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Assay and purity evaluation of 5-chlorooxindole by liquid chromatography

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Abstract

5-Chlorooxindole (5-CO) is a starting material for tenidap sodium, a pharmaceutical drug candidate produced by Pfizer. To insure potency and purity of the drug substance, it is necessary to demonstrate that commercial supplies of 5-CO are free from elevated levels of chemical analogs that could be carried through the synthetic scheme. This is accomplished using a single highly specific normal-phase chromatographic system that allows the quantitation of 5-CO concomitantly with all of its potential positional isomers. This paper describes the chromatographic system and its supportive validation data.

Keywords: Normal-phase high-performance liquid chromatography; 5-Chlorooxindole; Assay; Purity evaluation; Crown ether

1. Introduction

Chromatographic systems developed for use in the pharmaceutical industry ideally accomplish several objectives. To maximize the efficiency of analytical laboratories, it is imperative to combine assays (of the main component) and purity evaluations (quantitation of trace impurities) into a single chromatographic system, whenever possible. Using the same injections for purity and potency is desirable but not essential. A second objective is that validation and ruggedness data must be generated to confirm that the method is suitable for use in quality control laboratories. The data must also be presented in a form to convince regulatory agencies that the method will work as described. Finally, the method must be designed and written to facilitate smooth technology transfers to other laboratories within and outside of the organization.

Summarized below are the validation data generated in support of the chromatographic assay and purity evaluation system for 5-chlorooxindole (5-CO) and an inter-laboratory comparison of data generated on three batches of 5-CO.

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2. Experimental

2.1. Materials

Standards of 4-chlorooxindole, 5-chlorooxindole, 6-chlorooxindole, 7-chlorooxindole, and 5,7-dichlorooxindole were prepared and characterized in-house at Pfizer. 15-Crown-5 and oxindole were purchased from Aldrich Chemical Co. HPLC grade tetrahydrofuran (THF) was purchased from Baxter, Burdick and Jackson. HPLC grade hexane was purchased from J.T. Baker.

2.2. Equipment

The HPLC system used for most of these studies consisted of a Perkin-Elmer model LC-600 autoinjector equipped with a 50 μl loop, a Waters model 510 pump, a Waters model 440 absorbance detector (fixed wavelength, 254 nm), and a BAS model LC-22A column temperature controller. Waters Nova-Pak silica columns (4 μm; 15 cm × 3.9 mm i.d.; P/N 10025) were used for all separations.

2.3. Chromatographic conditions

The injection volume was 50 μl, and the mobile phase was hexane–isopropanol–tetrahydrofuran–15-crown-5 (1000:6:6:0.5, v/v) (degassed under vacuum with sonication or stirring for about 20 s), with a flow rate of 3 ml min^{-1}. The temperature was 30°C, and detection at 254 nm was employed.

2.4. Sample preparation

2.4.1. 5-Chlorooxindole

A 20 mg amount was weighed into a 100 ml volumetric flask, and 25 ml of THF were added followed by sonication and dilution to volume with mobile phase.

2.4.2. Potential impurities

Solutions of potential impurities were prepared individually and as a mixed standard. The final composition of the injected solutions contained 25% THF, the potential impurities, and mobile phase. The relative concentration of each potential impurity was matched to its target level (defined below) and ranged from 0.1 to 1% (relative to 5-CO). Since the concentration for 5-CO was 0.2 mg ml^{-1}, the impurity concentrations ranged from 2×10^{-4} to 2×10^{-3} mg ml^{-1}.

The target level for an impurity is defined as the level above which the quality or economic value of the sample of 5-CO is questioned. For impurities that are purged in subsequent synthetic steps, target levels are based strictly on economics. Commercial supplies containing purgeable impurities will produce material with lower synthetic yields and larger amounts of by-products requiring disposal. If target levels are set very low, suppliers may not be able to cost-effectively produce material which is considered acceptable to the customer. For purgeable impurities, a compromise is made between what suppliers can routinely supply and the costs associated with lower yields.

For impurities that are not easily purged and generate impurities in subsequent steps, target levels are set low enough to insure quality of the final drug substance. Structures of 5-CO and its potential impurities are shown in Fig. 1.

3. Results and discussion

The chromatographic system used for these analyses was a highly specific normal-phase assay

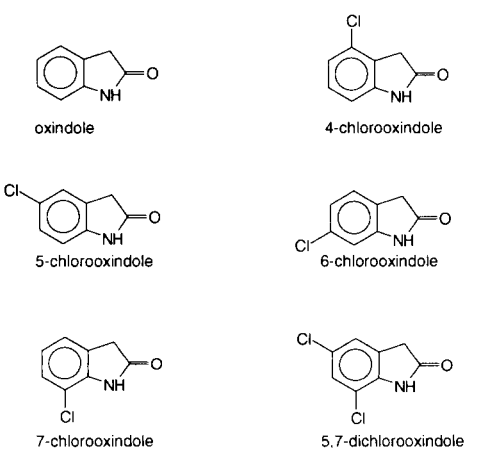

Fig. 1. Structures of 5-chlorooxindole and its potential impurities.

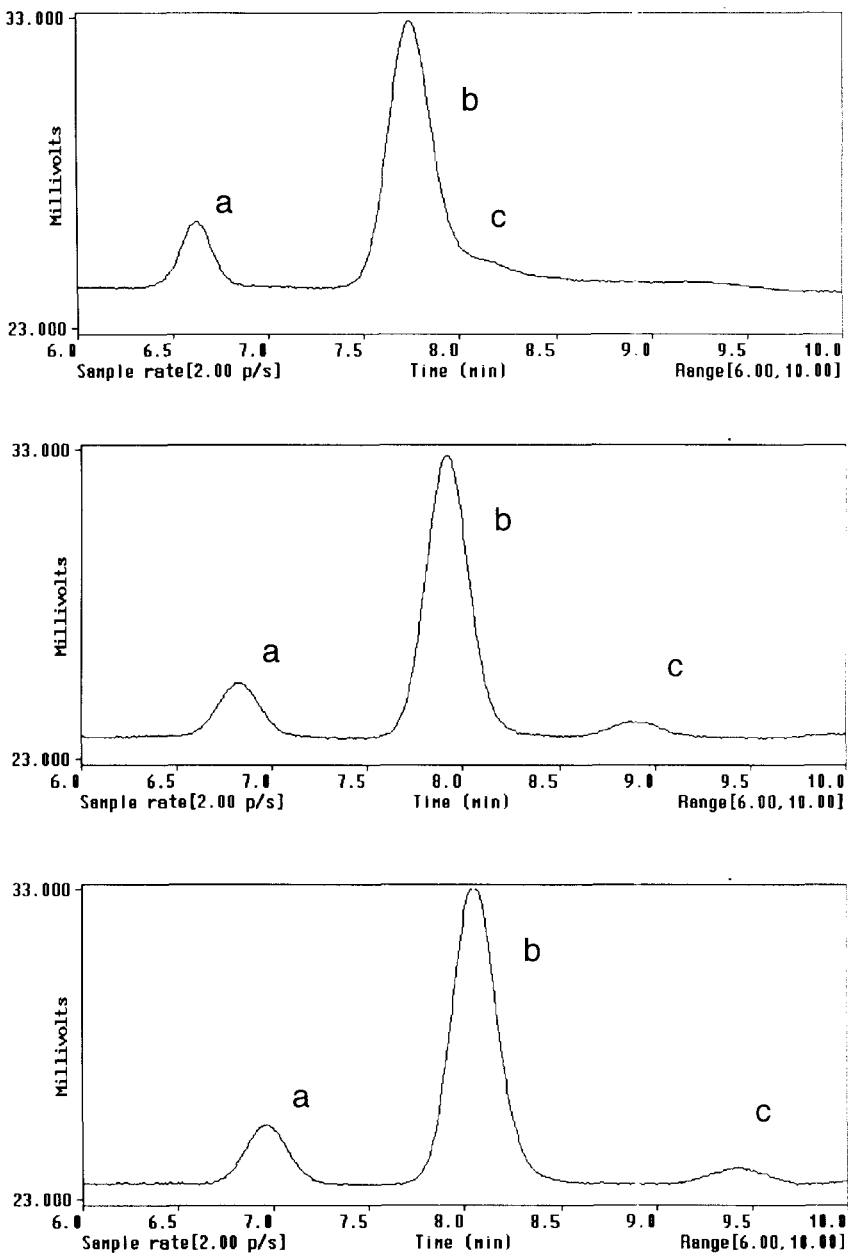

Fig. 2. Chromatograms illustrating the effect of equilibration time on the resolution of (peak a) 4-chlorooxindole, (peak b) oxindole, and (peak c) 6-chlorooxindole. Top, 0 h; middle, 12 h; bottom, 24 h.

system. The mobile phase contained 15-crown-5 which was used as a selectivity and retention modifier. 15-Crown-5 was selected over other crown ethers because it caused the greatest enhancement in retention for most of the compounds studied. Presumably, this crown ether formed the most stable complexes with these analytes. The effect of crown ether type and concentration on the chromatography of oxindoles and isatins has been described [1]. The use of crown ethers in liquid chromatography was recently reviewed [2].

Table 1
Specificity

Compound	Relative retention[a]
5-Chlorooxindole	1.00
6-Chlorooxindole	0.88
Oxindole	0.66
4-Chlorooxindole	0.58
5,7-Dichlorooxindole	0.37
7-Chlorooxindole	0.32

[a] Not corrected for void volume.

Table 3
Recovery of 5-CO and potential impurities

Compound	Concentration (mg ml^{-1})	Percent recovery[a]
5-Chlorooxindole	0.2	102 (0.8)
6-Chlorooxindole	0.0002	92 (6.4)
Oxindole	0.002	96 (0.5)
4-Chlorooxindole	0.0004	96 (2.8)
5,7-Dichlorooxindole	0.002	98 (5.4)
7-Chlorooxindole	0.0002	96 (3.3)

[a] $n = 4$. The values given in parentheses are the % RSDs.

The chromatographic system described here was developed using a software–hardware package that systematically generated a series of chromatograms, each with a unique mobile phase. The mobile phases were generated by mixing solvents from up to four solvent reservoirs. At the conclusion of a solvent survey, peak resolutions were calculated and the best chromatogram based on resolution and run time was selected. Ruggedness data also were generated using this approach [3].

The flow rate of 3 ml min^{-1} was chosen to maximize sample throughput. Normal-phase solvents are less viscous than reversed-phase solvents and column back pressure is rarely a concern under normal-phase conditions. Back pressure under the conditions cited here never exceeded 1000 lb in^{-2} even at the relatively high flow rate. In addition, loss of chromatographic performance at high flow rates becomes insignificant when the particle size of the column packing is small [4]. Since the chromatographic performance of the 4 μm column packing employed for these studies was excellent as measured by theoretical plates and tailing factors (vide infra), lower flow rates were not evaluated.

The injection volume of 50 μl was chosen because of limited solubility of the 5-CO in the mobile phase, the desire to obtain assay and purity information from the same injection, and because large injection volumes are more reproducible on some autoinjectors, especially under normal-phase conditions. For sample preparation, the THF was used to rapidly dissolve and keep the 5-CO in solution. When smaller amounts of THF were used, some samples of 5-CO would slowly precipitate.

Normal-phase chromatographic systems are the systems of choice to resolve positional isomers [5], but compared to reversed-phase systems, they require longer times to equilibrate. For the system described here, equilibration was not reached un-

Table 2
Precision of injection and limit of quantitation

Compound	Concentration (mg ml^{-1})	Relative concentration (%)	% RSD of peak area[a]	Limit of quantitation[b] (%)
5-Chlorooxindole	0.2	(100)	0.5	–[c]
6-Chlorooxindole	0.0002	0.1	12	0.03
Oxindole	0.002	1	1.7	0.02
4-Chlorooxindole	0.0004	0.2	9.3	0.02
5,7-Dichlorooxindole	0.002	1	1.1	0.02
7-Chlorooxindole	0.0002	0.1	6.0	0.02

[a] $n = 4$.
[b] The percentage (relative to 5-CO) that, if present, would yield a chromatographic peak with a signal-to-noise ratio of 10.
[c] Not applicable.

Table 4
Linearity of 5-CO impurities

Compound	Concentration (mg ml^{-1})	Relative concentration[a] (%)	% Recovery[b]	
			80%	120%
6-Chlorooxindole	0.0002	0.1	106	106
Oxindole	0.002	1	102	101
4-Chlorooxindole	0.0004	0.2	98	99
5,7-Dichlorooxindole	0.002	1	102	98
7-Chlorooxindole	0.0002	0.1	98	99

[a] Relative concentration of 5-chlorooxindole, 100%.
[b] The percent recovery of the response factor (area/concentration) at the largest level. $n = 3$.

Table 5
Solution stability of 5-CO and potential impurities

Compound	Concentration (mg ml^{-1})	Relative concentration[a] (%)	% Recovery[b]	
			6 h	1 day
5-Chlorooxindole	0.2	(100)	98	100
6-Chlorooxindole	0.0002	0.1	108	107
Oxindole	0.002	1	100	100
4-Chlorooxindole	0.0004	0.2	107	96
5,7-Dichlorooxindole	0.002	1	100	96
7-Chlorooxindole	0.0002	0.1	109	108

til approximately 4 l of mobile phase were consumed. Shorter equilibration times may be possible if higher flow rates are used. The effect of equilibration time on the resolution of 4-chlorooxindole, oxindole, and 6-chlorooxindole is shown in Fig. 2. Between analyses, the columns were stored in mobile phase. After the initial equilibration, the stored columns equilibrated within about 30 min of initiating a new batch of mobile phase.

Chromatographic specificity is summarized in Table 1. The compound retained to the greatest extent was 5-CO, the parent compound. Having all the potential impurities elute before the parent compound was ideal because the detectability of trace potential impurities was not adversely affected by long retention times or elution on the tail of the main peak.

The precision of injection was determined using a solution of 5-CO at its assay concentration and the potential impurities at their target levels. The percent relative standard deviations (% RSD) of the peak areas are summarized in Table 2. The precision for the 5-CO peak was well within acceptable limits for a main band assay. Impurity levels are measured against their target levels as limit tests for each impurity. The precision of the injections was lower for the low-level impurities than for the main component (5-CO), but still well within acceptable limits. The lowest precision measured was for the 6-chlorooxindole peak (12% RSD), but this still would easily allow the differentiation of 0.1 and 0.2% levels of this impurity, and thus, the requirements for a limit test were met.

Table 2 also summarizes the limit of quantitation (LOQ) for each potential impurity. The LOQ is defined as the lowest amount of an impurity (relative to 5-CO) that can be determined with acceptable precision and accuracy under the cited chromatographic conditions. LOQs were determined by measuring the signal (peak height) of each impurity at their target levels and the background noise of a chromatogram. The LOQs cited in Table 2 represent the percentage of each impurity (relative to 5-CO) that would yield a signal-to-

Table 6a
Inter-laboratory comparison

Compound	Batch A			Batch B			Batch C		
	Lab. 1	Lab. 2	Lab. 3	Lab. 1	Lab. 2	Lab. 3	Lab. 1	Lab. 2	Lab. 3
5-Chlorooxindole	98	99	98	100	98	99	100	102	98
6-Chlorooxindole	NSL	NSL	NSL	NSL	NSL	NSL	NSL	NSL	NSL
Oxindole	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6
4-Chlorooxindole	NSL	NSL	NSL	NSL	NSL	NSL	NSL	NSL	NSL
5,7-Dichlorooxindole	0.6	0.5	0.6	0.3	0.3	0.3	0.3	0.3	0.3
7-Chlorooxindole	NSL	NSL	NSL	NSL	NSL	NSL	NSL	NSL	NSL

Key: NSL, no significant level was detected (below 0.1%).

Table 6b
Individual assay results for laboratory no. 3

Compound	Batch A				Batch B				Batch C			
5-Chlorooxindole	98.1	98.1	98.3	97.0	99.0	99.2	98.9	99.3	97.8	98.6	98.5	98.7
Oxindole	0.71	0.72	0.73	0.68	0.66	0.66	0.67	0.65	0.59	0.62	0.62	0.61
5,7-Dichlorooxindole	0.59	0.59	0.59	0.59	0.27	0.28	0.28	0.28	0.28	0.27	0.28	0.28

Key: NSL, no significant level was detected (less than 0.1%).

noise ratio of 10. Since the LOQs cited in Table 2 are well below the target levels, accurate quantitation of each impurity can be readily accomplished.

The recovery was measured using a solution of 5-CO at its assay concentration and the potential impurities at their target levels. A recovery of 100% would indicate that the peak areas of that individual component were not affected by concomitants in the same solution. Recoveries were determined using external standard solutions of 5-CO and a mixed impurity standard. These results (Table 3) indicated quantitative recovery (within experimental error, vide infra) of each component and demonstrated that the measured signal of each component was not affected by the presence of potential concomitants. The recovery of individual components from a mixed impurity standard had previously been demonstrated.

The experimental error for this chromatographic system can be estimated by using the errors associated with weighing external standards and the precision of injection for each component (Table 2). The chromatographic test procedure, which was written for quality control laboratories, specifies that two individual weighings of the 5-CO working standard are made and injected into the chromatographic system. If the standard response from the first weight is within 2% of the response from the second weight, the analyst can proceed by injecting samples and one of the working standard solutions. When comparing data, assay values of the main component that are within 2% are considered consistent and within the experimental error of the technique.

For trace components whose abundance is reported to one significant figure (i.e. 0.2%), the recoveries, which ranged from 92 to 98%, can be considered quantitative. The main source of experimental error here was the variability that resulted from the integration of small peaks (Table 2).

The linearity of the 5-CO response was measured as a function of concentration. Five solutions ranging in concentration from 0.08 to 0.24 mg ml^{-1} were each injected four times. Average areas were used to construct a calibration plot. These data were consistent with a linear relationship between peak area and concentration ($r^2 = 0.9999$). The Y intercept was -2% of the peak area of the nominal 5-CO concentration of 0.2 mg ml^{-1}. The slope of the line was 1.036×10^8 area counts (mg 5-CO $ml^{-1})^{-1}$.

Because this procedure serves as a limit test for

Table 7
Typical system suitability data

Statistical parameters	Retention time (min)	Plates (tangent)[a]	Plates (Foley)[b]	Tailing factor	Resolution[c]
7-Chlorooxindole					
Mean ($n = 4$)	3.8	1600	1900	1.0	1.3
Range	(3.8–3.8)	1500–1700	1700–2200		
% RSD	0.29	5.2	11		
5,7-Dichlorooxindole					
Mean ($n = 4$)	4.4	1200	1500	1.0	5.1
Range	(4.4–4.4)	1200–1200	1500–1600		
% RSD	0.21	0.20	1.5		
4-Chlorooxindole					
Mean ($n = 4$)	7.0	3400	3800	1.0	2.0
Range	(6.9–7.0)	3200–3500	3300–4100		
% RSD	0.63	4.4	9.0		
Oxindole					
Mean ($n = 4$)	7.9	4200	4300	1.0	4.3
Range	(7.9–8.0)	4200–4300	4200–4400		
% RSD	0.46	1.5	1.5		
6-Chlorooxindole					
Mean ($n = 4$)	10.5	3500	2500	1.2	2.1
Range	(10.5–10.5)	2900–4000	2100–3200		
% RSD	0.33	17	19		
5-Chlorooxindole					
Mean ($n = 5$)	12.0	4200	3800	1.2	
Range	(12.0–12.1)	4100–4500	3600–4100		
% RSD	0.48	4.1	4.9		

[a] USP method.
[b] Ref. [6].
[c] Resolution $Rs = 2(t_2 - t_1)/(W_1 + W_2)$ is the resolution between the compound of interest and the next eluting peak, where t_1 and t_2 are the retention times of two components ($t_2 > t_1$), and W_1 and W_2 are the peak widths of two components.

several potential impurities, extensive validation of the linearity for each impurity was not necessary. Linearity was demonstrated between peak area and concentration in the vicinity of the target level of each impurity. This was done by determining the recovery of response factors at the target level of each impurity and at 80 and 120% of each target level. These data (Table 4) demonstrated adequate linearity in the vicinity of the target level for each potential impurity.

The stability of a solution of 5-CO at its assay concentration and the potential impurities at their target level was determined after 6 h and 1 day under normal laboratory storage conditions. Recoveries from initial values were determined using the average peak areas from 4 injections after each time point. Since the recovery data (Table 5) are within experimental error of the method, it is concluded that 5-CO and all of its potential concomitants are stable in solution for at least 1 day. These stability data give the analyst the opportunity to reinject sample solutions on the following day if there were equipment or other chromatographic problems.

The results of an inter-laboratory study are summarized in Table 6a. Laboratory no. 1 was the laboratory that originated and validated the methodology; laboratories no. 2 and 3 analyzed

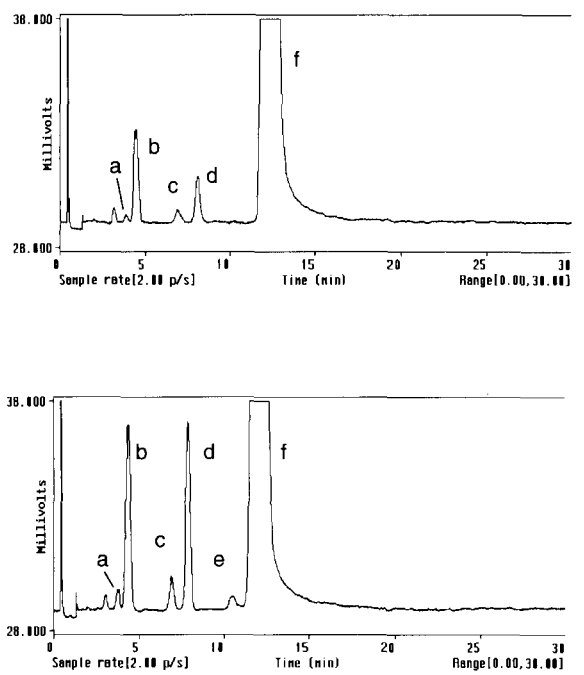

Fig. 3. Chromatograms of a typical lot of 5-chlorooxindole purchased from a potential supplier (top) and 5-chlorooxidole spiked with impurities at their target levels (bottom). Peak a, 7-chlorooxindole; peak b, 5,7-dichlorooxindole; peak c, 4-chlorooxindole; peak d, oxindole; peak e, 6-chlorooxindole; peak f, 5-chlorooxindole.

the same three samples of 5-CO as part of a laboratory certification process without previously knowing the assay results of laboratory no. 1. Agreement of the three data sets is excellent. Assay results of the main component which are within 2% are considered equivalent or within experimental error of a chromatographic assay. The impurity levels found by laboratories no. 2 and 3 also were consistent with the data generated by the originating laboratory.

Table 6b illustrates the individual assay values generated by laboratory no. 3. Two sample solutions were prepared for each of the three 5-CO samples, and each sample was injected in duplicate. The first two assay values were from the first sample solution; the second two assay values were from the second sample solution. These data are typical and illustrate the variability that can be expected during an assay.

System suitability data for a typical column are summarized in Table 7. All peaks were baseline-resolved within a 15 min run time. It is interesting to note that the chromatographic performance (measured as theoretical plates) of the first two eluting components was lower than the chromatographic performance of the later eluting peaks. This was a result of the large amount of THF in the sample solutions. The elutropic strength of the sample solutions was stronger than the strength of the mobile phase. As a result, all peaks would experience some degree of distortion, but the extent would increase with decreasing retention.

The tailing factors listed in Table 7 for the first four eluting compounds were all 1.0, indicating completely symmetrical peaks. The tailing factor of 1.2 for 5-CO can be attributed to the high concentration of this component injected into the column. Although the tailing factor for 6-chlorooxindole (6-CO) also was 1.2, the tailing can be attributed to the small peak size and the fact that the large 5-CO peak started to elute before the 6-CO peak reached the baseline (see Fig. 3, bottom).

The reproducibility of the chromatographic system is summarized in Table 8. The data

Table 8
System reproducibility

Column no.	5-CO retention (min)	Plates (Tangent)[a]	Plates (Foley)[b]	Tailing factor
1	12.0	4200	3800	1.2
2	11.5	4300	3600	1.2
3	11.6	4800	4000	1.2

[a] USP method.
[b] Ref. [6].

were generated using three different chromatographic columns and three different batches of mobile phase prepared on three different days. These results are indicative of a rugged, well-defined system.

Fig. 3 shows a chromatogram of 5-CO spiked with impurities at their target levels. In addition, there is a chromatogram of a typical lot of 5-CO purchased from a potential supplier. Impurity levels for this lot were all below their target levels. Pending other test results, this lot would be suitable for use as a starting material.

4. Conclusions

A normal-phase chromatographic system for the assay and purity evaluation of 5-chlorooxindole is described. The method has been validated and successfully transferred.

Acknowledgment

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