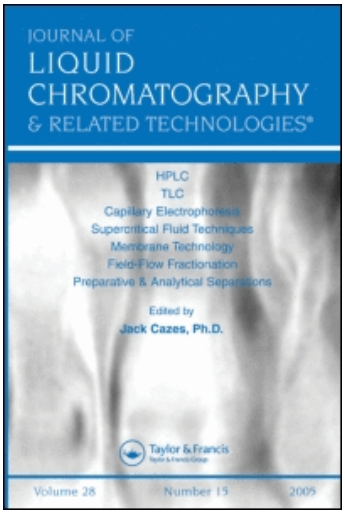

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MIXED-MODE GRADIENT HPLC ANALYSIS OF A TYROSINE KINASE INHIBITOR, ITS ISOMERS AND OTHER POTENTIAL IMPURITIES

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ABSTRACT

A mixed-mode high performance liquid chromatographic (HPLC) method has been developed for the determination of RG-14620 and its potential impurities and degradation products in a topical formulation. The separation of geometric and other structural isomers of RG-14620 as well as potential process impurities and degradation products was achieved by gradient elution from a Supelcosil LC-SCX cation exchange column (5 μm particle size, 250 x 4.6 mm i.d.) in series with a Nova-Pak dimethyloctadecylsilyl analytical column (4 μm particle size, 150 x 3.9 mm i.d.). The gradient components were 25mM sodium phosphate buffer (pH=3.5) and acetonitrile. The flow rate was 1.1 mL/min. with UV absorbance detection at 260 nm. The implementation of the mixed-mode technique is especially useful for pharmaceutical products whose collective mixture of precursors and potential degradation products often span a wide range on the polarity scale.

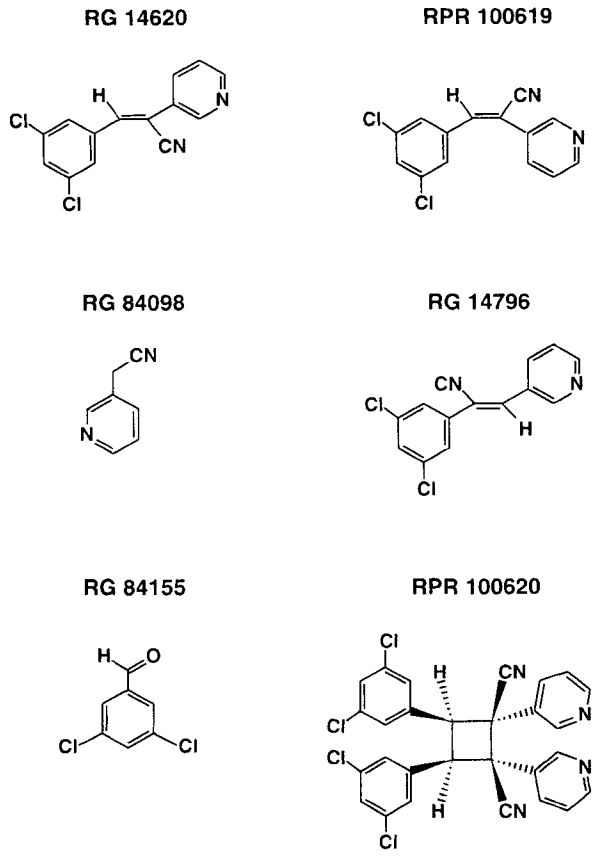

FIGURE 1. The chemical structures of RG-14620 and related substances.

INTRODUCTION

Cellular mitogenesis (cell growth) may be stimulated by a variety of serum growth factors. These growth factors interact with cell-surface receptors that possess an intrinsic, ligand-sensitive, protein tyrosine kinase activity [1]. Synthetic compounds known as tyrphostins are inhibitors of protein kinase activity specific for epidermal growth factor-receptor kinase [2]. RG-14620 is a novel

tyrphostin which may be useful in treating specific pathological conditions involving cellular proliferation including different types of cancers. The structures for RG-14620 and related compounds are given in Figure 1. RG-14620 has been formulated in a hydrophobic petrolatum base at a level of 5% (w/w).

Mixed-mode or mixed-interaction chromatography has found utility in many separations including the separation of small molecule organic acids [3], oligonucleotides [4] and other natural products [5]. Although a variety of techniques exist, the two major categories of mixed-mode chromatography are off-line and on-line chromatography. Off-line chromatography involves collection of solutes at the detector exit of the first column followed by re-injection of the captured eluent onto a second column. On-line chromatography entails coupling the two columns either directly or by means of a switching valve. The use of mixed-bed supports has also been successfully used [4].

Pharmaceutical products often have a varied mixture of potential degradation products and process impurities which collectively may not be suitably analyzed by either reversed-phase or ion-exchange chromatography alone. While the use of ion-pair reagents is a common solution to the separation of multiple species, the need for gradient elution in the present case made the use of ion-pair reagents less desirable due to difficulties in maintaining constant levels of ion-pair reagent on the column.

EXPERIMENTAL

Instrumentation and Operating Conditions

The liquid chromatograph consisted of an auto sampler (Waters' WISP, Model 710B), a gradient HPLC pump (Hewlett Packard, Model 1050), a variable wavelength detector (Applied Biosystems, Model 783), and a Supelcosil LC-SCX cation exchange

column (5 μm particle size, 250 x 4.6 mm i.d.) in series with a Nova-Pak dimethyloctadecylsilyl analytical column (4μm particle size, 150 x 3.9 mm i.d.). The gradient components were (A) 85/15 25mM sodium phosphate buffer (pH=3.5)/ acetonitrile and (B) acetonitrile. The initial mobile phase conditions were 22% B followed by a linear gradient of 22 to 35% B from 0 to 10 minutes . The percentage of component B was further increased from 35 to 54% from 10 to 32 minutes. The mobile phase was then returned to initial conditions and held for 12 minutes to re-equilibrate the column. The flow rate was 1.1 mL/min. with UV absorbance detection at 260 nm. The injection volume was 20 microliters. Peak Integration was performed using Waters' 860 Chromatography software on a DEC computer system. Photodiode array analysis was performed using a Waters' 990 Photodiode array detector and Waters' 600E system controller and pump. The pKa value for RG-84098 was determined using a Sirius PCA 101 autotitrator.

Chemical and Reagents

Acetonitrile (HPLC grade), tetrahydrofuran (HPLC grade), sodium phosphate, monobasic, monohydrate (ACS grade) and phosphoric acid, 85% (HPLC grade), were obtained from Fisher Scientific, Fair Lawn, N.J. Distilled water was further purified by passing it through a Milli-Q water purification system (Millipore Inc., Milford, MA). All pharmaceutical compounds were supplied by Rhône-Poulenc Rorer Central Research.

Sample and Standard Solution Preparation

RG-14620 converts to the intra-molecular cycloaddition product, RPR-100620, in the presence of light and therefore both sample and standard solutions were prepared in low-actinic glassware. The working sample diluent was prepared by adding 50

mL of acetonitrile to 50 mL of 25 mM sodium phosphate, monobasic, monohydrate buffer (pH adjusted to 3.5 with 85% phosphoric acid). The working standard diluent was prepared by adding 120 mL of 25 mM sodium phosphate monobasic, monohydrate buffer (pH adjusted to 3.5 with 85% phosphoric acid) to 60 mL of tetrahydrofuran. A stock standard solution was made by accurately weighing 24 mg of RG-14620 standard into a 100 mL low-actinic volumetric flask. Fifty mL of acetonitrile were added to the flask and the solution was sonicated for 3 minutes and diluted to volume with acetonitrile. The working standard solution was prepared by diluting 20.0 mL of the stock standard solution to 50.0 mL with the working standard diluent. A stock sample solution was made by weighing 0.24 grams of 5% (w/w) RG-14620 in petrolatum into a 50 mL screw-cap test tube. Twenty-five mL of tetrahydrofuran were accurately added to the test tube and the tube was shaken for 15 minutes. The working sample solution was prepared by diluting 5.0 mL of the stock sample solution with 20.0 mL of the working sample diluent and the resultant solution was mixed well and centrifuged for 15 minutes at 4000 r.p.m. in a bench-top centrifuge to obtain a clear supernatant.

RESULTS AND DISCUSSION

Development of a Chromatographic Method

Initial attempts to develop either isocratic or gradient methods to control RG-14620 and its potential impurities and degradation products resulted in the column's inability to provide for appropriate retention ($k' < 0.2$) of the polar precursor, RG-84098 (pKa=4.07). Although adequate reversed-phase methods had been readily developed for the control of the drug substance, early eluting excipient components in the drug product co-eluted with RG-84098. In order to increase the column retention of RG 84098,

ion-pair chromatography was pursued. The ion-pair phenomena entails the association of oppositely-charged species. At pH=3.0 more than 90% of RG-84098 molecules exist as the positively-charged species; however, attempts at ion-pairing RG-84098 with a variety of ion-pairing reagents, including hexane sulfonic acid and octane sulfonic acid, proved ineffectual. In addition, it became evident that a gradient method would be necessary to provide for separation of the complex mixtures of geometric isomers and potential degradation products. The use of ion-pairing reagents in a gradient system was not desirable due to the difficulty of keeping the column equilibrated with a constant level of ion-pairing reagent.

Initial work with a mixed-bed (reversed-phase/cation exchange) column demonstrated a k' value for RG 84098 of > 1.5. The column was a RP-C8/cation exchange column (Alltech M/M, RP8/Cation, 5 µm particle size, 150 x 4.6 mm i.d.). In spite of this column's ability to greatly increase the retention of RG-84098, the column's low theoretical plate count, as reflected in the poor resolution of the structural isomers (Figure 2) of RG-14620, precluded the use of the mixed-bed column alone. Further attempts to improve the chromatography, including placing the mixed-bed column in series with a Supelcosil LC-18 DB column (3 µm particle size, 150 x 4.6 mm i.d.), also resulted in inadequate resolution.

Replacement of the mixed-bed column with a Supelcosil LC-SCX cation exchange column (250 x 4.6 mm i.d.) in series with the Supelcosil LC-18 DB column provided both adequate retention of RG-84098 and resolution of impurity and degradation products of RG-14620. Subsequent systematic adjustments of the mobile phase gradient, as well as the pH of the mobile phase buffer component, were made to insure optimum resolution of all of the components.

Ultimately, the Supelcosil LC-18 DB column was replaced with a Waters' Nova-Pak C18 column (4 µm particle size, 150 x 3.9 mm i.d.). The Nova-Pak C18 column provided the best peak shape for RG-84155, a peak which previously had shown a propensity to

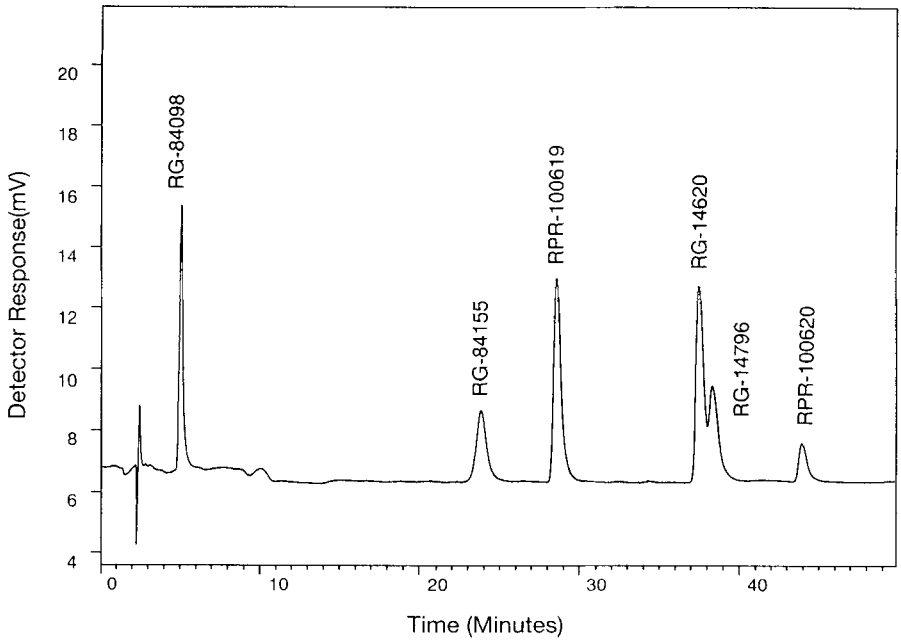

FIGURE 2. Chromatogram of an injection of RG-14620 standard solution spiked with 3% (w/w) related substances on an Alltech M/M, RP8/Cation column (150 x 4.6 mm i.d.).

front on the Supelcosil LC-18 DB column. Figure 3 displays a chromatogram of an injection of a mixture of RG-14620 and related compounds at ~3% (w/w) levels. Table 1 lists the retention time (RT, minutes), relative retention time (RRT) and resolution factor between adjacent peaks. Two base degradation peaks are also noted in Figure 3. Mass spectrometry suggested that base degradation product 1 was the 2,3-epoxide degradation product of RG-14620. The identity of base degradation product 2 was not determined but formed readily in solutions of base degradation product 1. The base degradation products were generated as part of accelerated degradation testing described below.

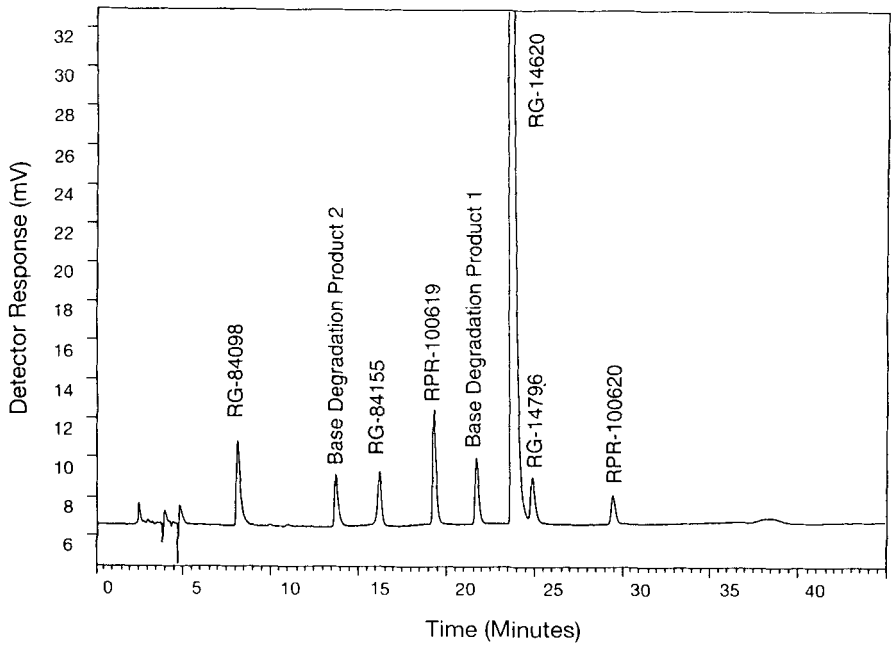

FIGURE 3. Chromatogram of an injection of RG-14620 standard solution spiked with 3% (w/w) related substances and solution degradation products on a Supelcosil LC-SCX cation exchange column (250 x 4.6 mm i.d.) in series with a Waters' Nova-Pak C18 column (150 x 3.9 mm i.d.).

The ruggedness of the chromatographic method was evaluated, in-part, by determining the chromatographic reproducibility of two columns sets. A comparison of retention times, relative retention times and resolution factors showed only minor differences. Additional studies were performed in order to determine the effect of changes in the pH of the aqueous component of the mobile phase on the chromatography. The resolution between RG-14620 and RG-14796 (the closest eluting

TABLE 1
Chromatographic Data for Mixed Mode Gradient Method on a Supelcosil LC-SCX Cation Exchange Column (250 x 4.6 mm i.d.) in Series with a Waters' Nova-Pak C18 Column (150 x 3.9 mm i.d.).

Compound	RT	RRT	Rs
RG-84098	8.10	0.34	---
Base Product 1	13.6	0.57	14.1
RG-84155	16.1	0.68	6.68
RPR-100619	19.3	0.81	8.63
Base Product 2	21.7	0.91	6.54
RG-14620	23.8	1.00	5.33
RG-14796	24.8	1.04	2.47
RPR-100620	29.4	1.24	10.1

RT = Retention Time (minutes)
RRT = Retention time relative to RG-14620
Rs = Resolution between component peak and previously eluting peak

peak to RG-14620) was monitored while changes in the pH were made. Results indicated no significant difference in resolution between these two components as the pH was modified by ±0.2 units. Table 2 displays the retention time for each component of the impurity mixture at a given pH of the mobile phase aqueous component. Over the range of pH 3.3 to 3.7, only two components demonstrated a strong retention time dependency on pH, namely RG-84098 and base degradation product 1. The retention time behavior of these components likely indicates the more prevalent role of the cation exchange column in the retention of these components. In summary, a change in pH of the aqueous component of the mobile phase over the range of pH 3.3 to 3.7 did not compromise the specificity of the method with

TABLE 2
Mobile Phase Aqueous Component pH Effects on Retention Time for Mixed Mode Gradient Method on a Supelcosil LC-SCX Cation Exchange Column (250 x 4.6 mm i.d.) in Series with a Waters' Nova-Pak C18 Column (150 x 3.9 mm i.d.).

Compound	Retention Time (minutes) (pH=3.3)	(pH=3.5)	(pH=3.7)
RG-84098	9.4	8.1	7.1
Base Product 1	15.0	13.6	12.5
RG-84155	15.8	16.1	15.8
RPR-100619	19.0	19.3	18.8
Base Product 2	21.4	21.7	21.1
RG-14620	23.7	23.8	23.1
RG-14796	24.7	24.8	24.1
RPR-100620	28.9	29.4	28.7

regard to RG-14620; however, the pH sensitivity of the retention time of two impurity components required control of the mobile phase aqueous component pH to within ±0.1 pH units.

Linearity and Response Factor Determination

The linearity of detector response for RG-14620 was tested over the range of 0.04581 to 0.1527 mg/mL. The detector response for RG-14620 was found to be linear from 47 to 159% of target sample concentration with a correlation coefficient of 0.99999 (n=8). Deviations of the observed values from the predicted values of the area responses (at the 95 percent confidence limit) derived from the regression analysis were not significant. The null hypothesis was adopted to examine the probability of systematic error in the

determination of the y-intercept. For a y-intercept equal to zero, P = 0.577 indicating that the y-intercept is not statistically different than zero. The linearity of detector response was also tested for RG-84098, RG-84155, RPR-100619, RG-14796 and RPR-100620 at levels of 0.3 to 2% (w/w) of RG-14620 levels. Deviations of the observed values from the predicted values of the area responses (at the 95 percent confidence limit) derived from the regression analysis were not significant. Regression analysis of the related substances showed satisfactory linearity with correlation coefficients of 0.999 or greater (n=6).

The relative response factors (RRF) for several of the related compounds are listed in Table 3. In order to calculate the RRF, the individual response factor (RF) for each substance was first determined from the harmonic mean of the (area/concentration) ratios. The harmonic mean was calculated by determining the anti-log of the average log value of the ratios. The harmonic mean was used as this mean represented the minimum variance unbiased estimator for the ratio. These responses were then compared with the working standard solution response of RG-14620 in order to calculate the relative response factors. The calculation of the relative response factors is shown at the bottom of Table 3.

Recovery of Drug Substance from Sample Matrix

The literature contains a variety of methods for the extraction of drugs from petrolatum [6], [7], [8]. These methods include chloroform, water/chloroform and hexane/water extraction for some water soluble drugs. RG-14620, however, has poor water solubility (<1 µg/mL) and shows affinity for both hexane and chloroform. Initial studies using THF as the extracting solvent and a working sample diluent of 30/70 sodium phosphate buffer (25 mM, pH=4.4)/acetonitrile, showed a mean percent recovery of 96.6% over the range of 50 to 150% of the target working sample concentration. The sample diluent was later modified based upon

TABLE 3
Relative Response Factors for Related Compounds of RG-14620 at 260 nm

Compound	Relative Response Factor*
RG-84098	1.1
RG-84155	1.3
RPR-100619	0.66
RG-14796	0.99
RPR-100620	2.2

The response factor for each component has been calculated using the following equation: *relative response factor = A_{14620}/ (C_{14620} x RF), where A_{14620} = peak area of RG-14620, C_{14620} = concentration of RG-14620 (mg mL^{-1}) and RF = response factor (harmonic mean) from peak area vs. concentration for each component.

observations of the pH effect on the mixed-mode column retention of RG-14620 (i.e. increased retention at lower mobile phase pH values). It was apparent that the increased retention of RG-14620 was due to the greater cationic character of RG-14620 at lower pH values. It was reasoned, therefore, that lowering the pH of the buffer component of the sample diluent would perhaps enhance the solubility of RG-14620 and therefore improve the recovery. Using a sample diluent of pH=3.5 (described in the sample and standard preparation section), the recovery values ranged from 99.8 to 101.6% for a set of 11 samples, with a mean recovery of 100.7% (SD=0.6).

Accelerated Sample Degradation

Photodiode array detection may be useful in evaluating the homogeneity of chromatographic peaks and has been shown to be able to detect impurity levels of 0.5 percent (w/w) for structurally

similar compounds [9]. Photodiode array detection is commonly used as evidence of method specificity.

In order to provide evidence of method specificity for the present procedure, accelerated degradation of the 5% petrolatum formulation of RG-14620 and placebo was performed. Sample and placebo were placed in separate glass test tubes with plastic screw-cap tops (glass weighing bottles with ground-glass stoppers were used for heat-treated samples) for three days and exposed to one of the following conditions: 25 mM sodium phosphate, pH=2; 25 mM sodium phosphate, pH=12; 3% hydrogen peroxide; heat (70°C) and light (295 foot-candles.) Three additional experiments performed to induce degradation entailed modifying the working sample diluent by replacing the 25 mM sodium phosphate buffer (pH=3.5) with either 25 mM sodium phosphate buffer (pH=2), 25 mM sodium phosphate buffer (pH=12) or 3% hydrogen peroxide.

Each of the above sample and placebo solutions were analyzed on a Waters' HPLC system equipped with a model 990 photodiode array detector. The placebo chromatograms were analyzed for the presence of any peaks which would potentially interfere with the quantitation of RG-14620 or related compounds. In addition, the RG-14620 peaks of the sample solution injections were examined by superimposing the UV -visible spectra derived from the upslope, apex and downslope of each peak. No significant differences were seen between the resultant spectra providing additional evidence to support the specificity of the method. It should be noted that the data obtained from the accelerated degradation study may not necessarily reflect the potential degradation of actual stability samples. The accelerated degradation study is performed in an attempt to demonstrate the specificity of the method in the absence of long-term stability data.

Method Precision and Solution Stability

The method precision of the 5% formulation assay procedure was determined by analyzing six separate sample preparations

from a composite sample of RG-14620 drug product versus an external standard. The data showed acceptable method precision with an RSD of 0.80% for six separate assays (assay mean = 101.3% of label claim).

Working sample and standard solution stability was carried out in low-actinic flasks at refrigerated (4°C) and room temperature (23°C) conditions. The standard solution appeared stable for three days both at room temperature and refrigerated conditions. No additional peaks were noted in the standard solution chromatograms and the standard areas of RG-14620 were within ±2% over the course of the study. The room temperature sample solution chromatograms showed the appearance of an unidentified peak at a retention time of 35.9 minutes (RRT = 1.49) after one day. The peak was present at a level of 0.04% by area. In contrast to the room temperature sample solution stability results, the refrigerated sample solution showed no additional peaks or change in peak area for RG-14620 after two days. The three-day refrigerated sample showed a minor peak near the limit of detection (0.03% by area) at RRT = 1.39. Therefore, the standard solutions were stored at room temperature and used within three days and the sample solutions were analyzed within 24 hours or stored refrigerated and analyzed within two days.

CONCLUSION

Mixed-mode chromatography provides a simple technique to analyze complex mixtures of components which have remarkably different chromatographic behavior due to a wide range of compound polarities. The coupling of two chromatographic modes provides a solution to separation challenges which may not be readily resolved by conventional single-mode separation techniques.

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