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Assay and purity evaluation of CP-93,393-1 by reversed-phase liquid chromatography
A snapshot of current practices for liquid chromatography methods development and validation

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Abstract

CP-93,393-1 is a new chemical entity under development for the treatment of anxiety and depression. This paper describes the selection and validation of a chromatographic system which was engineered to: (1) ensure acceptability by world-wide regulatory agencies, (2) maximize sample throughput, (3) simplify the analysis, (4) minimize ambiguity in peak assignments, and (5) facilitate the technology-transfer process. These objectives were achieved using a single, compound-specific and purity-indicating, isocratic, reversed-phase chromatographic system that allows quantitation of CP-93,393 concomitantly with all potential impurities from the same injection. A novel experimental matrix was employed to validate the chromatographic system.

Keywords: Pharmaceutical analysis; CP-93,393

1. Introduction

During the development of a drug candidate, chromatographic systems are developed and used for a variety of purposes. These systems evolve throughout the development process as additional knowledge about potential impurities is accumulated. The amount and type of validation also evolve as a drug candidate develops [1,2]. Methods developed for late-stage drug candidates must have high throughput to accommodate the huge numbers of samples associated with late-stage drug development activities and be sufficiently robust to facilitate technology transfers. Other desirable attributes of these chromatographic systems include the following: (1) compatibility with mass spectrometric detection to facilitate identification of unknown degradants and process by-products, (2) minimization of analysis time, especially sample preparation, (3) minimization of mobile-phase costs, and (4) minimization of the consumption of working standards which often are expensive to synthesize, characterize and maintain.

Several reports have been published that contain validation guidelines for analytical methods used for pharmaceutical analyses [3–9]. This manuscript summarizes the development and validation of the chromatographic system used to assay and determine the purity of the CP-93,393-1 drug substance. A novel experimental matrix designed with 32 indi-

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vidual sample solutions was employed to validate the chromatographic system. This system allows concomitant quantitation of the main component (CP-93,393) and potential impurities from the same sample solution. This approach of combining purity evaluation and assay of the main component will save time and resources in analytical laboratories. The validation and ruggedness data generated for the chromatographic system described here are consistent with: (1) the current USP/NF guidelines for quantitation of the major component of a bulk drug substance and with analytical methods developed for the determination of impurities in finished pharmaceutical products [10], (2) a text on the validation of analytical methods: definitions and terminology issued from the International Conference on the Harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use [11], and (3) prescriptive guidelines issued by the Canadian regulatory authorities [12].

2. Experimental

2.1. Chemicals

Standards of CP-93,393-1 and its potential impurities (Fig. 1) were prepared and characterized at Pfizer, Groton, CT, USA. CP-93,393 is the free base form of the compound; CP-93,393-1 is the hydrochloride salt of CP-93,393. HPLC-grade acetonitrile was obtained from Fischer Scientific, Fair Lawn, NJ, USA; HPLC-grade methanol and reagent-grade glacial acetic acid were obtained from J.T. Baker, Phillipsburg, NJ, USA; A.C.S. reagent-grade ammonium acetate was obtained from Aldrich, Milwaukee, WI, USA.

2.2. Equipment

The HPLC system used for the validation studies consisted of a Waters (Milford, MA, USA) model 717 autoinjector, a Waters model 510 pump, a Waters model 486 absorbance detector and a BAS (W. Lafayette, IN, USA) model LC-22A column temperature controller. Waters Puresil C_{18} columns, 5 μm particles, 150×4.6 mm, part no. WAT044345 were used for all separations except where noted. A Brownlee (Foster City, CA, USA) NewGuard scrubber column, p/n 140-601, containing a Brownlee NewGuard C_{18} insert, p/n G18-013, was placed between the pump and the injector. Scrubber columns have increased the lifetime of chromatographic columns developed for other pharmaceutical products and are routinely used as a preventive measure [13].

2.3. Chromatographic conditions

The conditions listed below were used for all separations, except where noted.

Mobile phase:	Acetonitrile–MeOH–buffer* (6:3:91, v/v/v) *=aqueous 0.05 *M* ammonium acetate, pH 4.6
Flow rate:	2 ml/min
Detection:	238 nm
Injection volume:	10 μl
Temperature:	30°C
Sample preparation:	1 mg CP-93,393-1/ml in mobile phase

3. Results and discussion

3.1. Method development

CP-93,393, its penultimate precursor (Compound 1), and two CP-93,393 degradants are shown in Fig. 1. These potential impurities were chosen for mobile-phase optimization studies based on purposeful degradation experiments and the synthetic pathway used to produce CP-93,393-1. Mobile-phase optimization employed systematic exploration of ternary mobile phases [14]. Initial separations were generated using alcohols as organic modifiers and sodium acetate as the buffer.

Degradant 1 tailed badly on some Puresil C_{18} columns with these mobile phases. Since substituting ammonium acetate for sodium acetate minimized the tailing, further studies using sodium acetate buffers were abandoned. Because adequate resolution of the four compounds injected was unsuccessful using alcohols exclusively as organic modifiers, separations including acetonitrile were attempted, with

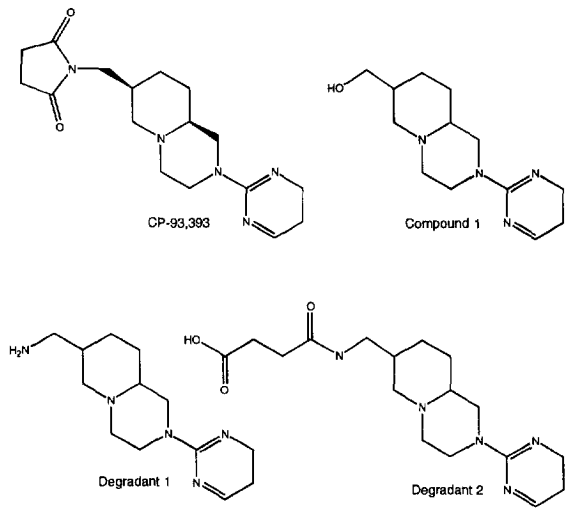

Fig. 1. Chemical structures of CP-93,393, Compound 1, Degradant 1 and Degradant 2.

much better success. The chromatographic conditions cited in Section 2 were eventually chosen for validation and ruggedness studies based on a compromise between resolution and time. A chromatogram illustrating the separation of CP-93,393 and the three potential impurities is illustrated in Fig. 2 confirming specificity with respect to CP-93,393.

The remaining chromatographic conditions listed in Section 2 were chosen for the following reasons: The flow-rate of 2 ml/min was chosen to maximize sample throughput. The column back pressure, under the conditions cited, ranged between 1500 and 2000 p.s.i. (1 p.s.i.=6894.76 Pa). This is well within the upper limit of 3000 p.s.i. specified by the manufacturer. Although the optimum flow-rate for the Puresil column is lower than 2 ml/min, loss of chromatographic performance is minimized at linear velocities (flow-rates) higher than the optimum velocity when the particle size of the column packing is small (below 10 μm) [15]. Higher flow-rates were not evaluated because of the potential problems associated with elevated back pressures. The UV detector was set at 238 nm, the UV maximum for CP-93,393. The UV spectra of the three potential impurities shown in Fig. 1 are very similar to the UV spectrum of CP-93,393 with UV maxima at or close to 238 nm. Column temperature was held at 30°C although separations at column temperatures of 25°C and 35°C indicated that slight variation in temperature did not have a significant effect on retention, resolution or peak shape. The injection volume of 10 μl and sample concentration of 1 mg CP-93,393-1/ml in mobile phase were chosen to simplify sample preparation and minimize solvent consumption (further dilution is not needed). This concentration of CP-93,393 allows both assay (of the main component) and purity evaluation (of trace impurities). At this concentration, the CP-93,393 peak is well within the linear range for UV detection, and trace components are readily detectable.

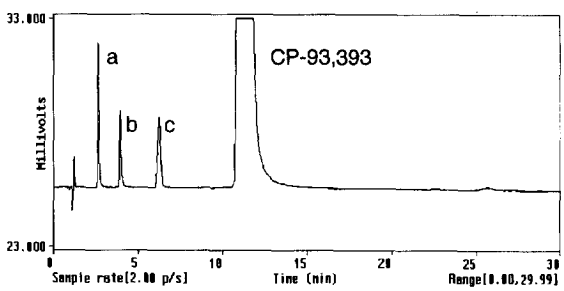

Fig. 2. Chromatograms of CP-93,393-1 and 0.2% spikes of (a) Degradant 1, (b) Compound 1, and (c) Degradant 2.

To maximize sample throughput, assay and purity evaluations ideally are determined from the same injection. To accomplish this, a compromise between peak shape of the main component and detectability of trace impurities is needed. Retention of CP-93,393 decreased from 11.8 to 11.5 min as the concentration of CP-93,393-1 was increased from 0.6 to 2.0 mg/ml. Chromatographic performance, determined theoretically, deteriorated from 6200 (at 0.6 mg/ml) to 3900 (at 2.0 mg/ml), but not to an extent that precluded accurate integration of the CP-93,393 peak.

3.2. Ruggedness testing

Analytical methods developed for use in quality control (QC) laboratories ideally are rugged. Retention times for the analytes of interest will not change significantly from day-to-day or from laboratory-to-laboratory if the method is considered rugged. To determine the ruggedness of the chromatographic methodology developed for CP-93,393-1, experimental conditions were purposely altered and chromatographic characteristics were evaluated.

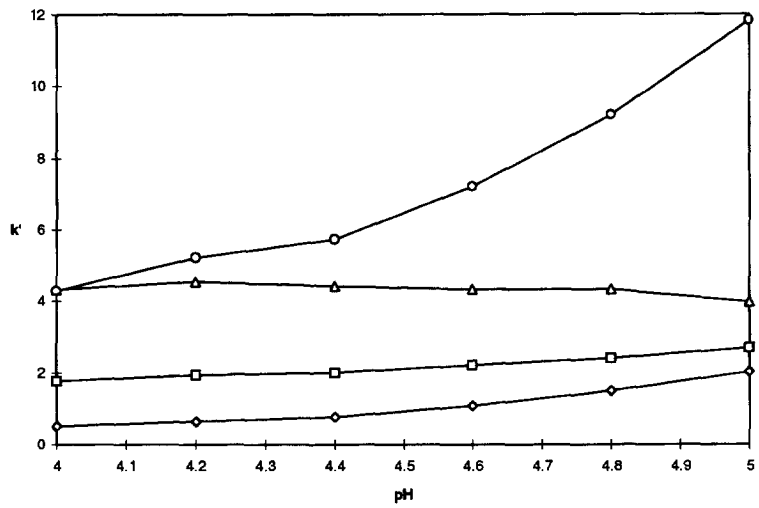

Fig. 3. The effect of mobile phase pH on the retention (k') of CP-93,393 and three potential impurities. ○=CP-93,393, Δ=Degradant 2, □=Compound 1, ◇=Degradant 1.

The effect of mobile phase pH on the retention (capacity factor, k') of CP-93,393 and three potential impurities is illustrated in Fig. 3. Although adequate resolution of these four compounds was accomplished at any pH at or above 4.2, a mobile phase of pH 4.6 was selected to accommodate a larger number of analyte peaks that may occur in degraded or crude samples of CP-93,393-1.

Additional studies were undertaken to determine the effect of varying the mobile phase percentages of methanol and acetonitrile on the retention of CP-93,393. The percentage of methanol was varied from 0 to 8% while the other mobile phase components were held constant at the levels listed in Section 2. The percentage of acetonitrile was then varied from 2 to 10% while the other mobile phase components remained constant. These data, which are summarized in Fig. 4, illustrate that small changes in the level of acetonitrile have a much larger effect on the retention of CP-93,393 than similar changes in methanol content. Acetonitrile is included as a modifier because adequate resolution of CP-93,393 and Degradant 2 was not achievable without it.

To demonstrate the reproducibility of CP-93,393 retention, two studies were undertaken. Initially, a single column was used over a period of 5 months with six batches of mobile phase. The average retention time of CP-93,393 was 10.9 min and ranged from 10.3 to 11.5 min with an R.S.D. of 4.4%. In a separate study, the variation in retention using five individual columns and one batch of mobile phase was studied. The average retention time of CP-93,393 was 10.2 min and ranged from 10.0 to 10.6 min with an R.S.D. of 2.5%. This excellent reproducibility of retention is consistent with a rugged system.

To determine if other columns would provide a separation equivalent to the Waters Puresil C_{18} column specified in this procedure, a batch of CP-93,393-1 containing several impurities was analyzed on six columns including a MetaChem Intersil C_{18}, a Waters Symmetry C_{18}, a Waters μBondapak C_{18}, a Waters Nova-Pak C_{18} and a Zorbax C_8 column. The mobile phase used for all columns is specified in Section 2. No attempts were made to fine-tune the mobile phase for each specific column.

The first chromatogram in Fig. 5 used a Waters Puresil C_{18} column. The first three peaks after the solvent front are Degradant 1, Compound 1 and Degradant 2 (see Fig. 1 for structures). The two remaining peaks that elute before the main component (CP-93,393) and the peak that elutes after CP-93,393 are reaction by-products that are not present at significant levels (≥0.1%) in purified CP-93,393-1. The second chromatogram in Fig. 5 used a Symmetry C_{18} column which was not avail-

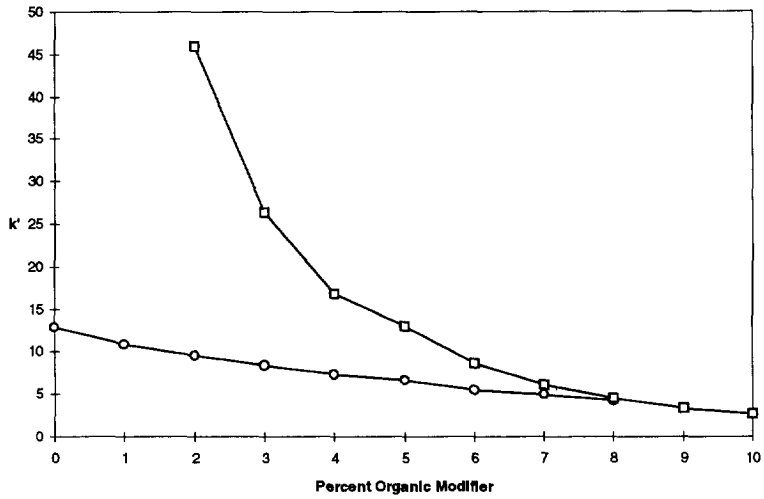

Fig. 4. Effect of the percentage of mobile phase modifiers on the retention (k') of CP-93,393. □=acetonitrile, ○=methanol.

able when this assay was developed and actually had essentially equivalent resolution in shorter time. The separation generated using the MetaChem was consistent with the Puresil column; the separation generated using the μBondapak column was inferior in efficiency and resolution. The last two columns chosen for evaluation (a Waters Nova-Pak C_{18} and a Zorbax C_8), yielded separations significantly different from the first four columns evaluated.

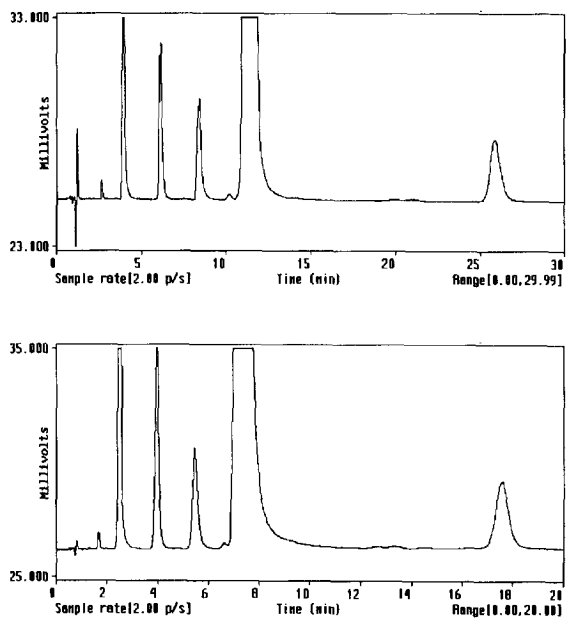

Fig. 5. Chromatograms of crude CP-93,393 using Puresil and Symmetry C_{18} columns.

3.3. Validation

The experimental matrix shown in Table 1 was used to validate selectivity, precision, linearity and accuracy/recovery. Each of the matrix entries represents an individual sample weighing. For instance, $W_{1,40}$ represents the first weighing at 40% of the nominal concentration of 1.0 mg CP-93,393-1/ml. By using multiple masses to validate linearity instead of serial dilutions from a stock solution, the true reproducibility of the assay is determined. For chromatographic assays, the largest source of variability may often be associated with sample preparation (weighing, transferring and diluting standards and samples).

System suitability and selectivity are summarized in Table 2. These data are typical and were generated on a column that had been used sporadically and stored in mobile phase for more than 2 years. These data were generated from six injections of a solution containing 1.0 mg/ml CP-93,393-1 and 0.2% spikes of Compound 1 and Degradant 1, and a 0.5% spike of Degradant 2. All peaks were well resolved and the precision of injection for all peaks was acceptable (see below). Selectivity also was studied over extend-

Table 1
Matrix used to validate selectivity, precision, linearity and accuracy/recovery

% Nominal concentration	Weighing number					
	1	2	3	4	5	6
40	$W_{1,40}$	$W_{2,40}$	$W_{3,40}$	$W_{4,40}$		
60	$W_{1,60}$	$W_{2,60}$	$W_{3,60}$	$W_{6,40}$		
80	$W_{1,80}$	$W_{2,80}$	$W_{3,80}$	$W_{8,40}$		
100	$W_{1,100}$	$W_{2,100}$	$W_{3,100}$	$W_{4,100}$	$W_{5,100}$	$W_{6,100}$
100[a]	$W^{a}_{1,100}$	$W^{a}_{2,100}$	$W^{a}_{3,100}$	$W^{a}_{4,100}$	$W^{a}_{5,100}$	$W^{a}_{6,100}$
120	$W_{1,120}$	$W_{2,120}$	$W_{3,120}$	$W_{4,120}$		
150	$W_{1,150}$	$W_{2,150}$	$W_{3,150}$	$W_{4,150}$		

$W_{1,40}$=The first weighing at 40% of the nominal concentration (1.0 mg/ml).
[a]These samples contain 0.2% spikes of Compound 1 and Degradant 1, and a 0.5% spike of Degradant 2.

ed time using several columns and many different batches of mobile phase. Relative retention time ranges (r_G=retention time of the peak of interest/retention time of CP-93,393) were as follows: CP-93,393=(1.00); Degradant 2 (0.50–0.67); Compound 1 (0.34–0.40); Degradant 1 (0.18–0.29). These data indicate that the retention time windows for each impurity are unique and do not overlap. For rugged separations such as this, impurity identification could be based on relative retention time alone; it would not be necessary to inject an authentic standard of each impurity to confirm identification.

To demonstrate a linear relationship between peak area and concentration of CP-93,393, the first weighing replicate (Table 1, column 1) was plotted against concentration. The regression coefficient (r^2) was

Table 2
System suitability, selectivity and precision of injection

Compound	t_R (min)[a]	Plates Tangent[b]	Plates Foley and Dorsey[c]	Tailing Factor	R_s[d]
Degradant 1					
Mean (n=6)	2.8	3700	1700	1.2	4.8
Range	(2.8–2.8)	3500–3900	1200–2200		
%R.S.D.	0.21	3.7	24		
Compound 1					
Mean (n=6)	3.8	3700	1900	1.5	6.4
Range	(3.8–3.9)	3400–3900	1200–2300		
%R.S.D.	0.11	5.5	23		
Degradant 2					
Mean (n=6)	5.7	4700	4000	1.1	9.7
Range	(5.7–5.7)	4500–4900	3700–4100		
%R.S.D.	0.39	3.4	3.9		
CP-93,393					
Mean (n=6)	10.3	4500	2500	1.6	NA[e]
Range	(10.3–10.3)	4400–4600	2500–2500		
%R.S.D.	0.053	1.3	0.4.7		

[a]t_R=retention time.
[b]Theoretical plates (tangent)=USP method.
[c]Theoretical plates (Foley and Dorsey [17]).
[d]R_s=resolution=$[2(t_2-t_1)]/(W_1 + W_2)$=resolution between the compound of interest and the next eluting peak; t_1,t_2=retention times of the two components ($t_2>t_1$); W_1,W_2=peak widths of two components.
[e]NA=not applicable.

1.0000; the x intercept was 0.5% of the nominal concentration, and all residuals were within 2% of the response for all six concentrations. The slope of the line was 1.3637×10^{7} area counts/(mg CP-93,393-1/ml). Recovery of each weighing in Table 1 was determined using the nominal concentration as the reference. Individual recoveries ranged from 99.1 to 100.5 and reflect the variability associated with weighing and the chromatographic system. The average recoveries at each concentration ranged from 99.4 to 100.1%. Experimental error for this chromatographic and other systems can be estimated by using the errors associated with preparing external standards and the precision of injection. For assays developed for the pharmaceutical industry, response factors for replicate standards that are within 2% are considered consistent and within the experimental error of the technique [16]. The recovery data generated for this chromatographic system are well within this requirement.

The stability of a 1 mg/ml solution of CP-93,393-1 prepared using the conditions cited in Section 2 was evaluated. The solution was stored in a capped volumetric flask on a laboratory bench under normal lighting conditions. Recovery as a function of time was measured using a freshly prepared standard of CP-93,393-1 (Table 3). Based on these data which show quantitative recovery through two weeks, 1 mg/ml solutions of CP-93,393-1 may be assayed within 2 weeks of preparation. The area percentages of Degradant 2, a CP-93,393 hydrolysis product, are also summarized in Table 3. Based on these data which show an insignificant amount of Degradant 2 formed within 1 day, quantitation of Degradant 2 should be made on solutions that are no more than 1 day old.

Levels of unidentified impurities are estimated by their peak areas relative to the CP-93,393 peak. To accommodate potential non-linearity for trace analytes relative to a large parent peak, a diluted CP-93,393-1 standard is employed, consistent with USP guidelines [10]. For this application, the concentration of the diluted standard was 0.2% relative to the 1 mg/ml solution of CP-93,393-1. To support the use of a diluted standard, the stability of this solution was determined. At 2×10^{-3} mg/ml CP-93,393-1, the trace levels of Degradant 2 that were generated in solution were below its detection limit. Consistent with the 1 mg/ml data, the 2×10^{-3} mg/ml solution of CP-93,393-1, which showed quantitative recovery (97.6–102.5%, average=99.9, R.S.D.=2.0%, n=6) through 2 weeks, may be used to estimate levels of unknowns if used within 2 weeks of its preparation.

Table 3
Stability of CP-93,393-1 in solution

Time	Percent recovery	Area % Degradant 2
0 days	100.4	
1 day	100.8	0.02
2 days	100.6	0.04
1 week	100.5	0.17
2 weeks	100.2	0.34

Intra- and inter-day method precision were measured using drug substance stability data. Samples of CP-93,393-1 were challenged at several temperatures and humidities for 6 and 12 weeks and at 6 months. Intra-day precision was determined three times by measuring the response factor [peak area/(mg CP-93,393/ml)] of the CP-93,393-1 standard throughout single chromatographic runs on three separate days. The percent relative standard deviation (%R.S.D.) of the standards were 0.4, 0.2 and 0.6 for the 6-week (n=11), 12-week (n=11) and 6-month (n=13) time points, respectively.

The stability study discussed above indicated no significant degradation (<0.1%) of CP-93,393-1 through 6 months, regardless of storage condition. Since all samples can be considered equivalent (in terms of CP-93,393 content), the average potencies at the three time points were used to measure the inter-day precision of the technique. The average potencies at 6 weeks, 12 weeks and 6 months were 89.5, 89.4 and 89.9, respectively. The theoretical potency of CP-93,393-1 is 90.0% (10.0% represents the hydrochloride counter-ion portion of the CP-93,393-1 salt).

Because this procedure serves as a limit test for several potential impurities, extensive validation of the linearity for each impurity was not necessary. Linearity (range) was demonstrated between peak area and concentration in the vicinity of the target level (defined below) of each impurity. This was done by determining the recovery of response factors at the target level of each impurity and at 50% and 150% of each target level. The target level for an impurity is defined as the level above which the

Table 4
Linearity (range), precision, LOQ and recovery of potential CP-93,393-1 impurities

Compound	Concentration (mg/ml)	Relative concentration[a]	% Recovery[b]		% R.S.D. of peak area	Limit of quantitation[c]	Recovery from concomitants[d]
			50%	150%			
Degradant 1	0.002	0.2%	101	100	0.5	0.005%	96%
Compound 1	0.002	0.2%	100	101	0.5	0.009%	102%
Degradant 2	0.005	0.5%	99	100	0.6	0.01%	109%

[a]relative concentration of CP-93,393-1=(100).
[b]% recovery is the average percent recovery of the response factor (area/concentration), $n=4$.
[c]Limit of quantitation=the percentage (relative to CP-93,393-1) that, if present, would yield a chromatogram with a signal-to-noise ratio of 10.
[d]Recovery of the response factors from a solution of CP-93,393-1, Degradant 1, Compound 1 and Degradant 2.

quality of the CP-93,393-1 sample is questioned. These data (Table 4) demonstrated adequate range in the vicinity of the target level for each potential impurity.

The precision of injection for each of the potential impurities was determined using a solution of the potential impurities at their target levels. Individual sample weighings were not used to determine precision of the chromatographic system because for trace analysis wherein impurity levels are reported to one significant figure the errors associated with preparing standard solutions becomes insignificant compared to the errors associated with accurately integrating small peaks. The % R.S.D.s of peak areas, which are summarized in Table 4, were excellent for each component and reflect a well-behaved and stable system.

Table 4 also summarizes the limit of quantitation (LOQ) for each potential impurity. LOQ is defined as the lowest amount of an impurity (relative to CP-93,393-1) that can be determined with acceptable precision and accuracy under the cited chromatographic conditions. LOQs were estimated by measuring the signal (peak height) of each impurity at 0.2% relative to a 1 mg/ml solution of CP-93,393-1 and the background noise (RMS) of the chromatogram. The LOQs cited in Table 4 represent the percentage of each impurity (relative to CP-93,393-1) that would yield a signal-to-noise ratio of 10. Since the LOQs cited in Table 4 are well below the target levels, accurate quantitation of each impurity can be readily accomplished.

Accuracy/recovery of the potential impurities were measured using a solution of CP-93,393-1 at its assay concentration and the potential impurities at their target levels. A recovery of 100% would indicate that the peak area of that individual component was not effected by solution concomitants. Recoveries were determined using external standard solutions of each impurity. These results (Table 4) indicated quantitative recovery (within experimental error, see below) of each component and demonstrated that the measured signal of each component was not significantly affected by the presence of potential concomitants. For trace components whose abundance is reported to one significant figure (i.e. 0.2%), the recoveries, which ranged from 96% to 109%, can be considered quantitative. The main source of experimental error here was the variability that resulted from the integration of small peaks.

4. Conclusions

A reversed-phase chromatographic system for the assay and purity evaluation of CP-93,393-1 is described. Studies have been conducted that demonstrated the system is rugged and the system has been extensively validated.

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