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Determination of a process intermediate of celiprolol and its potential impurities by gradient high-performance liquid chromatography — application of high–low chromatography*

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Abstract: 3-(3-acetyl-4-hydroxyphenyl)-1,1-diethylurea (A-1354), is a synthetic intermediate of the β-adrenergic blocker, celiprolol hydrochloride. A liquid chromatographic method has been developed and validated for the determination of bulk A-1354 and its potential impurities. High–low chromatography was used to improve the detectability of trace impurities. Enhanced chemical detectability was achieved by comparing the detector response of trace-impurity peaks from a stock sample solution (high-concentration) with the detector response for the A-1354 peak in a quantitatively diluted working sample solution (low-concentration). Chromatographic separation was achieved by gradient elution of A-1354 and its known impurities using an Ultrasphere C18 analytical column (5 μm, 250 × 4.6 mm i.d.). The gradient mobile phase components were methanol and 0.1% triethylammonium phosphate, pH = 4.0. The flow rate was 0.9 ml min^{-1} with UV absorbance detection at 236 nm. The method was determined to be specific, linear, precise and accurate for A-1354 and its known impurities. Known impurities of A-1354 are quantitated to 0.05% (w/w).

Keywords: *High–low chromatography; HPLC; gradient chromatography; celiprolol hydrochloride intermediate.*

Introduction

Celiprolol hydrochloride is a cardio-selective β-adrenergic blocking agent [1] which has been shown to be effective in treating coronary heart disease and hypertension. Stimulation of the β_1 receptors increases the heart rate and force of contraction of the heart muscle.

Presently, there are more than 30 β-blockers marketed world-wide and while the antihypertensive effect of celiprolol is similar to that seen in other β-blockers [2], there are several advantages which exist to promote use of celiprolol for the treatment of cardiovascular disease. Celiprolol has no adverse effects upon serum lipids [3] or glucose metabolism [3, 4] and has not been demonstrated to have an undesirable effect on the liver, intestine, kidney or CNS functions [5]. In addition, non-selective β-blockers can produce bronchoconstriction which can limit their use in many patient populations. Celiprolol possesses a bronchodilator activity which is not mediated by partial effects on the β_2 receptors [6].

In the synthesis of the celiprolol process intermediate A-1354, seven known potential impurities have been identified (Fig. 1). These related substances represent both an intermediate (A-1511) and side products (A-1534, A-1788, G-516, G-690, RG-14381 and RG-14382) in the synthesis of A-1354. The challenge in developing a satisfactory method derived from the need to quantitate very low levels of impurities (0.05% w/w) while separating multiple compounds which have quite different chromatographic behaviours. In order to improve the detection limits of trace components in the bulk substance, high–low chromatography [7–8] was employed. This detection amplification is achieved by comparing the detector response of trace-impurity peaks from a stock sample solution (high-concentration) with the detector response for the A-1354 peak in a quantitatively diluted working sample solution (low-concentration).

The literature contains assay methods for celiprolol hydrochloride in biological matrices using colorimetric [9], HPLC [10, 11], and potentiometric methods [12]. These methods,

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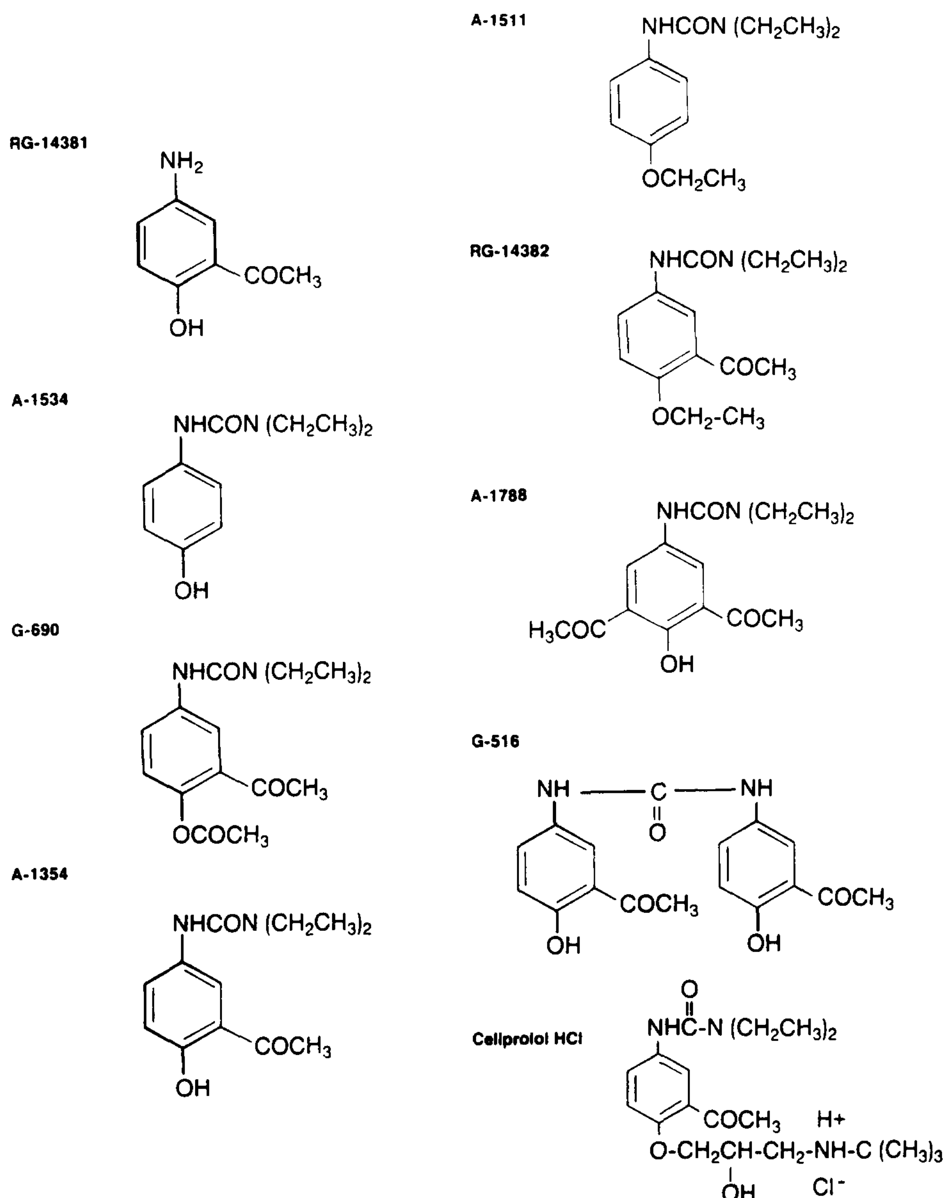

Figure 1
The chemical structures of celiprolol hydrochloride and related substances.

however, do not address the requirements of separating the multiple potential trace components of the process intermediate, A-1354.

Experimental

Apparatus

The liquid chromatograph consisted of a Waters WISP, Model 710B autosampler (Millipore, Bedford, MA, USA); a gradient HPLC pump, Hewlett–Packard, Model 1050 (Hewlett–Packard Co., Avondale, PA, USA); a variable wavelength detector, Applied Biosystems, Model 783 (Applied Biosystems Inc., Ramsey, NJ, USA) and an Ultrasphere C18 analytical column, (5 μm, 250 × 4.6 mm i.d.) (Beckman, San Ramon, CA, USA). Peak integration was performed using Waters Chromatography Software on a DEC computer system monitoring a UV absorbance detector at 236 nm. Statistical analysis was performed using RS/1® software (BBN Software Products Corporation, Cambridge, MA, USA) on a Compaq® 628/20E PC (Compaq Computer Corporation, Houston, TX, USA).

Reagents

Methanol, dimethylsulphoxide, phosphoric

acid (85%) and triethylamine (Fisher Scientific, Fair Lawn, NJ, USA) were HPLC grade. Water was obtained from a Milli-Q water purification system (Millipore). A-1354, A-1511, A-1788, G-516, G-690, RG-14381 and RG-14382 were synthesized by the Rhône–Poulenc Rorer Process Chemistry Department.

Mobile phase

Eluent A was HPLC grade methanol. Eluent B was 0.1% (v/v) triethylammonium phosphate buffer and was prepared by quantitatively transferring 1 ml of triethylamine reagent to a 1-l volumetric flask containing 980 ml of water. The pH was adjusted to 4.0 with phosphoric acid (85%). The solution was then brought to volume by adding the appropriate amount of water.

Chromatographic conditions

Various flow rates were tested during method development until 0.9 ml min^{-1} was found to be optimum. Prior to the first injection, column equilibrium was established by conditioning the column for 30 min with a mobile phase of 50% A–50% B. The linear gradient profile was 50% A–65% A for 10 min and followed by holding at 65% A from 10 to 20 min. At 20.1 min, initial chromatographic conditions (50% A) were re-established and held for 15 min prior to the next injection. The injection volume was 20 μl.

Preparation of standards and samples

Sample diluent was prepared by mixing 50 ml of dimethylsulphoxide with 950 ml of methanol. A stock solution of A-1354 standard was prepared by accurately weighing approximately 40 mg of A-1354 reference standard into a 100 ml volumetric flask. Five millilitres of dimethylsulphoxide were added to the flask and the flask was sonicated for 5 min or until the standard dissolved. The solution was then cooled to room temperature and diluted to 100 ml with methanol. The working standard solution was prepared by pipeting 6.0 ml of the stock standard solution into a 100 ml volumetric flask and diluting to volume with sample diluent. Sample solutions were prepared in a similar manner with the stock sample solutions used to estimate the impurity levels and the working sample solution used to assay the A-1354.

Results and Discussion

Separation considerations

The Ultrasphere C18, 5 μm particle size analytical column gave lower back-pressures than other 5 μm particle size, C18 columns. Column back-pressure is particularly important when viscous mobile phase mixtures such as 50:50 (v/v) methanol–buffer are used. A Waters μBondapak C18 column (10 μm particle size, 300 × 3.9 mm i.d.), as expected, gave lower back-pressures than the 5 μm particle size columns, however, the column efficiency did not allow for adequate resolution of all of the impurities or for trace impurity detection.

A-1354 is an ortho-substituted aminophenol derivative. With the exception of RG-14381, each of the related compounds have an amide functional group. RG-14381 instead possesses a primary amine substituent and demonstrated HPLC retention times with the greatest sensitivity to mobile phase pH. As the pH of the aqueous component of the mobile phase is decreased, the retention of RG-14381 also decreased. The optimum pH for the aqueous component of the mobile phase was determined by examining the effect of pH on peak tailing and the retention of RG-14381.

A-1354 and each of the identified related substances, with the exception of G-516, were readily soluble in methanol. To improve the solubility of G-516, dimethylsulphoxide was added to the sample diluent at a concentration of 5% (v/v).

A gradient mobile phase was necessary because of the differences in on-column behavior of several of the components. RG-14382 and A-1788 are baseline resolved only with isocratic mobile phases which gave inadequate resolution of G-690 and A-1354. Thus, a lower concentration of the organic solvent in the mobile phase was necessary, initially, to ensure resolution of G-690 from A-1354. A higher concentration of the organic solvent in the mobile phase was needed to provide adequate resolution of the later-eluting peaks RG-14382 and A-1788. The use of a mobile phase gradient also reduced the peak width of the last-eluting compound, G-516.

Detection considerations

At concentrations of A-1354 necessary to determine impurities at the 0.05% level (stock solution), the detector response for A-1354

was not linear. Therefore, a second more dilute solution of the sample (working solution) was necessary to quantitate A-1354. The high and low concentration solutions were examined separately and the detector response of the trace impurities in the stock solution was compared with the response of A-1354 in the working (high) solution. This method was suitable for routine analysis of A-1354 and for monitoring trace levels of related substances in bulk material.

The wavelength of maximum absorption for A-1354 is 236 nm. The related compounds have absorption maxima at or near this wavelength. The relative response factors (RRF) and relative retention times for the compounds are listed in Table 1. 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) values. The harmonic mean was calculated by determining the anti-log of the average log value of the ratios. The harmonic mean was used because this mean represented the minimum variance unbiased estimator for the ratio. These responses were then compared with the working sample solution response of A-1354 in order to calculate the relative response factors. The calculation of the relative response factor is shown at the bottom of Table 1.

Figure 2 displays a chromatogram of the A-1354 stock solution spiked with 0.25% (w/w) impurities. The limit of quantitation was defined as the lowest concentration of the determined linear range. For the assay of A-1354, this amount was determined to be 8.4 μg ml^{-1}. The limit of detection was defined as the minimum amount of analyte detectable by the method at three times the signal-to-noise ratio. For the assay of A-1354, this amount was determined to be 7.2 ng ml^{-1} or 0.07 ng injected.

Table 1
Relative response factors and relative retention times for A-1354 and related compounds

Compound	Relative response factor*	Relative RT†
RG-14381	1.13	0.427
A-1534	1.83	0.513
G-690	1.17	0.909
A-1354	1.00	1.00
A-1511	1.74	1.18
RG-14382	1.26	1.33
A-1788	1.05	1.39
G-516	1.03	1.60

The response factor for each impurity has been calculated using the following equation: *relative response factor = $A_{1354}/(C_{1354} \times RF)$, where: A_{1354} = peak area of A-1354, C_{1354} = concentration of A-1354 (mg ml^{-1}) and RF = response factor (harmonic mean) from peak areas vs concentration analysis for each impurity.
†Relative RT = relative retention time.

System precision and linearity

System precision was determined for several different solutions. Six replicate injections of A-1354 working solution gave a relative standard deviation (RSD) of 0.11% (Table 2). An impurity mixture at 0.05% (w/w) of the A-1354 stock solution concentration was prepared and six replicate injections made. At these trace

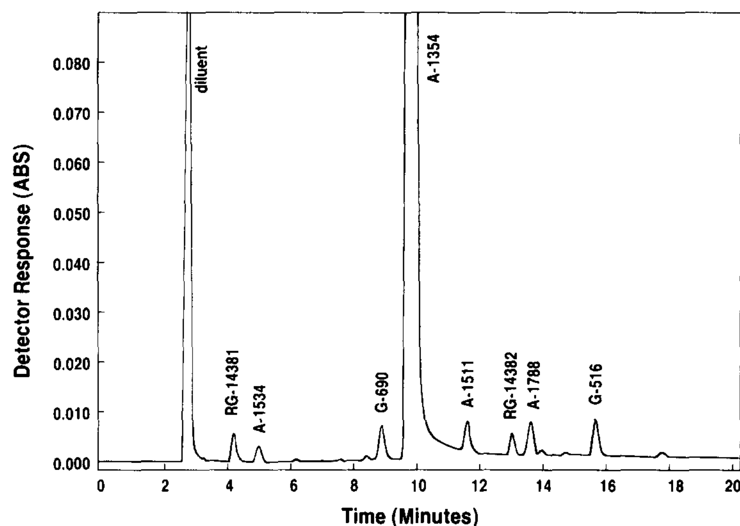

Figure 2
Chromatogram of an injection of the A-1354 stock solution spiked with 0.25% (w/w) related substances.

Table 2
A-1354 working solution injection precision

Injection no.	Area response
1	1476314
2	1475523
3	1476982
4	1476700
5	1473121
6	1477817
Mean	1476076
SD	1633
% RSD	0.11
Concentration of A-1354	0.024 mg ml^{-1}

levels, the RSD for each of the related substances was less than 3% (Table 3). At 0.5% (w/w) impurity levels, the RSD values for each of the impurities was less than 1%. A further test of the precision was made by determining the precision of multiple injections of the impurity which was detected with the lowest sensitivity (A-1534) and the least resolved impurity (A-1511) at levels of 0.05% (w/w) in the sample matrix. A-1534 had the smallest extinction coefficient of the related compounds and gave an RSD for six injections of 3.4% in the sample matrix. A-1511 eluted near the tail of the major peak, A-1354, in the stock solution injection. Tangential integration gave an RSD of 4.7% which was equivalent to the error limits at the 95% confidence interval (2σ) of $\pm 0.005\%$; that is, a peak at the 0.05% level would be reported as $0.05\% \pm 0.005\%$.

The linearity of detector response for A-1354 was tested for both the stock solution and the working solution. At the higher concentration of 0.40 mg ml^{-1} (stock solution), the detector response for A-1354 was non-linear. The stock solution was therefore diluted to 0.024 mg ml^{-1} (working solution) and the detector response for A-1354 was found to be linear from 50 to 150% of the target concentration of 0.024 mg ml^{-1}. The correlation coefficient was 0.99999 ($n = 9$). The linearity of detector response was also tested for each of the related substances at levels of 0.05–0.5% (w/w) of the A-1354 stock solution and found acceptable. Deviations of the observed values from the predicted values of the area responses (at the 95% confidence limit) derived from the regression analyses were not significant. Regression analysis of the related substances showed satisfactory linearity with correlation coefficients of 0.999 or greater ($n = 10$).

The method precision was determined by analysing six separate sample preparations of A-1354 versus standard A-1354. An RSD of 0.5% for these six separate assays of a sample of A-1354 was obtained.

Method ruggedness was evaluated by determining the chromatographic reproducibility of two Ultrasphere C18 columns. Table 4 shows little variation in the retention times, relative retention times, k' values, resolution factors and tailing factors [13] of the related compounds for the two columns. A comparison of the system suitability parameters for both columns shows little variation.

Solution stability

The stability of the potential impurities and the A-1354 solutions stored at both ambient temperature and refrigerated (below 10°C) was investigated. A 0.25% impurity standard solution (consisting of the seven known impurities at levels of 0.001 mg ml^{-1}) remained stable at ambient conditions for 3 days. A working sample solution of A-1354 (0.024 mg ml^{-1}) remained stable at ambient conditions for 3 days. The A-1354 stock solution (0.40 mg ml^{-1}) showed slight instability at the 0.05% level over 10 h at ambient conditions. Stock solutions of A-1354 stored in the refrigerator for 3 days showed no instability. It is, therefore, recommended that the stock solution

Table 3
Impurity mixture injection precision at the 0.05% level

Injection no.	RG-14381	A-1534	G-690	A-1511	RG-14382	A-1788	G-516
1	10806	6479	10553	7029	9676	11840	12927
2	10535	6417	10519	7025	9775	11763	12391
3	10570	6359	10172	6982	9652	11778	12204
4	10585	6255	10320	7107	9565	11929	12105
5	10476	6260	10083	7170	9566	11713	12240
6	10394	6586	10359	7149	9689	11802	12047
Mean	10561	6393	10344	7077	9657	11804	12319
SD	139	129	186	76	81	74	320
% RSD	1.32	2.02	1.80	1.07	0.849	0.627	2.59

Table 4
Column equivalency study results

Compound	RT	RRT	k'	Rs	Tf
Column no. 1					
RG-14381	4.15	0.427	0.496	19.0	1.38
A-1534	4.99	0.513	0.800	15.8	1.22
G-690	8.84	0.909	2.19	2.78	1.17
A-1354	9.73	1.00	2.51	—	1.17
A-1551	11.5	1.18	3.15	5.80	1.16
RG-14382	12.9	1.33	3.66	10.2	1.13
A-1788	13.5	1.39	3.87	10.6	1.09
G-516	15.5	1.60	4.60	18.7	1.22
Column no. 2					
RG-14381	4.25	0.442	0.543	18.7	1.40
A-1534	4.95	0.514	0.797	15.6	1.17
G-690	8.76	0.911	2.18	2.79	1.07
A-1354	9.62	1.00	2.49	—	1.18
A-1551	11.4	1.19	3.15	6.05	1.11
RG-14382	12.8	1.33	3.66	11.2	1.09
A-1788	13.4	1.39	3.85	12.1	1.15
G-516	15.3	1.59	4.54	17.5	1.33

RT = Retention time (min).
RRT = Retention time relative to retention time of A-1354.
k' = Capacity factor.
Rs = Resolution between A-1354 and compound of interest.
Tf = Tailing factor.

either be analysed within 10 h of being dissolved or be stored in the refrigerator.

System suitability

The system suitability was determined for each chromatographic assay using the following criteria as outlined in the USP [13]: tailing factor of A-1354 peak (A-1354 working standard solution) not greater than 2.0, RSD of six replicate injections of the working standard solution of A-1354 not greater than 2.0%, capacity factor (k') for A-1354; 2–3 and resolution between A-1354 and G-690 should be greater than 1.5.

The tailing factor value was selected to ensure the maximum chromatographic efficiency achievable with this method. The limit for instrument precision ensured adequate precision and is based on injections made within the same day on the instrument. The range for the capacity factor was selected after validation of the method. The range represented normal day to day variability encountered for this parameter.

Conclusions

A simple approach, high–low chromatography, was successfully applied to the quantitation of related substances in a raw material. This approach may be applied to improve the sensitivity of detection of low levels of impurities. The present method is sensitive to levels of 0.05% (w/w) of known impurities of A-1354. Due to the slow formation of an unidentified solution degradate, stock solutions of A-1354 should be analysed within 10 h of being dissolved or stored under refrigerated conditions. The method was determined to be specific, linear, precise and accurate for the quantitation of A-1354 and its known impurities.

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