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Automated assay for GV104326, a novel tribactam antibiotic, in human plasma by high-performance liquid chromatography and solid-phase extraction

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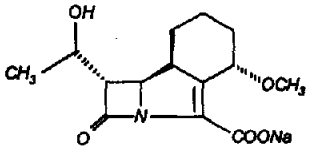
ABSTRACT

A highly automated, rapid, robust and specific plasma assay for GV104326, a novel tribactam antibiotic, has been developed to monitor human volunteer trials. The method involves automated solid-phase extraction with a strong anion-exchange phase and HPLC on a reversed-phase column with ultraviolet detection. The calibration range for the assay is 0.05–2 μg/ml. The assay is linear over this range and is specific with respect to endogenous interference and likely metabolites of GV104326. Both intra- and inter-assay variability were <8% and intra- and inter-day bias <10%.

INTRODUCTION

GV104326 (Fig. 1) is the first member of a new class of tricyclic β-lactam (tribactam) antibiotics, possessing a potent and particularly broad spectrum of activity against Gram-positive, Gram-negative, and anaerobic bacteria [1]. In addition, it has a remarkably good resistance to hydrolysis by either β-lactamases or renal dehydropeptidases [2].

Beta-lactam antibiotics have traditionally been assayed by microbiological methods [3,4], and by HPLC [5–14]. HPLC is the generally preferred technique because of its sensitivity, speed, robustness and above all specificity. The determination of GV104326 in biological fluids during preliminary animal studies was performed by an HPLC method utilizing protein precipitation with acetonitrile, evaporation of the supernatant and direct injection of the redissolved residue. Although sufficiently sensitive (100 ng/ml) and specific, the method was rather labour-intensive and was also highly dependent on the selectivity of the HPLC column because of the critical separation of co-extracted endogenous components. This separation could vary between batches of columns and also during ageing of the column.



GV104326B

Fig. 1. Structure of GV104326B.

An improved assay method was required to monitor GV104326 plasma levels in volunteers after intravenous administration. Of importance was a more robust, rapid and automated method to efficiently assay the large number of samples generated. Another important issue with GV104326, as with most β-lactam antibiotics, is the limited stability in plasma, hence the speed of the assay is of prime importance. Techniques considered were direct injection onto restricted access stationary phases, column-switching HPLC and solid-phase extraction (SPE). The first two techniques were considered to pose

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more problems because of the high polarity of the molecule. Very little retention was obtained on a Pinkerton column at pH 6. In order to obtain more retention by hydrophobic mechanisms it would be necessary to use pH values <4 to protonate the carboxylic acid, however the stability of GV104326 is very poor at these pH values. Column switching using reversed-phase enrichment precolumns would suffer from the same problem of low retention, and alternative anion-exchange precolumns tend to have low capacity, restricting the number of samples that can be injected before repacking is necessary. SPE was attempted initially with C_{18} packing, but recovery was very low, again because of poor retention. Attention was switched to a strong anion-exchange (SAX) phase (trimethyl quaternary ammonium) and we here describe the assay developed using this SPE phase. The assay is highly automated, as a result of the use of a Gilson Aspec (automated sample preparation with extraction columns) and the direct injection of the SPE eluate onto the column. We also present the results from the assay validation and compare them with those obtained using the acetonitrile deproteinization assay in human plasma.

To our knowledge, this approach to the HPLC assay of β-lactam antibiotics has not been published in the literature.

EXPERIMENTAL

Materials and reagents

GV104326B (sodium salt) was synthesized by Chemical Development, Glaxo. The structure is shown in Fig. 1. Acetonitrile (HPLC grade) and glacial acetic acid (analytical grade) were obtained from Carlo Erba (Milan, Italy). Sodium hydroxide (1 *M*), ammonium acetate and trisodium citrate 2-hydrate (all analytical grade) were obtained from Merck-Bracco (Milan, Italy). Water was purified through a Milli RO/Milli-Q (Millipore, Milan, Italy) system.

Chromatography

The HPLC system consisted of a Waters 590 (Milan) isocratic pump, an Aspec (Gilson/Biolabo, Milan, Italy) containing a Rheodyne 7010 injection valve with a 100-μl loop, a SM4000 (Milton Roy, Milan) UV detector and data acquisition by an Access-Chrom (PE-Nelson, Milan, Italy) VAX-based system.

The chromatography column was stainless steel and packed with 5-μm Hypersil ODS (Hewlett-Packard, Milan, Italy). Internal dimensions were 100 × 4.6 mm. A precolumn packed with the same phase and of dimensions 20 × 4.6 mm was used. Mobile phase consisted of 7% acetonitrile in 0.1 *M* ammonium acetate at pH 5.7 with glacial acetic acid (15.42 g in 2 l of Milli-Q water and 1 ml of glacial acetic acid added and filtered through a 0.45-μm filter). The flow-rate was 1 ml/min and the column temperature ambient. Retention time of GV104326 was approximately 7 min. The UV detector was set at 268 nm.

Standard preparation

Plasma calibration standards in the range 0.05–2 μg/ml were prepared by spiking heparinized control human plasma (700 μl) with standard solutions of GV104326B (sodium salt) dissolved in 50 m*M* 2-(N-morpholine)ethanesulphonate (MES) buffer at pH 6.5, and diluting to 1.4 ml with the same buffer. The standards were vortex mixed and immediately placed in the aluminium Aspec rack at 4°C.

Sample extraction

Plasma samples, previously stored at −80°C, were thawed at 4°C and centrifuged at the same temperature to remove fibrin. A 700-μl aliquot was transferred to a 3-ml glass vial, 700 μl of 50 m*M* MES buffer added and the mixture vortexed and placed immediately in an aluminium Aspec rack maintained at 4°C. Completely automated SPE and injection were performed by the Aspec. The Aspec programme, which was performed in sequence mode so that the HPLC separation of the extracted sample was carried out during the preparation of the next sample, is shown in Table I.

Assay validation

Both intra- and inter-day validations were performed, the former by assaying six replicates of spiked samples at the same concentration

TABLE I

ASPEC PROGRAM FOR FULLY AUTOMATED SOLID-PHASE EXTRACTION AND HPLC INJECTION

SPE cartridges	500 mg Bond-Elut SAX
Conditioning	1 × 3 ml 100 m*M* ammonium acetate (pH 6.5)
	1 × 6 ml water
Sample	1 ml (+2 ml air flush)
Wash	1 ml water (+2 ml air flush)
Elution	2 × 0.5 ml trisodium citrate (0.5 *M*) pH 7.9 (+2 ml air flush)
Mixing	Two cycles of aspiration and dispensing of the sample
Injection	0.5 ml onto the 0.1-ml loop (full-loop injection)

levels as the calibration standards, and the latter by assaying, on four different days, duplicate spiked samples at four different concentration levels in the calibration range. On each occasion, the calibration curve was constructed from single standards at each of six concentrations, using independent standard solutions.

In addition, linearity, specificity, recovery and total analysis time were determined. The data were compared with those obtained by the preliminary acetonitrile deproteinization method. The accuracy was expressed by the bias (difference from the theoretical) and the precision expressed by the relative standard deviation (R.S.D.). Both bias and precisions are presented as percentages.

Stability

The stability of GV104326 under four different storage conditions was studied as follows:

(a) Stability in plasma diluted either in MES buffer (1:1) or in water (1:1) at 4°C (in the Aspec rack) at a concentration of 1 μg/ml over 24 h after preparation.

(b) Stability in a pooled plasma spiked sample, after extraction, at ambient temperature (in the Aspec collection rack) at a concentration of 1 μg/ml up to 24 h after preparation.

(c) Stability, in frozen plasma, at −25°C, at a concentration of 0.5 μg/ml, for up to 1 month after preparation.

(d) Stability, in frozen plasma, at −80°C, at a concentration of 0.5 μg/ml, for up to 7 months after preparation.

Spiked samples were prepared by addition of aqueous solutions, except study (a), of GV104326, minimizing the dilution of the sample to 5%.

RESULTS AND DISCUSSION

Solid-phase extraction

A strong anion-exchange phase was selected in order to ensure complete retention of GV104326, through interaction with the relatively weak carboxylic anion. It was thought essential to dilute the plasma to some extent, to ensure disruption of protein binding and also to stabilize GV104326. The use of pH 6.5 was optimum for stability and also was high enough to ensure good retention on the packing ($pK_a \approx 4$). Also, the use of a zwitterionic buffer (MES) should minimize the competition for retention with GV104326. The SPE packing was washed with a concentrated acetate buffer initially to ensure that the phase was in the acetate form and then washed with water to remove excess competing anions. Initial studies using 100-mg packing and 100-μl plasma loadings gave reasonable recoveries, but washing, even with a small volume of water, could not be performed without loss of recovery. A wash of some kind was considered essential to eliminate as much as possible of the plasma remaining on the packing. Therefore attention was switched to 500-mg packings and 500 μl of loaded plasma. Again retention was good and up to 1 ml of water could be used as a wash. Use of a wash with low-concentration buffers at pH 6–8 always caused loss of recovery. Possibly the low capacity of the phase, the presence of retention mechanisms

other than ion exchange or the presence of competing anions in plasma could explain the loss of GV104326 on washing. In order to obtain complete recovery, elution with high salt-containing buffers or acids could be used but, considering the instability of GV104326 in acid, salts were studied for elution. The highest recovery was obtained with two 0.5-ml elutions with 0.5 *M* citrate at pH 7.9. The direct injection of 100 μl of this is fully compatible with the reversed-phase conditions, but unfortunately the stability in this solvent is somewhat limited (3 h at ambient temperature), making reinjection of the eluted sample, the morning after an overnight run, impossible.

Chromatography and specificity

Typical chromatograms of blank and spiked plasma standards are shown in Fig. 2. Control human plasma from six different volunteers did not reveal any measurable interference in GV104326 peak-area measurement. The chromatograms are exceptionally clean, owing to the use of two different mechanisms of retention for SPE (anion exchange) and chromatography (reversed phase) and the reasonably long UV wavelength monitored. The use of anion exchange as the SPE clean-up enables removal of lipophilic and/or basic endogenous components from plasma, improving the selectivity of the clean-up compared with using a reversed-phase mechanism and, importantly, eliminates the need to wash the analytical column after every injection to remove late-eluting components.

The retention time of GV104326 is approximately 7 min. This could probably be shortened; however, the rate-limiting step is the Aspec clean-up, which takes approximately 14 min. At present, no information is available on possible metabolism of GV104326 in man, but studies of pure solution degradation and metabolism in rat urine suggest that the major metabolite would be the open lactam form of GV104326, as observed elsewhere for β-lactam antibiotics [15,16]. This product is well separated from GV104326 on reversed-phase columns. Furthermore, intact GV104326 is excreted extensively in rat urine and the urine GV104326 peak has been shown, by particle beam mass spectrometry, to be pure.

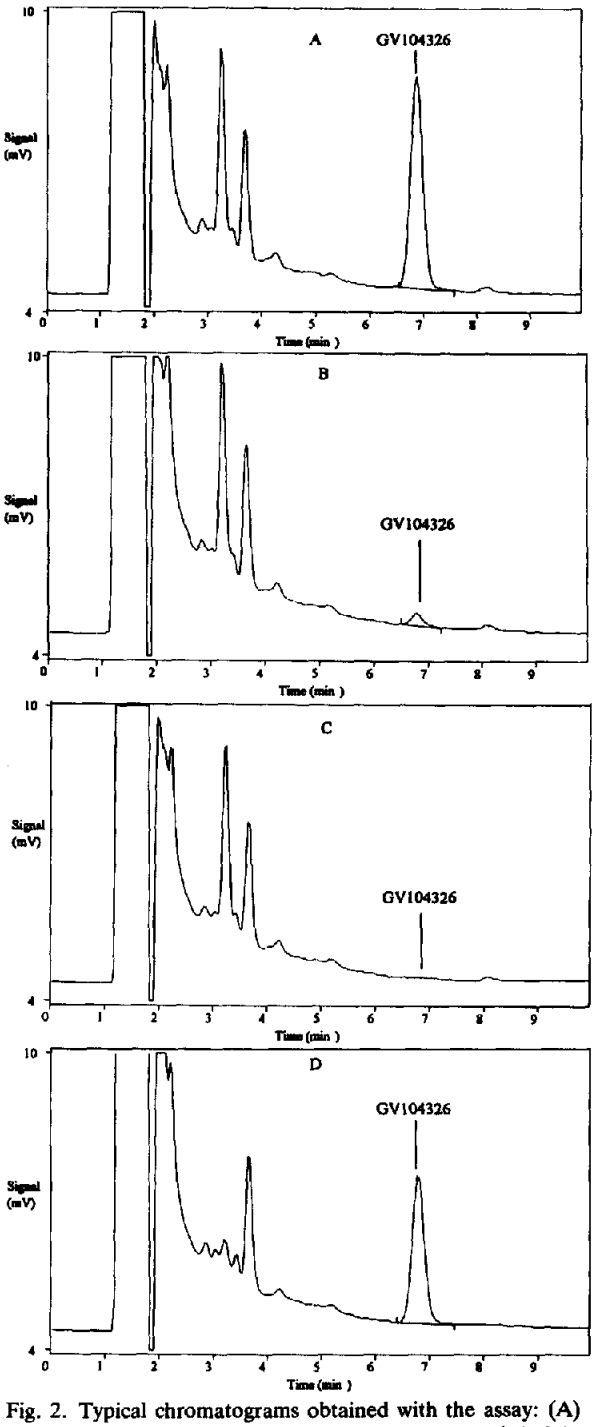

Fig. 2. Typical chromatograms obtained with the assay: (A) 1 μg/ml GV104326 plasma calibration standard; (B) 0.05 μg/ml GV104326 plasma calibration standard; (C) human plasma blank standard; (D) human plasma sample obtained 2 h after a single intravenous administration of 250 mg/kg GV104326B (concentration = 0.67 μg/ml).

Putting all this information together suggests that it is very unlikely that possible metabolites of GV104326 would interfere in the human plasma assay.

Lifetime of the HPLC column

As reported elsewhere [17,18] in assays in which neat plasma is added directly to SPE cartridges without prior protein precipitation, column efficiency has a tendency to decrease steadily with the number of injections of extracted plasma, to a point where integration of the lowest concentration standard becomes problematical. The lifetime of the column is extended by using a precolumn containing the same phase as the analytical column, and when changed approximately every 100 injections the lifetime of the column is approximately 300 injections. Alternatively, if a sensitivity of 100 ng/ml is sufficient, 50 μl of the final extract can be injected and the column life is doubled. As an exercise, an intra-day validation was performed with a column containing, 15,000 theoretical plates/m (as measured from the peak height/area ratio), and acceptable results were obtained (*i.e.* accuracy and precision < 10%). The column was discarded when the number of plates dropped below 15,000/m. Some extended column life could be obtained by reversing the direction of flow. The column was not washed with strong solvents but washed with about 100 column volumes of mobile phase at the end of every analytical batch.

Validation

The results of the intra- and inter-day variability are shown in Tables II and III. Intra-day and inter-day bias ranged from 4 to 9% and from 1 to 5%, respectively. Intra-day and inter-day variability ranged from 1 to 5% and from 4 to 7%, respectively. The validation results are compared with those obtained with the preliminary acetonitrile deproteinization method in Table IV. With both intra-day and inter-day studies, the precision of the Aspec method was superior to the preliminary method. In addition, the manual labour time and total analysis time were considerably shorter using the Aspec method, liberating the analyst to perform other tasks.

Standard lines were all calculated using linear regression, weighted by the reciprocal of the concentration and not forcing through zero. Also, the blank standard was not included in the regression and only served as an interference

TABLE II

INTRA-DAY VARIABILITY

Calibration concentration (μg/ml)	Observed concentrations (μg/ml)	Mean concentration (μg/ml)	S.D.	R.S.D. (%)	Bias (%)
0.05	0.052, 0.054, 0.054 0.053, 0.056, 0.053	0.054	0.014	2.6	8.0
0.1	0.111, 0.109, 0.110, 0.106, 0.100, 0.105	0.107	0.004	3.7	6.7
0.2	0.214, 0.210, 0.218, 0.213, 0.190, 0.210	0.209	0.010	4.6	4.6
0.5	0.570, 0.544, 0.541, 0.547, 0.540, 0.536	0.546	0.012	2.3	9.2
1	1.08, 1.04, 1.06, 1.05, 1.05, 1.06	1.06	0.014	1.3	5.7
2	2.18, 2.18, 2.16, 2.18, 2.13, 2.15	2.17	0.020	0.9	8.3

TABLE III

INTER-DAY VARIABILITY

Calibration concentration (μg/ml)	Observed concentrations (μg/ml)	Mean concentration (μg/ml)	S.D.	R.S.D. (%)	Bias (%)
0.05	0.049, 0.051, 0.049, 0.050 0.052, 0.055, 0.055, 0.048	0.051	0.003	5.5	2.0
0.1	0.091, 0.099, 0.110, 0.093, 0.092, 0.097, 0.109, 0.091	0.098	0.076	7.7	−2.0
0.5	0.467, 0.481, 0.492, 0.491, 0.419, 0.469, 0.509, 0.473	0.475	0.027	5	−5.0
2	1.90, 1.91, 1.98, 2.07, 1.82, 1.90, 2.04, 2.01	1.96	0.086	4.4	−2.0

check. All lines were linear with intercepts always <10% of the peak area of the lowest standard. Regression coefficients were always >0.995 and typically >0.998.

Recovery was determined at three concentration levels, in the calibration range, by comparing mean calculated plasma concentrations of two standards spiked before extraction with the mean of two control human plasma standards spiked after extraction, at each concentration level. The mean recovery was 87%, 84% and 86% at 0.05, 0.5 and 2 μg/ml, respectively.

The limit of reliable quantification is defined as the lowest concentration of GV104326 determined with acceptable accuracy and precision and hence is 0.05 μg/ml (100-μl injection) or 0.1 μg/ml (50-μl injection).

Stability, in the short-term studies at 4°C, was defined as the time after which the GV104326 peak area decreased by 5% of its initial area, at the time of spiking. Data were fitted to a log peak area/time regression and the stability determined from the regression line. GV104326 was stable in water-diluted plasma for 7 h and MES-diluted plasma for 11 h, at a concentration of 1 μg/ml. After SPE extraction, GV104326, at a concentration of 1 μg/ml, was stable for 3 h at room temperature. The use of a zwitterionic

TABLE IV

COMPARISON OF VALIDATION PARAMETERS BETWEEN ASPEC METHOD AND MANUAL DEPROTEINIZATION METHOD

Validation test	Aspec method	Deproteinization method
Intra-day precision	1–5%	2–8%
Intra-day bias	4–9%	1–8%
Inter-day precision	4–8%	4–16%
Inter-day bias	2–5%	−5–1%
Linearity of calibration	>0.995	>0.995
Recovery	84–87%	90–91%
Limit of quantification	0.05 μg/ml	0.1 μg/ml
Speed (total time 50 samples including standards and quality controls)	13 h	22 h
Manual labour time (50 samples)	1.5 h	4 h

buffer (MES) has been shown to stabilize β-lactam antibiotics compared with anionic buffers such as phosphate and acetate [7,19,20]. Previous studies have shown that the optimum pH for GV104326 stability in plasma is pH 6.5. Also, studies showed that the use of a more concentrated buffer solution (0.5 *M*) improved GV104326 plasma stability from 11 h to 4.5 days at 4°C, but unfortunately this concentration of MES drastically affected the SPE recovery, hence a compromise concentration of 50 m*M* was used. The limited stability of GV104326 in diluted plasma restricted the batch size to around 45 samples, including standards and quality controls, although a batch size of 50 in the validation studies always gave good results.

For the studies at −25 and −80°C, the data were fitted to an unweighted linear regression of log plasma concentration with time. The 95% lower confidence limit of the regression line was calculated using a one-sided test. GV104326 was considered to be stable in plasma up to a time when the concentration was greater than or equal to 90% of the initial concentration. This was shown by the time at which the lower 95% confidence limit intersected 90% of the initial concentration.

GV104326 was stable in human plasma for 10 days at −25°C and at least 7 months at −80°C.

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