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Quantitation of PGE9509924, a novel, nonfluorinated quinolone, in rat plasma using liquid chromatography electrospray-tandem mass spectrometry following solid-phase extraction sample clean-up in a 96-well format

P.H. Zoutendam *, M. Gavin, M.J. Martin, M.K. Dirr

Procter and Gamble Pharmaceuticals, Inc., Health Care Research Center, 8700 Mason-Montgomery Rd, Mason, OH 45040, USA

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Abstract

PGE9509924, a novel nonfluorinated quinolone, is a potent antibacterial agent with a broad spectrum of activity. A semi-automated method using 96-well format, solid-phase extraction has been developed for quantitating PGE9509924 in rat plasma. The Waters Oasis HLB extraction plate containing a polymeric packing material was found to give the best overall recoveries. All liquid transfer steps other than aliquoting the plasma are accomplished using a 96-channel pipettor. Reverse-phase HPLC with electrospray/MS/MS detection using selective reaction monitoring is used to quantitate the samples. Stable isotopically labeled PGE9509924 is used as the internal standard. The assay is linear over the range from 0.01 to 10 ug/ml. Excellent precision is obtained within a single run and between multiple runs performed on different days. CVs of $<6\%$ were observed. The combination of the semi-automated, 96-well parallel sample processing and the short runtime on the LC/MS/MS results in a high throughput assay with reduced operator interaction.

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Keywords: 96-well format; Solid-phase extraction; Quinolone; Electrospray HPLC/MS/MS; Plasma assay; Antibacterial agent

1. Introduction

PGE9509924, (**I**) 1-cyclopropyl-7-(3′-aminopiperidine)-8-methoxy-1,4-dihydro-4-oxo-3-quinolone, is a novel, nonfluorinated quinolone (see Fig. 1). It is a potent antibacterial and has a broad spectrum of activity against clinically important bacteria, including gram-positive, gram-negative, and atypical bacteria [1–6]. It maintains its potency against a number of drug resistant bacteria [1–3]. To evaluate the pharmacokinetic behavior of this compound, a reliable plasma assay was needed.

A high throughput type assay in which large numbers of samples could quickly and accurately be assayed was desired. One of the limiting factors

* Corresponding author. Tel.: +1-513-622-0757; fax: +1-513-622-0523.

E-mail address: zoutendam.ph@pg.com (P.H. Zoutendam).

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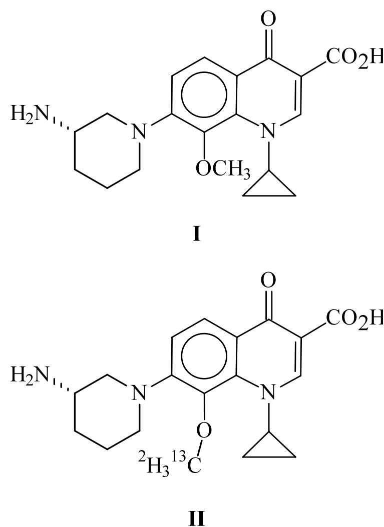

Fig. 1. Structures of PGE9509924 (**I**) and the internal standard, stable isotopically labeled PGE9059924 (**II**).

in assaying plasma samples is the runtime of the chromatographic separation. The vast majority of published assays for quinolones in biofluids utilize HPLC with UV or fluorescence (FL) detection [7–17]. Carlucci [7] reviewed bioanalytical methods for quinolones in 1998 and HPLC with UV or FL detection were the predominant detection methods used. Employing a highly selective detector such as a mass spectrometer permits the chromatographic runtime to be greatly reduced, compared with using FL or UV detection where longer runtimes are needed to separate the compound of interest from interfering substances. The advantages of using HPLC with tandem mass spectroscopy detection for increasing throughput while maintaining the selectively and sensitivity of biofluid methods are well known.

Another major limitation on the throughput of any plasma assay is the sample preparation procedure. Protein precipitation [7–10], liquid–liquid extraction [7,11–13], solid-phase extraction [14,15], and direct injection of plasma with on-line column switching [7,16,17] have all been used as sample preparation procedures for various quinolones in biofluids. The availability of 96-channel pipettors and the use of 96-well format procedures for sample preparation provides a means of increasing the throughput of the sample processing procedure by permitting large numbers of samples to be prepared in parallel. Protein precipitation, liquid-liquid extraction, and solid-phase extraction are all compatible with 96-well formats, however, 96-well format procedures were not used in any of the referenced methods. To maximize sample preparation throughput we decided to develop this method using 96-well format methodology and to automate the procedure as much as possible. Several different types of solid-phase extraction media are commercially available in 96-well format plates. Based on this and the author's familiarity with solid-phase extraction procedures, solid-phase extraction was selected for the sample preparation procedure.

2. Experimental

2.1. Chemicals and reagents

PGE9509924 (**I**) and $^{2}H_3^{13}C$-PGE9509924 (**II**) were synthesized in house [18]. Methanol (HPLC grade), 2-propanol, phosphoric acid, and ammonium hydroxide were from J.T. Baker (Phillipsburg, NJ, USA). Ammonium acetate was from Eastman Kodak Co (Rochester, NY, USA). Formic acid was purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). Rat plasma was obtained from PelFreez Biologicals (Rogers, AR, USA). Purified water was obtained from a Milli-Q UV Plus system (Millipore, Bedford, MA, USA). The extraction plate used was the 10 mg/well, Oasis HLB 96-well plate manufactured by Waters Corp. (Milford, MA, USA).

2.2. Instrumentation

The Quadra 96-Multipipettor, Model 320 with the vacuum module (Tomtec, Inc., Hamden, CT, USA) was used for transferring samples and performing the solid-phase extraction. Samples were taken to dryness in the SpeedVac, Model DSC250 with VaporNet (Savant Instruments, Inc., NY, USA). The mass spectrometer was a PE-Biosystems API 3000 triple-stage quadrapole mass

spectrometer (Concord, Ontario, Canada). The HPLC system on the mass spectrometer consisted of a Gilson Gradient System (Model 305 pumps, Model 805 Manometric Module, Module 811C Analytical Mixer) (Gilson, Inc., Middleton, WI, USA), a Waters Solvent Degasser (Waters Corp.) and a LEAP HTS PAL autosampler (Leap Technologies, Carrbora, NC, USA). A divert valve (ThermoQuest Corp., San Jose, CA, USA) was installed between the outlet of the column and the mass spectrometer. The HPLC used for UV detection consisted of a Waters Alliance HT 2790 with a Waters 996 Photodiode Array detector.

2.3. Chromatographic conditions

On the LC/MS/MS system, a Waters Symmetry Shield RP18 column (2.1 ID × 50 mm, 3.5 um) with a rapid gradient separation was used. Mobile-phase A consisted of 50 ml methanol, 50 ml 2-propanol, 900 ml purified water, 1.0 ml of formic acid, and 0.154 g of ammonium acetate (2 mM). Mobile-phase B consisted of 600 ml methanol, 200 ml 2-propanol, 200 ml purified water, 1.0 ml formic acid, and 0.154 g ammonium acetate (2 mM). For the initial 1.5 min, 100% mobile-phase A was used, then the mobile-phase was changed in a linear manner over the next 1.5 min to 100% B. The system was maintained at 100% B for 0.5 min, then it was changed back to 100% A over 0.1 min. The system was equilibrated for 1.4 min prior to the next injection. The flow into the mass spectrometer was diverted to waste for the first 1.5 min after each injection. The flow rate was 0.4 ml/min and the injection volume was 10 ul. The LEAP autosampler permits two rinse solvents to be used. Normally, a high organic solvent and a low organic solvent are used. The high organic rinse solvent consisted of 0.2% formic acid, 10% 2-propanol, 70% methanol, and 20% water. The low organic rinse consisted of 0.2% formic acid, 10% 2-propanol, 40% methanol, and 50% water. The presence of the 2-propanol in the mobile-phase and the autosampler rinse solvents, reduced the amount of carryover seen from ~0.3 to <0.05%.

A Zorbax Rx-C8 column (4.6 mm × 250 mm, 5 μm) was used with the HPLC/UV system. Mobile-phase A was methanol and mobile-phase B was 50 mM phosphoric acid adjusted to pH 3.1 with ammonium hydroxide. For the first minute the mobile-phase was held at 25% A/75% B. It was then linearly ramped to 75% A/25% B over 15 min, held at these percentages for 1 min, ramped back to the initial conditions over 0.5 min, and re-equilibrated for 4.5 min. The flow rate was 1.0 ml/min and the injection volume was 25 ul. The retention time of PGE9509924 is 8.2 min. Chromatograms were extracted at 296 nm for determining PGE9509924. Chromatograms at additional wavelengths can be extracted as needed.

2.4. ESI-MS/MS conditions

Solutions of the analyte and IS were infused to determine the optimized MS/MS conditions for each compound. The TurboIonspray source temperature was set to 400 °C. The Ionspray voltage was 1500 V, the orifice was set to 60 V, the ring was set to 140 V, and the ST3 setting was −66 V. The nebulizer, curtain, and collision gases were set to 8, 6, and 5, respectively. The MS/MS detection scheme utilized collisionally activated dissociation (CAD) with selective reaction monitoring (SRM) of m/z 358–340 and 362–344 for the analyte and IS, respectively, at optimized collision energy of −30 V (R02-Q0). The dwell time was 100 ms for each selected ion reaction. No interference for this SRM scheme, monitoring the loss of water (−18 amu), was detected in matrix blanks.

2.5. Preparation of standard solutions

Standards were prepared via serial dilution of a stock solution on a daily basis. Standards were prepared at the following concentrations: 0.01, 0.02, 0.04, 0.1, 0.4, 1.0, 4.0, 7.5, 8.5, 10.0 ug/ml. The standards, including the stock solution were prepared in 20% methanol/80% 50 mM phosphoric acid adjusted to pH 3.1 with concentrated ammonium hydroxide. A stock solution of the internal standard (~50 ug/ml) was prepared in 1:1, methanol:water. The final internal standard solutions were prepared fresh daily at a concentration of 0.4 ug/ml, using 20% methanol/80% phosphate, pH 3.1 as the diluent. The internal standard stock solution was stored in the refrig-

erator between runs. No change in its concentration or purity was noted over a period of 3 months.

2.6. Preparation of quality control standards

Stock solutions were prepared in the same buffer used for the standards at ten times the concentration of the QC samples. Quality Control samples were prepared by spiking aliquots of plasma with these stock solutions. Two sets of QC samples were prepared. The first set consisted of QCs at four concentrations, 0.02, 0.04, 0.8 and 8.0 ug/ml. A second set was prepared that included four additional, higher concentrations. The second set consisted of QCs at 0.01, 0.0375, 0.75, 7.5, 15, 75, 150 and 375 ug/ml.

2.7. Semi-automated SPE procedure

Each well was conditioned with two aliquots of 400 ul of methanol and two aliquots of 400 ul of water. Each aliquot was drawn through the extraction plate by placing the plate on a vacuum manifold and applying vacuum. For these solutions and the solutions described below, the vacuum was adjusted to between 1 and 5 mmHg such that each solution was drawn through the wells in less than 1 min. Next, an aliquot of the internal standard solution (50 ul) followed by either an aliquot of standard solution (50 ul) or an aliquot of buffer (50 ul) was added to each well. The appropriate standard solution was added to the standards. Blank buffer was added to the samples, QCs, and blanks. Finally, aliquots of plasma (50 ul) (blank plasma to the standards and sample plasma to the samples and QCs) were added to each well. The samples were then drawn through the extraction plate by applying a vacuum. This method of preparing the standards was compared with spiking blank plasma with stock standard solutions, then sampling the spiked plasma. Equivalent results were obtained. The method described here was considered more convenient and was routinely used. The wells in the plate were then washed sequentially with 400 ul of water and twice with 400 ul of 30% methanol/70% water. The samples were eluted with two 300 ul aliquots of 1% formic acid in methanol. The combined elution fractions were taken to dryness in a SpeedVac vacuum centrifuge set to medium heat (43 °C) with the lamps off. Samples were reconstituted in 500 ul of 1:1, water:mobile-phase A. Aliquots of plasma were transferred with a manual pipettor, while all other liquid transfers were performed by the Quadra 96. Plasma samples were transferred manually because samples were to be received in small cyrovials that were not compatible with the 96 channel pipettor. To avoid handling the samples twice, they were directly, but manually pipetted into the 96 well SPE plate.

2.8. Validation

Standard curves were prepared and assayed on 4 separate days. For assessing the precision of the assay, two sets of QC samples were each assayed on 2 days. Both sets contained QCs at nominal concentrations of 0.02, 0.0375, 0.75 and 7.5 ug/ml. The set second contained four additional quality control samples prepared at higher concentrations (15, 75, 150 and 375 ug/ml). The 15 and 75 ug/ml samples were diluted 1 to 10 with blank plasma, then assayed as previously described. The 150 and 375 ug/ml samples were diluted 1 to 10 and again 1 to 5 with blank plasma, before being assayed. To assess the stability of the prepared samples, a double set of samples were prepared with run number 3. The second set was carried through the SPE procedure, taken to dryness, and stored in the dry state at room temperature. Three days later these samples were reconstituted and assayed versus freshly prepared standards. The original, reconstituted samples prepared 3 days earlier and which had been stored at room temperature were also reassayed. Recovery was estimated by comparing the peaks areas of the analyte peaks to the peak areas of a set of standards which were not carried through the SPE procedure. HPLC with UV with detection at 296 nm was used to estimate recovery. Only a representative sampling of the higher concentration samples (>1 ug/ml) were used.

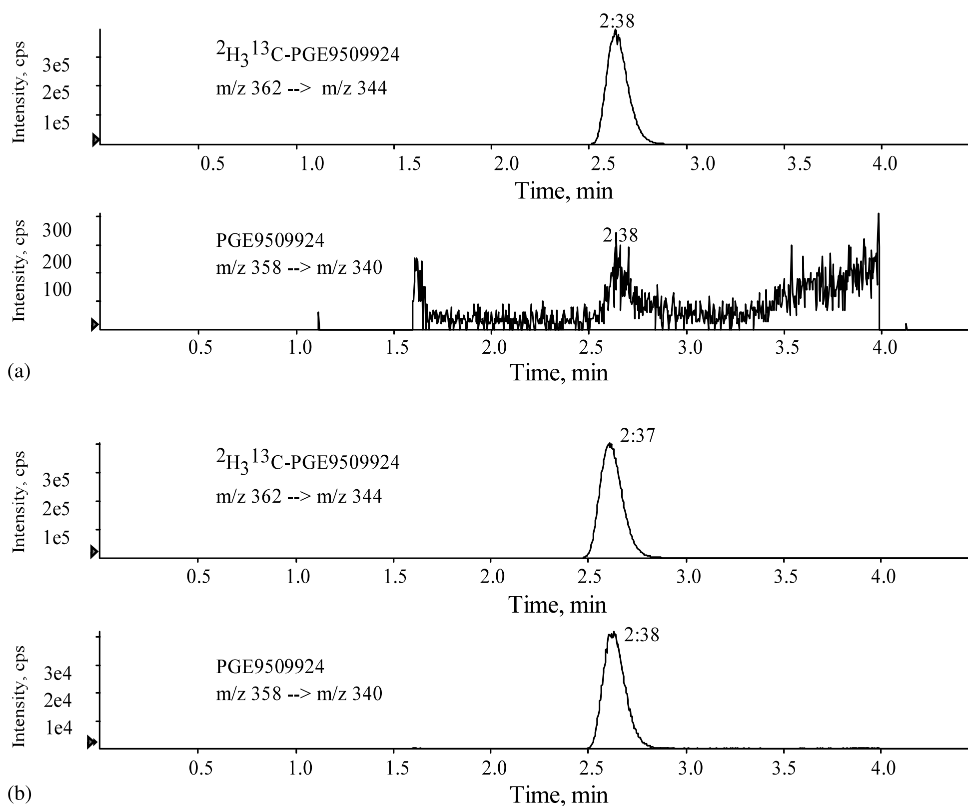

Fig. 2. Chromatograms of (a) a plasma blank spiked with internal standard and (b) a standard spiked with 0.1 ug/ml of PGE9509924. Conditions are listed in the experimental section.

3. Results and discussion

3.1. Optimization of SPE conditions

Several different brands and types of 96-well extraction plates were evaluated. For this compound, better overall recovery was observed with the Waters Oasis HLB extraction plate. The Waters Oasis HLB material is a polymeric reversed phase material. The other plates we evaluated contained various silica based reversed phase materials. PGE9509924 was very strongly retained on these materials. Multiple elution steps with strong solvents were required to quantitatively elute it. On either the 10 or 30 mg HLB extraction plate, PGE9509924 could be quantitatively eluted with two 300 ul portions of methanol containing 1% formic acid.

Water was used for the initial rinse, to elute proteins and salts. To optimize any additional rinses, various rinse solutions and the number of rinses used were evaluated. Rinses containing from 0 to 40% methanol and 0 to 20% acetonitrile mixed with water were evaluated. Rinses containing phosphate buffer at both pH 3 and 7.4 were also tried. The rinses and elution fractions were collected and analyzed by reverse-phase HPLC with UV detection at 230 nm. This method detected most materials, which might be present in the sample. While the MS detector is selective for the analyte of interest, the presence of other materials can affect the amount of suppression, thereby affecting the response for that particular sample. A cleaner sample will help insure that the method is accurate, rugged and reliable. The elution fractions obtained from the samples with the rinses containing 30 and 40% methanol were noticeably

Table 1
Standard curve data

Day	b	Standard error b	m	Standard error m	Correlation coefficient
Run 1	0.00474	0.00585	0.01798	0.00026	0.99914
Run 2	0.00496	0.00662	0.01962	0.00030	0.99908
Run 3	0.00382	0.00489	0.01877	0.00021	0.99958
Run 4	0.00129	0.00113	0.01934	0.00006	0.99997

A linear curve, $y = mx + b$, was used, where x = concentration (ng/50 μl of plasma) and y is the ratio of the area of the analyte peak to the area of the internal standard peak. A weighting factor of $1/x$ was used.

cleaner than the others. Little improvement was observed when the methanol content was increased from 30 to 40%. In all cases, two rinses gave cleaner elution fractions than one rinse, however, three rinses did not result in any further improvement. No improvement was noted when phosphate buffer at pH 3 or 7.4 was used in place of water. Based on this we decided to use two rinses using 30% methanol/70% water. Overall recovery throughout the SPE procedure was as good with the two 30/70 methanol/water rinses as it was with weaker rinse solvents. Overall recovery averaged greater than 80%.

3.2. Adsorption

When dissolved in 100% aqueous media at concentrations below 0.5 ug/ml, losses in the concentration of PGE9509924 in both glass and plastic vials were observed. The loss was greatest at neutral pH, but was still significant at acidic pH (3.1). At higher concentrations, such losses were not noticeable. Adding methanol to the solution decreased the amount of absorptive losses. A mixture containing 20% methanol and 80% phosphoric acid adjusted to pH 3.1, eliminated adsorptive losses and was selected as the diluent for preparing stock solutions and standard solutions.

3.3. Chromatography

Peaks with good peak shape were observed with the gradient method used. Isocratic methods were investigated, however, the gradient method gives sharper peaks with less tailing compared with the peaks observed when an isocratic method is used. No loss in peak shape was observed with the relatively high flow rate used (0.4 ml/min) and it decreased overall runtime. Fig. 2 shows sample chromatograms of a plasma blank containing the internal standard and a standard spiked with 0.1 ug/ml of PGE9509924. A trace amount of PGE9509924 is present in the stable isotope labeled internal standard, as can be seen in the PGE9509924 channel in Fig. 2a. Because this is present in all samples and standards and is very small, it was not found to have any measurable effect on accuracy or precision. No peaks were observed in plasma blanks without internal standard.

3.4. Linearity and dynamic range

The linear range of this assay was validated over the range from 0.01 to 10.0 ug/ml. Standard curves were calculated by performing linear regression on the concentration versus area ratio data using a weighting factor of $1/x$, where x is the concentration. The slopes, intercepts, standard errors, and correlation coefficients for the standard curves are listed in Table 1. The curves from all four runs were linear, giving correlation coefficients (r) of > 0.999. The lower limit of quantitation was 0.01 ug/ml. The limit of detection (LOD) was 0.005 ug/ml. A reconstitution volume of 500 ul was selected to ensure that the response was linear and quantifiable over this range of concentrations. If needed, a lower LOD could easily be obtained by reconstituting the sample in a smaller volume and/or injecting a larger volume. The upper end of the range is limited by the amount of carryover observed and the fact that at higher concentrations the response of the HPLC/MS/MS tends to deviate from linearity. The upper limit of the linear range

Table 2
Precision of quality control samples

	n	Concentration added (μg/ml)	Concentration found (μg/ml)	Recovery (%)	CV (%)
QC Level 1					
Run 1	4	0.0218	0.0211	96.8	3.5
Run 2	3	0.0218	0.0207	95.1	1.6
Run 3	4	0.0104	0.0082	78.8	7.5
Run 4	4	0.0104	0.0102	98.1	0.7
Pooled CV					4.4
QC Level 2					
Run 1	4	0.0435	0.0460	106	3.4
Run 2	3	0.0435	0.0471	108	3.7
Run 3	4	0.0378	0.0377	99.7	5.4
Run 4	4	0.0378	0.0388	103	3.5
Pooled CV					4.1
QC Level 3					
Run 1	4	0.816	0.873	107.	0.9
Run 3	4	0.756	0.799	106.	3.3
Run 4	4	0.756	0.771	102.	2.1
Pooled CV					2.3
QC Level 4					
Run 1	4	8.16	8.14	99.7	0.9
Run 2	3	8.16	8.12	99.5	1.9
Run 3	4	7.56	7.59	100.	3.0
Run 4	4	7.56	7.77	103.	3.6
Pooled CV					2.6
QC Level 5					
Run 3	4	15.1	16.5	109.	3.5
Run 4	4	15.1	16.3	108	4.4
Pooled CV					4.0
QC Level 6					
Run 3	4	75.6	76.2	101.	5.2
Run 4	4	75.6	77.2	102.	1.9
Pooled CV					3.9
QC Level 7					
Run 3	4	151	165	109.	5.7
Run 4	4	151	155	103	4.8
Pooled CV					5.3
QC Level 8					
Run 3	4	378	361	95.6	4.6
Run 4	4	378	363	96.1	2.6
Pooled CV					3.7

could be extended by diluting the sample more, but at the expense of increasing the limit of quantitation. The method was validated over this range because most samples were expected to fall within this range, with the option of diluting very high concentration samples with plasma prior to sample preparation.

3.5. Precision

Replicate samples of two sets of quality control samples were each assayed in two runs. Excellent within run precision was obtained at all levels. The within run coefficients of variation (%CV) were all less than 6%. The pooled CVs [19] were also all less

than 6%, demonstrating the excellent day-to-day precision of this method. Relative recoveries were very good, ranging between 90 and 110% in all cases except for one of the level 1 QCs in one run. Results are presented in Table 2.

3.6. Stability of prepared samples

When assayed versus freshly prepared standards, processed samples that had been stored at room temperature for 3 days in either the dry state or reconstituted, showed no loss in concentration.

4. Conclusions

The method described in this report is capable of accurately determining PGE9509924 in rat plasma. The method works equally well with mouse and dog plasma (data not shown). The method is linear over the range from 0.01 to 10 ug/ml and is highly reproducible. The combination of the 96-well format, semi-automated sample preparation procedure and the use of HPLC/MS/MS results in a high throughput method. To date, up to 140 samples (not including standards and QCs) have been prepared and assayed in 1 day. Potentially, the throughput could be increased even further, by automating the aliquoting of the plasma samples.

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