Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 601–607
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

LC–MS–MS determination of brostallicin in human plasma following automated on-line SPE

Sara Calderoli [a,*], Emiliana Colombo [a], Enrico Frigerio [a], Christopher A. James [a], Martin Sibum [b]

[a] *Pharmacia Corp., Viale Pasteur 10, Nerviano, Milan 20014, Italy*
[b] *Spark Holland Instrumenten, P. De Keyserstraat 8, P.O. Box 388, 7800-AJ Emmen, The Netherlands*

Received 24 April 2002; received in revised form 20 January 2003; accepted 23 January 2003

Abstract

LC–MS–MS method using automated on-line solid-phase extraction (SPE) has been developed and validated for the quantitation of brostallicin (**I**), a new distamycin derivative, in human plasma. **I** is a DNA minor groove binder currently under phase I–II clinical evaluation as an anticancer drug. Plasma (0.4 ml) was spiked with 0.2 ml stable label **IS** solution and placed in a 96-well plate maintained at +4 °C. Aliquots of 0.1 ml of prepared samples were loaded into the on-line SPE HySphere Resin SH cartridges (10 mm × 2 mm ID) and the analytes back eluted with the mobile phase into LC–MS–MS system. A Platinum Cyano column (100 mm × 4.6 mm, 3.6 μm) was used to perform the chromatographic analysis. The mobile phase was acetonitrile–ammonium formate buffer (pH 3.5; 20 mM) (70:30, v/v) at a flow rate of 1.0 ml/min. LC flow was split so that 300 μl/min was directed toward the mass spectrometer interface. Retention time of **I** was 2.6 min and the total cycle time was 8 min. MS detection used an Applied Biosystems/MDS SCIEX API 365 with a TurboIonSpray® interface and MRM (*m/z*: 725/257 for **I** and *m/z*: 729/257 for **IS**) operated in positive ion mode. The method was validated over the calibration range 0.124–497 ng/ml. A negligible carry-over effect from the system was observed. In spite of the known instability of **I** in human plasma (about 20% decrease over 12 h), the ratio analyte/**IS** peak area showed good stability over the analysis time required for 96 samples. The automated on-line SPE method can be considered as a valid alternative to the off-line manual SPE procedure previously developed.

Keywords: Brostallicin; On-line SPE; Prospekt 2™; LC–MS–MS; Human plasma

1. Introduction

Brostallicin (**I**) shown in Fig. 1, laboratory code PNU-166196A, *N*-(5-{[(5-{[(5-{[(2-{[amino(imino)methyl]amino}ethyl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)amino]carbonyl}-1-methyl-1H-pyrrol-3-yl)-4-[(2-bromoacryloyl)amino]-1-methyl-1H-

* Corresponding author. Tel.: +39-02-48385515; fax: +39-02-48383012.
E-mail address: sara.calderoli@pharmacia.com (S. Calderoli).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00167-5

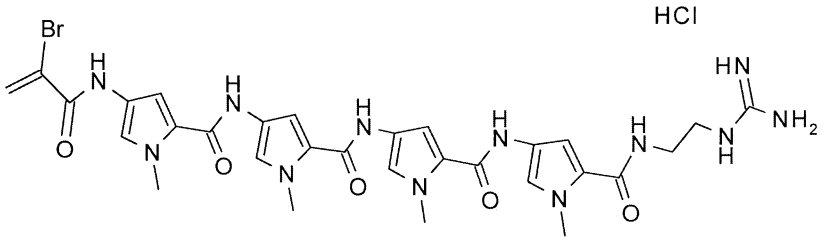

Fig. 1. Chemical structure of **I**.

pyrrole-2-carboxamide hydrochloride, is a new synthetic DNA minor groove binder [1–3]. The compound is currently under phase I–II clinical evaluation as an anticancer drug. Two LC–MS–MS methods for the determination of **I** in plasma utilizing a simple protein precipitation step for sample preparation, or a manual solid-phase extraction (SPE) in the 96-well plate format, have been already developed and validated in order to support toxicokinetic and clinical studies. Further attempts to automate the manual SPE procedure, using a Packard Multiprobe II robot, were not fully successful due to significant contamination across the wells, which was particularly noticeable when relatively large numbers of plasma samples were processed. The objective of this study was to develop and validate a sensitive and automated high throughput on-line SPE LC–MS–MS method for the determination of **I** in human plasma in order to support human clinical studies.

2. Experimental

2.1. Chemicals and solutions

I (purity 95.57%) and [$^2H_4$]-**I** (**IS**, purity $>90\%$) were supplied by Pharmacia, Nerviano, Milan, Italy. All other chemicals and solvents were of analytical grade and were obtained from Carlo Erba Reagents (Milan, Italy). Stock and working solutions of **I** were prepared by dissolving a weighed amount of **I** in acetonitrile–water (50:50, v/v) in silanized volumetric flasks. A stock solution of **IS** was prepared in acetonitrile–water (50:50, v/v) using a silanized volumetric flask to provide a final concentration of 50 μg/ml. The primary solution was diluted with water in a silanized volumetric flask to give an **IS** working solution with a concentration of 1.0 μg/ml. Corrections for purity were made and all reported concentrations are in term of free base.

2.2. On-line SPE system and HPLC conditions

Prospekt 2™ (Spark Holland, Emmen, The Netherlands), on-line SPE system, consisted of the following modules:

- Automated cartridge exchange (ACE) module for disposable cartridge exchange. ACE holds two trays each containing a maximum of 96 cartridges; each tray has a chip containing tray information on how often a cartridge is used and information about the sorbent and production date.
- High-pressure dispenser (HPD) module for handling of solvents. HPD transports liquids by way of 2-ml high-pressure syringe (up to 300 bars).
- The autosampler used was the Endurance v. 2.2, model 925 with a Peltier plate cooling and a 250-μl syringe (Spark Holland, Emmen, The Netherlands). It was equipped with a "serum needle" to avoid possible blockages in the system.

Prospekt 2™ was controlled using Spark Holland propriety software running SparkLink™ v. 2.01.

SPE cartridges: HySphere Resin SH (strong hydrophobic), 10 mm × 2 mm ID; this is a modified polystyrene-divinylbenzene phase, 15–25 μm particles, irregular in shape.

HPLC apparatus consisted of a HP1100 system (Agilent Technologies, Waldbronn, Germany) with pump, oven and solvent degasser. The chromatographic analysis was performed under isocratic conditions on a Platinum Cyano column (100 mm × 4.6 mm, 3.6 μm) equipped with a pre-column (Hypersil ODS C18, 7.5 mm × 4.6 mm, 5 μm, both from Alltech, Italy), which were both maintained at 45 °C. The mobile phase was acetonitrile–ammonium formate buffer (pH 3.5; 20 mM) (70:30, v/v) at a flow rate of 1.0 ml/min. LC flow was split so that 300 μl/min was directed toward the mass spectrometer interface.

2.3. Mass spectrometry conditions

MS detection was performed using an Applied Biosystems/MDS Sciex API 365 mass spectrometer (Toronto, Canada) with a TurboIonSpray® interface. Curtain gas (nitrogen) flow was set at 12 units, while the nebulizer gas (air) flow was set at 13 units. The turbo probe temperature was adjusted to 400 °C. Multiple reaction monitoring (MRM) detection in the positive ion mode was employed using nitrogen as the collision gas (set at 4 units) with collision energy of 61 eV. Precursor-to-product ion (MS–MS) transition of 725/257 (*m*/*z*) was used. Declustering and focusing potentials were set at 25 and 225 V, respectively. MS–MS transition of 729/257 (*m*/*z*) was selected to detect **IS**. The data were processed using Applied Biosystems propriety software running Analyst v. 1.1.

2.4. Sample preparation

2.4.1. Manual sample preparation

Plasma calibration standards were prepared daily and quality control (QC) samples and study samples, previously frozen at −80 °C, were quickly thawed in a water bath at 30 °C. Immediately after defrosting, samples were vortex-mixed and placed in an ice water bath and then centrifuged at 14 000 rpm (IEC Micromax RF) for 10 min at 4 °C. After centrifugation, all samples were immediately placed in an ice water bath. Aliquots (400 μl) from each human plasma sample were then transferred into a 96-well plate, followed by the addition of 200 μl of water containing approximately 1.0 μg/ml **IS** and finally vortex-mixed.

2.4.2. On-line SPE

The injection and the extraction were completely automated using Prospekt 2™ system.

2.4.2.1. Endurance autosampler. The syringe dispenser aspirated 90 μl of sample (flush volume) from the well to fill the lines with sample and remove wash solvents. Then, the injection valve was switched in LOAD position and the loop (100 μl) was quantitatively overfilled with 200 μl of the sample. At this point, the injection valve of the autosampler switched into INJECT position and HPD pump loaded the sample contained in the loop onto SPE cartridge (see step 5 in Prospekt 2™ method below).

After each injection, the autosampler was programmed to perform a wash of the needle with 1 ml of acetonitrile–formic acid (0.1 M) (20:80, v/v). To reduce carry-over, two extra-wash steps were used with 500 μl of acetonitrile–formic acid (0.1 M) (20:80, v/v). The injection valve was switched between LOAD and INJECT positions after the first wash.

2.4.2.2. Prospekt 2™ method. The method used consisted of 11 steps:

1) A new cartridge was moved from tray to the clamp.
2) The autosampler started loading the sample in the loop.
3) The cartridge was solvated with 1 ml of acetonitrile at 2.5 ml/min.
4) The cartridge was equilibrated with 1 ml of water at 2.5 ml/min.
5) HPD back eluted the filled loop of the autosampler onto SPE cartridge with 500 μl of water at 1 ml/min; the drug was retained onto the cartridge and plasma proteins went to the waste.
6) The cartridge was washed with 1 ml of water at 1 ml/min.
7) Input signal to Prospekt.
8) Output signal from Prospekt.

9) HPLC pump back eluted the cartridge with acetonitrile–ammonium formate buffer (pH 3.5; 20 mM) (70:30, v/v) at 1 ml/min for 2 min. The analyte was eluted to the analytical column and hence to MS.
10) After elution and during LC–MS run, HPD washed the cartridge with 2 ml of acetonitrile–water (20:80, v/v) at a flow rate of 1 ml/min.
11) The cartridge was put back into the tray.

2.5. Assay validation experiment

Linearity, intra- and inter-day precision and accuracy, lower limit of quantitation (LLOQ), specificity and recovery were assessed.

The linearity of the method was evaluated from three calibration curves of six calibration points prepared in duplicate and run on three different days over the concentration range 0.124–497 ng/ml of human plasma. The first curve was run at the beginning and the second at the end of each analytical batch. All chromatograms obtained were evaluated by peak area measurements. Calibration curves were constructed by plotting the peak area ratio (y) of the drug/**IS** against its concentration (x). Weighted linear regression ($1/x^2$) was used to fit the calibration line and hence to calculate **I** in calibration and QC samples. Precision and accuracy at LLOQ were assessed at the lowest calibration curve point by analysis of five-spiked plasma samples in one analytical run.

Precision and accuracy of the analytical method were evaluated by repeated analyses of QC samples at different concentrations (0.318, 254 and 445 ng/ml). Five replicates of each QC sample pool, prepared in a single batch at each concentration, were assayed in three validation runs to provide inter- and intra-assay precision and accuracy.

Specificity of the method was performed by comparison of LC–MS–MS chromatograms of **I** at LLOQ and blank plasma samples from three individual volunteers. Potential MS crosstalk between **I** and **IS** was assessed in terms of blank human plasma spiked with and without **IS**.

Recovery was evaluated comparing the peak area of extracted QC plasma samples to the peak area obtained with unextracted standard solutions dissolved in mobile phase, directly injected onto LC–MS–MS system.

3. Results and discussion

3.1. SPE and LC–MS–MS conditions

During the method development various type of cartridges (C2, C8, C8 EC, C18 and SH) were tested: C2, C8, C8 EC and C18 cartridges were not able to completely retain the compound. The limited retention may be due to the fact that at neutral pH the compound is ionized, as the estimated pK_a of **I** is 12. However, the HySphere Resin SH cartridges, which contain a strongly hydrophobic resin phase, gave a satisfactory recovery and were used for this study. The retention time of **I** and **IS** was about 2.6 min and the total cycle time was 8 min. As observed for the extraction cartridge in this study, and also as is reported for the chromatography of **I**, retention on standard "reverse phase" material is relatively weak, probably due to the high polarity of the compound. The Platinum Cyano column appears to be uniquely retentive for **I** and allows the use of a high organic content in the mobile phase, which improves the sensitivity of the method. It has also been noted that the compound exhibits an unusual chromatographic behavior: with acetonitrile content of over 60% in the mobile phase, the retention time increases with increase of percentage of acetonitrile. On the contrary, with a mobile phase containing less than 60% of acetonitrile, it showed a more typical "reversed phase" behavior with the retention increasing with decreasing percentage of acetonitrile.

A Q1 scan of **I** revealed the parent molecular ion $(M+H)^+$ at 725 m/z. MS–MS transition of 725/257 (m/z) was selected to assess quantitation of **I** in human plasma.

A Q1 spectrum of **IS** showed the protonated parent ions at 729 m/z as expected. MS–MS transition of 729/257 (m/z) was chosen to detect **IS**.

Table 1
Precision and accuracy of the method for the determination of **I** in human plasma using an automated on-line SPE LC–MS–MS analysis

QC concentration (ng/ml)	Intra-assay precision (%CV, $n = 5$)	Inter-assay precision (%CV, $n = 15$)	Intra-assay accuracy (%bias, $n = 5$)	Inter-assay accuracy (%bias, $n = 15$)
0.318	6.5	14.2	5.2	8.4
254	2.4	11.5	−3.5	−1.6
445	4.5	12.6	−9.1	−11.1

3.2. Linearity

The linear correlation coefficient (r) ranged from 0.991 to 0.996, using linear regression with a $1/x^2$ weighting factor. The back-calculated calibration standard values showed a bias ranging from −10.2 to 7.0% over the range from 0.124 to 497 ng/ml.

3.3. Precision and accuracy

The intra-day precision (expressed as %relative standard deviation (%RSD)) evaluated from QC samples ranged from 2.4 to 6.5%. The inter-day precision evaluated at the same concentrations ranged from 11.5 to 14.2%. The intra-day accuracy (expressed as %bias) evaluated on the same plasma

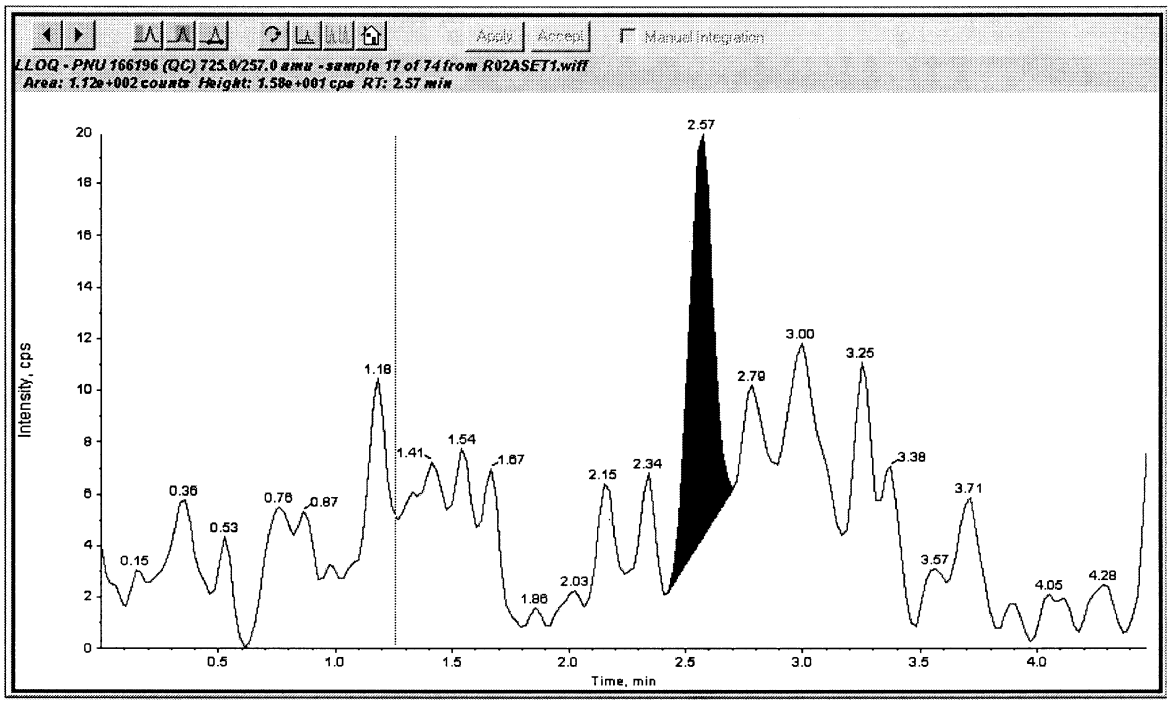

Fig. 2. Chromatogram of extracted lower calibration standard (0.124 ng/ml) for **I** in human plasma with an on-line SPE LC–MS–MS system.

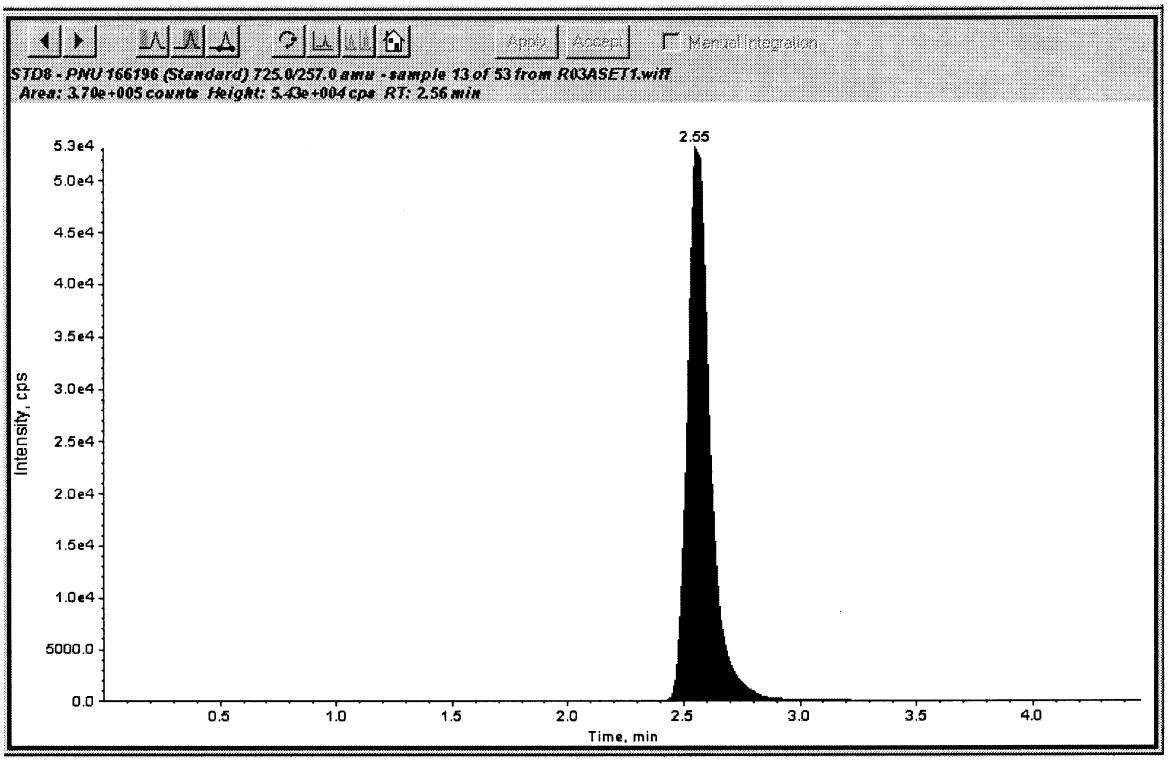

Fig. 3. Chromatogram of extracted upper calibration standard (497 ng/ml) for **I** in human plasma with an on-line SPE LC–MS–MS system.

samples ranged from −9.1 to 5.2%. The inter-day pooled accuracy ranged from −11.1 to 8.4% (Table 1).

3.4. Specificity

No interfering peaks were detected in any extracts from three individual blank human plasma samples spiked with and without **IS**. No significant crosstalk between **I** and **IS** was observed.

3.5. Lower and upper limits of quantitation

LLOQ was 0.124 ng/ml (Fig. 2). Signal-to-noise ratio, calculated by Analyst v. 1.1 software, was about 20. At this level, precision (expressed as %RSD, $n = 5$) was 18.2% and accuracy (expressed as %bias) was −11.3%. Upper limit of quantitation (ULOQ) was 497 ng/ml (Fig. 3).

3.6. Recovery

The mean total recovery of **I** evaluated from QC plasma samples ranged from 40.2 to 57.9%, while the recovery of **IS** was 68.7%.

3.7. Carry-over

During method development significant carry-over problems were found. However, after introduction of the extra-wash steps described in Section 2.4.2.2, only a negligible carry-over effect (about 0.04%) was observed after injection of a blank plasma extract following injection of the upper calibration standard.

3.8. Stability

The stability of **I** in human plasma has already been extensively assessed under a number of

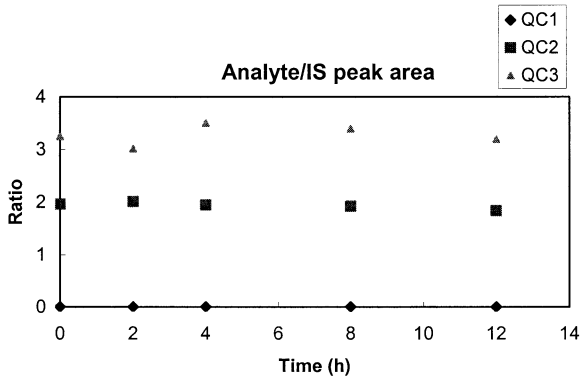

Fig. 4. Stability plot for QCs over 12 h storage period in the cooled autosampler.

different conditions. When placed at room temperature for 2 h, a moderate instability of the compound in human plasma was seen particularly with the low-concentration QC samples. However, the compound appears to be relatively stable after storage for 4 h in an ice water bath. It indicates that potential instability of the analyte in human plasma during sample processing procedure can be minimized with cooling of plasma samples. For this reason, the autosampler rack was refrigerated at 4 °C. Repeated injections at 0, 2, 4, 8 and 12 h of QC samples spiked with **IS** and left in the cooled autosampler plate holder showed a loss in the analyte peak area of about 10–30% after 12 h, which is approximately the time needed to analyze one full plate of 96 samples. Whilst instability of the compound could be noted, the ratio of analyte/**IS** peak was constant over 12 h period (Fig. 4) indicating that **IS** was compensating for any instability in the autosampler.

4. Conclusions

An analytical method using an on-line SPE system followed by LC–MS–MS detection was developed and validated for the quantitative determination of **I** in human plasma. No interference was observed for three individual blank human plasma samples. The intra- and inter-assay precision and accuracy of the method were judged adequate according to the current international requirements [4]. Utilization of an automated on-line SPE system, where 100% of the final extract is presented to the detector instead of only a small percentage like for the off-line SPE methods, resulted in not only adequate sensitivity to support further human clinical studies, but also in a simpler and faster sample preparation. Consequently, the analytical method here described can be considered as a valid alternative to the off-line SPE procedure previously developed.

References

[1] P. Cozzi, I. Beria, M. Caldarelli, C. Geroni, N. Mongelli, G. Pennella, Bioorg. Med. Chem. Lett. 10 (2000) 1273–1276.
[2] P.G. Baraldi, R. Romagnoli, I. Breria, P. Cozzi, C. Geroni, N. Mongelli, N. Bianchi, C. Mischiati, R. Gambari, J. Med. Chem. 43 (2000) 2675–2684.
[3] P. Cozzi, Farmaco 56 (2001) 57–65.
[4] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (12) (2000) 1551–1557.