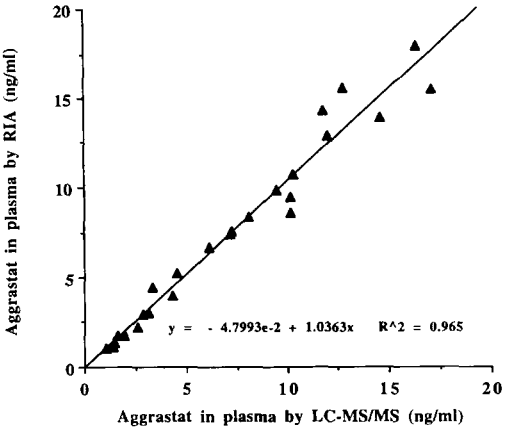


Fig. 6. Cross-validation of assays for the determination of Aggrastat in patients plasma by both RIA and LC–MS/MS.

4. Discussion

Radioimmunoassay has declined in popularity over the past decade for the determination of small drug molecules in biological fluids. There are several reasons for this: (1) many substances can now be conveniently determined at low ng ml^{-1} concentrations using techniques based on HPLC; (2) small molecules are not immunogenic and must be coupled to a carrier protein prior to immunization, which is simple enough if the substance in question contains functional groups which can be readily conjugated to the protein, but frequently additional resources are required to synthesize suitable haptens; (3) it can take 3–6 months to raise polyclonal antisera of good titer and, in the absence of a reasonable knowledge of the substance's metabolism, there is no guarantee that the antisera will prove specific for the antigen in the presence of closely related metabolites.

Table 2
Summary of statistics observed in the cross-validation of RIA and LC–MS/MS-based methods for the determination of Aggrastat in human plasma

Test	Statistic
(1) Ratio Analysis	
Ratio RIA/LC–MS	1.01
RSD	12.53%
(2) Paired t-test	
Observed t	1.04
t-critical ($p = 0.05$)	2.06
(3) Regression analysis	
Intercept	−0.05
(confidence interval)	−0.73 to 0.63
Slope	1.036
(confidence interval)	0.95 to 1.12
Fixed bias (RIA/LC–MS)	Not significant
Proportional bias (RIA/LC–MS)	Not significant

Potential lack of specificity with respect to metabolites is immunoassay's greatest drawback, but it is also suprisingly difficult to check. There is very rarely an alternative method of adequate sensitivity against which the RIA can be compared, and reliance has to be made on a series of never-completely definitive tests to assess specificity. These may include parallelism and standard addition experiments to detect potential cross-reacting metabolites with non-parallel displacement curves, along with the fractionation of plasma or urine preparations by HPLC with subsequent analysis of the fractions by RIA. The latter procedure, although useful, is tedious and is rarely applied to more than a few samples. Additionally, manipulative losses during extraction and recon-

stitution of fractions can be troublesome, especially at low concentrations.

The advent of LC–MS/MS has permitted the rapid development of equally sensitive and definitively specific methods against which immunoassays can now be cross-validated. Our criteria for method cross-validations are based on a few simple statistical treatments, such as *t*-test and regression analysis. Strictly, the use of a more complex weighted errors-in-variables regression should be used since a simple linear regression assumes no error in the reference method [17]. However, for the comparison of data ranging over orders of magnitude, the differences in confidence intervals using both types of regression are minimal [16].

The results of the present cross-validation experiments (Table 2) showed no significant differences between the assays and no fixed or proportional biases. Based on these results, we concluded that the RIA and LC–MS/MS-based assays yield essentially identical results when applied to the analysis of plasma from subjects dosed with Aggrastat and, accordingly, that the specificity of the immunoassay was acceptable.

LC–MS/MS has become established as an invaluable bioanalytical tool because of its high sensitivity and unparalleled specificity [18–20]. However, RIA is direct (requiring no sample extraction), convenient (allowing batch analysis of hundreds of samples), not labor intensive, extremely reliable (provided specificity can be demonstrated) and inexpensive (requiring little apparatus other than an automatic pipettor, a centrifuge and a gamma counter). The credibility of RIAs is much enhanced when they are successfully cross-validated against equally sensitive and intrinsically specific LC–MS/MS-based methods and such experiments are now recommended as part of our RIA validation package [14, 21].

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