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A Method for the Determination of Purity of PNU-10483 Bulk Drug by Capillary Zone Electrophoresis

Gavin Gibson
Western Michigan University, gangibson@yahoo.com

THE CARL AND WINIFRED LEE HONORS COLLEGE

CERTIFICATE OF ORAL EXAMINATION

Gavin Gibson, having been admitted to the Carl and Winifred Lee Honors College in 1994, successfully presented the Lee Honors College Thesis on April 24, 1998.

The title of the paper is:

"A Method for the Determination of purity of PNU-10483 Bulk Drug by Capillary Zone Electrophoresis"

Tore Ramstad

Dr. Tore Ramstad
Pharmacia & Upjohn

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A Method for the Determination of Purity of PNU-10483 Bulk Drug by Capillary Zone Electrophoresis

by Gavin Gibson

Abstract

In this investigation, a method was developed for the determination of purity of the drug PNU-10483 using the technique of capillary zone electrophoresis (CZE). CZE was seen as a potential alternative to the formerly used procedure of ion-pair reversed phase high-performance liquid chromatography (HPLC). Nearly five years earlier, a similar study was performed, in which an attempt was made to develop a method that used CZE to determine the purity of bulk drug and the potency of drug tablets. While this development was mostly successful, sporadically unpredictable results reduced the method's overall viability. These outliers were thought to be caused by inconsistent performance of the vacuum system on the first-generation capillary electrophoresis (CE) instrument used, a PE 270A CE system. As a second-generation instrument had been obtained since that investigation, the procedure that was developed on the original was investigated again and improved.

Separation was carried out by using an untreated fused silica capillary (72 centimeters (cm) total, 50 cm to detector, 50 micrometers inner diameter). Sample injection was via vacuum suction at 5" Hg for 2.5 seconds. The run buffer used was 50 millimolar (mM) sodium phosphate, monobasic, adjusted to pH 3.30 using 85% phosphoric acid. The sample buffer used was 2.5 mM sodium phosphate, monobasic. For an internal standard, pyridine was chosen, and both it and the PNU-10483 sample were added at a concentration of 0.3 milligrams/milliliter. Stabilization of migration times was induced by a rinse cycle of 0.5 molar sodium phosphate, monobasic adjusted to pH 2.5 with 85% phosphoric acid. The voltage applied was 25 kilovolts, and the current observed at this level was about 30 microamps. Detection was on-line at a wavelength of 200 nanometers. Adjusted-area quantitation was used.

The method was selective, as it could separate all the known process and degradation impurities of PNU-10483, as well as its internal standard. When using normal assay concentrations, the procedure was linear over the range 50-120%. For this method, mean recovery was 100.1%, the relative standard deviation (RSD) was 0.97%, and the correlation coefficient for the first-order regression of amount found versus amount added was 0.9982. Furthermore, neither the intercept nor the slope were statistically different from zero or one, respectively, at the 95% confidence level. Residuals were random, not accumulating at any one place. For replicate injections of a single sample solution, the RSD was 0.75%. For single injections of replicate sample solutions at the 100% level, mean recovery was 99.1% and the RSD was 0.82%. At the 80% level, mean recovery was 99.6% and the RSD was 0.87%. Finally, the developed procedure was applied to several research lots of PNU-10483. In this study, the developed method was not compared directly to HPLC, but results found in the earlier study found good agreement between the two techniques, suggesting that CZE could serve as a valid alternative to HPLC in the purity determination of PNU-10483.

Introduction

Capillary electrophoresis (CE) is a relatively new technique for the separation of molecules, having been introduced in principle in 1979 and first demonstrated in 1981 (1). CE has several different potential applications, including the identification of different chemicals and their structures, indication of substance purity, or assays of substance potency. As is obvious from its name, CE utilizes the phenomenon of electrophoresis, in which molecules with different masses and electric charges move at different rates when placed in an electric field. This process takes place within a narrow fused-silica capillary of from 30 to 100 centimeters (cm) in length. Substances migrate along a narrow bore within the capillary, which is typically from 50 to 75 micrometers (μm) in diameter. The entire capillary is usually about 375 μm in diameter. While the capillary itself is extremely delicate, it is almost totally covered with polyimide, so as to prevent breakage. In CE, a capillary is filled with an electrolyte solution, or, run buffer. The ions of this solution allow current to flow through the capillary. Also, as the run buffer comes into contact with the walls of the capillary, silanol groups from the fused-silica dissociate, releasing hydrogen ions into the solution. The silanol groups remain attached to the inside surface of the capillary, giving the walls a negative charge (2).

After a capillary has been rinsed and filled with run buffer solution, a small amount (typically around 10 nanoliters) of sample in a less concentrated electrolyte solution is injected into the positive end of the capillary by vacuum suction, electrokinetic force, or gravity injection, which is a siphoning effect. Once this has been achieved, high voltage (up to 30 kilovolts

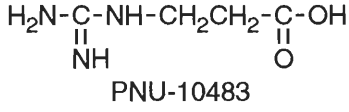
(kV)) is applied, causing an electric field to form within the capillary. This field acts upon a positively charged area near the inner walls of the capillary, setting up a flow toward the negative electrode. All of the run buffer solution within the capillary flows at the same speed, setting up what is known as the electroosmotic flow. The electroosmotic flow, in turn, induces a plug flow, which leads to a high separation efficiency. The sample substances being examined, however, travel at different speeds, bringing about separation. Generally, samples migrate in direct proportion to their charge/size ratio. Positively charged ions move faster than do negatively charged ions, which, in fact, migrate against the field. Migration velocity can be altered through the manipulation of the polarity of the electric field, temperature, applied voltage, or different electrolyte compositions (2).

A useful feature of capillary electrophoresis is its on-line detection capability. As samples migrate along a capillary once voltage is applied, at a certain point they pass through the capillary's detector window, which is a small portion of the capillary where the polyimide coating has been removed. A light source set at a certain wavelength (λ), either in ultraviolet (UV) or visible regions, shines through this window and into a photoreceptor. If the sample has specific chromophores, this would be enough to be registered by the photodetector. However, many of the ions being investigated do not have notable chromophores. In this case, a run buffer that absorbs light is used. As samples move through the detector window displacing the run buffer, more light is able to shine through, thus increasing the signal to the photoreceptor. The signals received by the photoreceptor are used to construct an electropherogram, a plot that shows the results of a CE run (2).

Capillary electrophoresis improves upon its predecessor, slab-gel electrophoresis, in several ways. The most notable advantage held by CE over slab-gel electrophoresis is its ability to rapidly dissipate the heat that was a major limitation of the older method. This is facilitated by the extremely narrow capillaries used in CE. Additionally, CE is capable of having far shorter analysis times and much higher efficiency than slab-gel electrophoresis. As such, several molecular types that were previously mainstays for investigation with slab-gel electrophoresis investigation have migrated to CE with excellent results. These include biopolymers, proteins, peptides, and nucleic acids, which can be sequenced easily using CE. In fact, CE has become a valuable tool for genomic characterization for the Human Genome Project. Finally, slab-gel electrophoresis is incapable of on-line detection, unlike CE (3).

While previous modes of electrophoresis did not show much promise for the characterization of small molecules, CE is quite capable in this area. As such, it has seen much use in this way, particularly by the chemical and pharmaceutical industries. Pharmaceutical applications exist that use different modes of CE, such as capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), and free-zone electrophoresis, but comparatively few met strict validation requirements until recently. Those methods that have shed the so-called "soft" status of previous CE applications are found mostly in the area of major-component determinations in pharmaceutical formulations (4).

In this study, a method for the determination of the animal health candidate drug PNU-10483 was developed. PNU-10483, whose molecular structure is shown below, was initially investigated as a human health candidate for the treatment of adult-onset (Type II) diabetes, but due to two adverse events in phase I trials, development ceased in 1993.



PNU-10483

More recently, it has returned as an animal health candidate for the potential treatment of obesity in dogs (5). In order to aid the earlier development of this candidate, many assays using HPLC had been developed, but CZE was also considered as a potential alternative. In CZE, the capillary is filled with a single buffer and separation occurs only according to the charge to size ratio. As a result, each analyte forms a zone which moves at a different rate than other zones (2). The first investigation into such a method met with some degree of success, but was frequently plagued with unpredictable results. Specifically, outliers were observed in nearly every series of runs. Investigation into the cause of these outliers suggested that they were the fault of inconsistent performance of the vacuum system within the instrument that was used, a first-generation ABI (now PE) 270A Capillary Electrophoresis System (3). Two years after the first study had been completed, a second generation instrument, the PE 270A-HT Capillary Electrophoresis System had been purchased. This instrument featured an improved vacuum system, a change which held the possibility of making the determination of purity of PNU-10483 by CZE truly viable (5).

Before any method can be used for the testing and/or analysis of substances such as pharmaceuticals, its performance must be evaluated with respect to several parameters. One such parameter is specificity, or, whether the method is specific to the component of interest, without interference from similar substances such as breakdown products or impurities. Another parameter important to method validation is sensitivity, which determines the limit of detection (LOD) and the limit of quantitation (LOQ) for the method. The LOD can be described as the lowest amount of the sample that can be distinguished from background "noise," whereas the LOQ is the smallest sample concentration at which reliable quantitation can be performed. Usually, these values are judged from the method's signal-to-noise (S/N) ratio. S/N ratios greater than or equal to 3:1 for the LOD and 10:1 for the LOQ are usually sufficient (2).

Linearity is also found for method validation. It is assessed by running samples for concentrations usually ranging from about 50% to 150% of normal. Ideally, the amounts of sample found through CE should correspond with the amounts added, forming a linear plot. The intercept of such a first-order regression plot should not be statistically different from zero, while the slope should not be statistically different from one. The correlation coefficient is also calculated in this test (2).

Finally, precision is another parameter important to method validation. To test precision, identical runs are repeated several times, either all from a single sample preparation or each from a different but identically prepared sample preparation. Results are obtained from the

amounts of sample found and the relative standard deviation (RSD) determined from these values. In CE, RSD values below 2% are generally acceptable (2).

If CE is ever to be considered as an equal to the technique of high-performance liquid chromatography (HPLC), more work must be done for it to match the performance of HPLC. When using HPLC, relative standard deviation values of ≤ 1% are commonplace, lower than the usual RSDs of less than or equal to 2% for major components obtained through CE. Furthermore, to obtain even this level of performance from CE methods requires that careful attention be paid to the regulation of several parameters, including temperature, pH, the state of the surface of the capillary, and the mode and length of the sample introduction. Additionally, an internal standard is usually required in order to attain low RSD values. If these criteria are properly adjusted and/or maintained, performance more in line with HPLC is possible. Also, CE performance will likely improve as the development of next-generation CE instruments continues (3).

Methods and Materials

Equipment

Early work in the development of this method was performed on a first-generation PE 270A Capillary Electrophoresis System, but validation experiments were all performed on the second-generation PE 270A-HT Capillary Electrophoresis System. The capillaries used were purchased already prepared by PE. They were 50 micrometers (μm) in diameter, composed

of fused silica, and precut to a length of 72 centimeters (cm) (=L_t). The detector window was at 50 cm from the start of the capillary (=L_d). Each capillary used was pretreated with 1 N NaOH for approximately 20 minutes, and rinsed in this manner again as required to restore proper functioning. The instruments' variable wavelength detectors, which utilize deuterium sources, were set to 200 nanometers (nm). This wavelength had been previously determined to be optimal partly as a result of the lack of notable chromophores in the PNU-10483 molecule. Sample injection was hydrostatic by vacuum at 5" Hg and sustained for 2.5 seconds. The capillaries were all maintained at a temperature of 30°C by the air-driven, thermostat-controlled compartments of both instruments used. Each run consisted of two 3 minute rinses, first with phosphate wash solution and secondly with the run buffer, followed by the injection of the sample and the application of voltage for from 10 to 12 minutes, as necessitated. Later in the study, septa were placed on top of both buffer and sample vials in an attempt to curb the evaporation of these solutions, particularly the sample, which contained semi-volatile pyridine. In addition, a 1:1 water:ethylene glycol refrigerant solution was circulated through the autosampler tray of the PE 270A-HT at 6°C by a Neslab RTE-5DD Refrigerated Circulating Bath. This was done to further reduce the effects of volatilization of the internal standard. The PE 270A-HT can be controlled one of two ways, either directly from its built-in microprocessor or by an external personal computer through an interface. The former method was found to be simpler and was used in this study. Data collection was carried out by a PE Nelson 900 Series Interface under the control of the program "AccessChrom," and was performed at the rate of 5 points per second.

Reagents

Phosphate buffers were prepared using sodium phosphate, monobasic, monohydrate, which was obtained from Mallinckrodt (AR grade). The buffers were adjusted to the required pH levels with 85% phosphoric acid, also acquired from Mallinckrodt (AR grade). Purified water, used to prepare buffer solutions, was obtained from a Milli Q system from Millipore. To minimize the chances of the capillaries becoming plugged, all solutions were filtered through 0.45 μm, HPLC-certified filters from Gelman Sciences. Each solution was also vacuum ultrasonicated, in order to remove dissolved gases. The internal standard, pyridine, was obtained from Burdick & Jackson, and new internal standard solutions were prepared daily, as the pyridine was subject to volatilization. In general, new solutions of all types were freshly prepared as often as possible, as the performance of the assay was quite sensitive to their ages. All known and possible process impurities of PNU-10483 were obtained from Aldrich, Mallinckrodt, Sigma, and P & U Chemical Research Preparations (now Chemical and Fermentation Operations). For each of the developmental and validation studies, milled PNU-10483 bulk drug from lot 24968-MAK-95 was used. PNU-10483 lot 24968-MAK-101 was used as the reference standard in the assay of research lots of PNU-10483. This lot was most recently characterized in 1996.

Methodology

Much of the validation work of the previous study laid the ground for this study, so a few areas did not need to be dealt with directly here. For example, checks on potential sources of

interference to assay stability were performed in more detail earlier, but were briefly confirmed in this study, nonetheless. A satisfactory internal standard, pyridine, had also already been indentified in the previous study. Furthermore, the compositions of the solutions used varied only slightly from those found to be optimal in the earlier study (3). One alteration was the adjustment of the concentration of PNU-10483 in the sample solution to 0.3 milligrams (mg)/milliliter (ml) from 0.2 mg/ml. This was carried out by adding pyridine to the sample buffer at 0.3 mg/ml, measuring out 10 ml of the resultant solution, and adding approximately 3.0 milligrams (mg) of PNU-10483 in a suitable vial. Also, sodium phosphate was used in the buffer solutions rather than potassium phosphate. The wash solution used, 0.5 molar (M) phosphate buffer at pH 2.5, did not need to be altered in any way.

Linearity and recovery results for this method were investigated for concentrations from 50-120% that of the original assay concentration. Precision, in turn, was measured for replicate injections of a single sample preparation and also for single injections of replicate sample preparations at 100% and 80% concentration levels. Quantitation was performed according to adjusted area, A_{adj}, in which $A_{adj} = A_{unadj}/t_m$; where t_m is the migration time (5). Furthermore, any one run buffer vial was not used more than two successive times, in order to prevent an inward drift of migration times. Also, the vials containing the 0.5 M phosphate wash solution were switched after every second run.

Results

Method Development

This study started at the optimal conditions established in the earlier study. Specifically, analyte concentration of 200 parts per million (ppm), 200 nm detection, sample introduction for 2.5 seconds via vacuum injection at 5" Hg, and application of voltage at 30 kilovolts (kV) (resulting in a current of approximately 30 microamps (μA)). With these conditions, a electrophoresis run was executed, generating an electropherogram seen as Figure 1. Surprisingly, response for the PNU-10483 peak was smaller than had been expected, showing a S/N ratio. As an initial effort to improve response, electrokinetic injection was used in the place of vacuum injection, applying 5 kV for 5-20 seconds. However, these conditions resulted in even smaller resultant peaks, most likely due to the fact that PNU-10483 had insufficient electrophoretic mobility. In addition, pyridine displayed a broader peak when injected electrokinetically.

The buffer and sample solution pH of 3.3 was found to be optimal in the earlier study, but some work was done in this study to confirm these results. Once again, pH 3.3 showed the most symmetrical peak shape of any of the pH levels tested. At pH < 3.30, the PNU-10483 peak displayed notable tailing, and at pH > 3.30, fronting became a problem with this peak's shape.

In the previous study, potassium phosphate was used in both the sample and run buffers and also in the wash solution. However, it has been noted in other CE studies that the closer the

match between the electrophoretic mobility of the analyte and of the buffer counter ion, the more symmetric peak shape is. In the interest of taking advantage of this behavior, the effect on peak shape of sodium, lithium, and potassium salts of dihydrogen phosphate were compared. Each run buffer was prepared at 50 mM and each sample buffer at 2.5 mM for the salt of each ion, and the pH of each was adjusted to pH 3.3 with 85% phosphoric acid (the pH of the sample buffer decreased to 3.2 when 200 ppm PNU-10483 was added). Table 1 shows the results found by this analysis. Potassium and sodium ions were observed to effect similarly desirable peak shapes. More careful scrutiny of these peaks at 5% or peak base values showed sodium to have slightly preferable peak shape, and, as such, $NaH_2PO_4 \bullet H_2O$ was used in the run and sample buffers and the wash solution.

As noted previously, signal-to-noise ratios were a bit questionable at the start of this study. In an attempt to rectify this situation, an investigation into the effects of higher analyte concentrations was initiated. Until this point, PNU-10483 in the sample solution was added at a concentration of approximately 200 micrograms (μg)/ml, which was found to be optimal in the previous study. PNU-10483 concentrations in a range of approximately 200-400 μg/ml were tested; the results are seen in Table 2. In column 6, an F parameter is calculated which shows about how quickly peak height increases in comparison to peak area. Peak height was seen to increase with concentration at a faster rate than did peak area. For each concentration above 200 ppm, overall performance decreased, but at a relatively slow rate until the concentration of 300 ppm was reached. As such, analyte concentration from this point on were increased to 300 ppm. It was worth noting that even after this adjustment, the S/N

ratios were still somewhat lacking, a point that would seem to show that this method would benefit from techniques that enhance the sensitivity of detection, such as "z-cell" capillaries, whose detector windows are in the shape of the letter "Z," thereby extending the path length for detection and improving response (2, 5).

Specificity was tested only briefly, as this had already been more extensively covered in the earlier study (3). Nevertheless, it was not completely certain that the changes made to the buffer solutions would not adversely affect selectivity. Therefore, a mixture of pyridine and PNU-10483 and the process and/or degradation impurities guanidine, PNU-10483 lactam (PNU-92576), β-alanine, cyanamide, and dicyandiamide were added to sample buffer and separated by CZE. The results of this run are seen in Figure 2. It is interesting to note that while dicyandiamide produced a peak in the earlier study, it did not here. Later, this was thought to be caused by the decomposition of dicyandiamide to cyanamide, as the dicyandiamide used was several years old. Also, cyanamide did not elute as it was uncharged under the assay condition of pH 3.3 and exhibited small electroosmotic flow was small. Regardless, when these results and those from the previous study were combined, it could be concluded that both pyridine and PNU-10483 exhibited separation from all other components. Table 3 gives additional information about each of the impurities tested.

Validation Results

Early series of runs in the validation process gave very high RSD values; these were subsequently attributed to the volatility of the pyridine internal standard. An example of the

degradation of results that often occurred from one day to the next can be observed in Figure 3, as well as a blank run which was added for comparison. In order to stop or slow the evaporation of pyridine, septa were placed over the sample vials and a refrigerated recirculator was used to maintain the temperature of the PE 270A-HT's autosampler tray at 6°C. Additionally, sample filtering, which had previously been used until early validation, was eliminated. It was found that this technique was not absolutely necessary and may or may not have had a negative effect on the results. Also, a greater amount of sample solution was added to each sample vial (approximately 300 µl or more), in order to decrease headspace and further counteract the volatility of the internal standard. After these measures had been enacted, performance improved notably, but could still show considerable variation from one day to the next, particularly in peak shape. This inconsistency in performance was later seen to be directly related to the ages of the buffer solutions used. As a result, solutions were prepared fresh daily from this point on. After all extra precautions were taken, the electropherogram seen in Figure 4 was produced. This separation was achieved with the use of a sample buffer of 2.5 mM, pH 3.30 $NaH_2PO_4 \bullet H_2O$ containing 0.3 µl/ml pyridine and 300 ppm PNU-10483, a run buffer of 50 mM $NaH_2PO_4 \bullet H_2O$, pH 3.30, and a wash solution of 0.5 M $NaH_2PO_4 \bullet H_2O$, pH 2.50.

Precision

Precision for this method was determined by performing six replicate injections of one sample preparation and single injections of six replicate sample preparations at 100% and 80% normal concentration (300 ppm). The results obtained from the replicate injection runs are shown in

Table 4. The RSD was found to be 0.75%. While each sample vial was sealed by a septum in these runs, the refrigerated recirculator was not utilized. The results of the replicate preparation injections at 100% are shown in Table 5. Mean recovery for this set was seen to be 99.1% and the RSD was 0.82%. This mean recovery value was just over one standard deviation away from 100%, an acceptable result, but one probably attributable to the variation in performance seen from day to day until the previously mentioned preventative measures were taken. The results for the replicate preparations at 80% sample concentration are shown in Table 6. Mean recovery for these runs was 99.6%, while standard deviation (SD) was 0.87% and RSD was 0.87% as well. The results of these investigations showed that the assay maintained precision at an acceptable level.

Linearity/Recovery

Table 7 shows linearity and recovery results for the range of 50-120% of normal sample concentration. The levels of 130% and 140% were also performed but exhibited uneven and unsatisfactory results. Specifically, recovery at 130% was approximately 85% while recovery at 140% was approximately 105%. A negative display of nonlinearity was not surprising at such high values, but as the 130% level value was low and the 140% level value was high, it is not certain what the cause of these nonlinear values was. Mean recovery for these runs over the range of 50-120% was 100.1% and the RSD was seen to be 0.97%.

A first-order regression plot made from the recovery results that were obtained is shown in Figure 5. The intercept found was 0.027 and was not statistically different from zero. The

slope found was 0.990 and was not statistically different from one. The lower and upper 95% intercept values were -0.0650 and 0.119 while the lower and upper slope values were 0.955 and 1.025. These were acceptable results, meeting usual validation expectations. Figure 5 also includes a plot of the residuals, which appeared to be distributed about the regression line without any bias.

Application of Developed Assay to Research Lots of PNU-10483

Having been fully developed at this point, the assay was next used to examine six research lots of PNU-10483, each of which was at least five years in age. The determination was made duplicate. The lot 24968-MAK-101 was used as the reference standard and upon its most recent examination in 1996, was assigned a purity value of 99.8%. The results obtained from these runs are displayed in Table 8. While no comparison to HPLC was made, all values were within expectations. Furthermore, the assay was well behaved.

Conclusion

While this study was very similar to one performed nearly five years ago, the previous study was hampered by the appearance of outliers in almost every data set. These anomalies were later attributed to the imperfect vacuum system of the first-generation CE system used. The problems experienced in this earlier study made it impossible to meet validation expectations at that time (3). However, second-generation CE systems that have been introduced since 1993 have far fewer of the weaknesses that handicapped their predecessors. In particular,

vacuum performance has been greatly enhanced. The method introduced in the previous study, when modified to perform on the newer instrument, shows no statistical outliers and passes validation requirements. While sensitivity could be improved somewhat, either by the use of z-cell capillaries or similar methods, it was sufficient enough to be deemed acceptable on a standard capillary. Therefore, when performed on more advanced CE systems, this method is now a viable and orthogonal alternative to HPLC for the determination of purity of PNU-10483.

References

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Table 1
Effect of Buffer Counter Ion on Separation Efficiency*

Ion(M)	$w_{1/2}$ (s)	$w_{5\%}$ (s)	w_{base} (s)	$N_{1/2}$	$N_{5\%}$
K	3.5	7.7	10.7	145107	130557
	3.1	8.1	11.2	178516	113077
Li	5.4	10.0	13.8	56220	72453
	5.1	9.4	12.1	62251	79475
Na	4.1	7.6	10.7	96257	122622
	4.1	7.5	10.2	99164	126325

* All values pertain to PNU-10483
Run buffer: 50 mM MH_2PO_4, pH 3.30
Sample buffer: 2.5 mM MH_2PO_4, pH 3.3

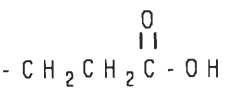
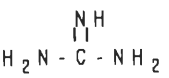
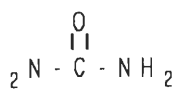
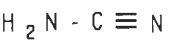
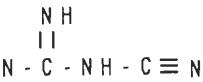
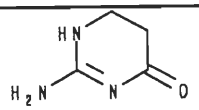
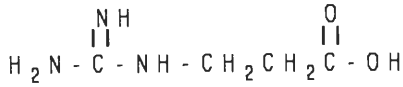
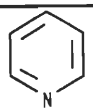
Table 2
Effect of Sample Prep Concentration on Assay Performance

Sample	Area	$A/A_{200\ ppm}$	H (μV)	$H/H_{200\ ppm}$	F*	$W_{1/2}$	$N_{1/2}$
200	42050		11478			0.061	123246
225	49238	1.171	13046	1.137	0.971	0.063	113096
250	55738	1.326	13050	1.137	0.857	0.072	87674
275	61542	1.464	14534	1.266	0.865	0.072	87219
300	69484	1.652	16316	1.422	0.861	0.072	85390
400	94268	2.242	20110	1.752	0.781	0.080	68089

* $F = (H/H_{200\ ppm})/(A/A_{200\ ppm})$

Table 3

Possible Interferences: Bulk Drug, Process Impurities, Degradation Products, and Internal Standard

Compound	Source	Structure
β-Alanine	Starting material	
Guanidine	Possible process impurity and degradation product	
Urea	Possible degradation product	
Cyanamide	Starting material	
Dicyandiamide (PNU-96,629)	Process impurity	
PNU-10,483 Lactam (PNU-92,576)	Degradation product	
PNU-10,483	Bulk drug	
Pyridine	Selected Internal Standard	

All runs were in 50 mM $NaH_2PO_4 \cdot H_2O$ buffer at pH 3.3; 25 kV applied potential; 2.5 s hydrostatic injection.

Table 4

Precision for Replicate Injections of a Single Sample Preparation

Run	Standard Factor*
1	4.35378
2	4.28313
3	4.31224
4	4.33969
5	4.27223
6	4.33033
	x = 4.31523 RSD = 0.75%

* Std. Factor = $(wt/A_{adj})_{10483}(A_{adj})_{IS}$

Table 5
Precision Results for Single Injections of Replicate Sample Preps at the 100% Level

Run	% Recovery
1	99.3
2	98.7
3	100.6
4	98.6
5	98.4
6	98.8
RSD for standards (n = 6) = 0.86%	x = 99.1% s = 0.81% RSD = 0.82%

Table 6
Precision Results for Single Injections of Replicate Sample Preps at the 80% Level

Run	% Recovery
1	98.8
2	98.8
3	100.5
4	100.2
5	100.5
6	98.9
RSD for standards (n = 6) = 0.77%	x = 99.6% s = 0.87% RSD = 0.87%

Table 7
Linearity/Recovery Results for PNU-10483

% of Assay Conc.	Amount Added (mg)*	Amount Found	% Recovery
50	1.447	1.446	99.9
60	1.857	1.849	99.6
70	2.154	2.149	99.8
80	2.356	2.367	100.5
80	2.306	2.339	101.5
90	2.619	2.624	100.2
100	2.946	2.946	99.4
100	3.022	3.002	100.0
110	3.271	3.325	101.7
120	3.750	3.690	98.4
RSD for standards (n = 8) = 0.67%			x = 100.1% s = 0.97% RSD = 0.97%

* in 10 ml

Table 8
Purities Determined for Research Lots of PNU-10483 Using the Developed CZE Assay

Sample	Result (%)
Sigma 86F0339	98.0
	98.6
Sigma 76F0608	99.3
	99.0
24272-MAK-18	99.6
	98.3
25944-MAK-12	98.5
	97.7
24958-MAK-95	100.6
	100.5
(A)0219-MAK-096	97.0
	98.0

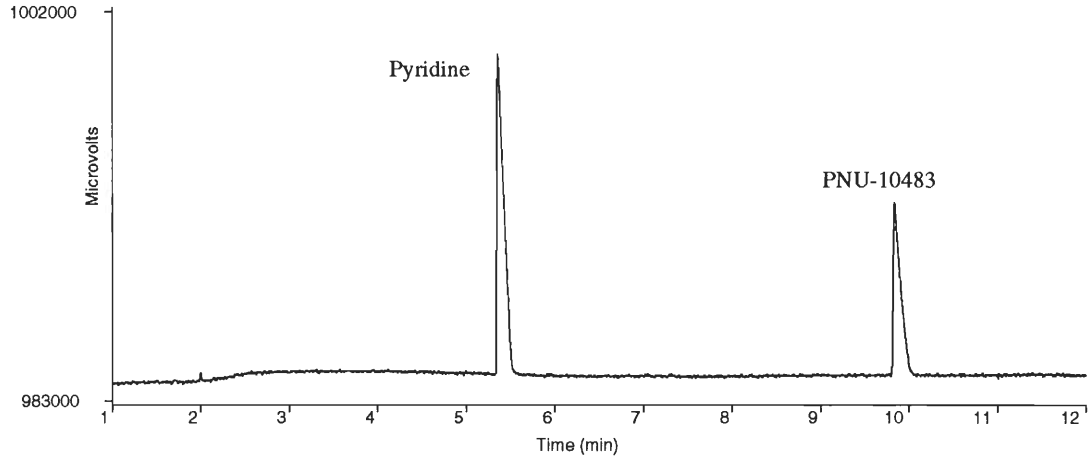


Figure 1. Results of capillary electrophoresis using the optimal conditions established by the earlier study, representing the state from which current development started. Conditions: capillary, 50/72 cm, 50 μm id; run buffer, 50 mM KH_2PO_4, pH 3.30; sample buffer, 2.5 mM KH_2PO_4, pH 3.3; internal standard, 0.3 μl pyridine/ml sample buffer; sample concentration, 200 ppm PNU-10483; injection, vacuum, 2.5 s; temperature, 30°C; voltage (V), 25 kV; wavelength (λ), 200 nm.

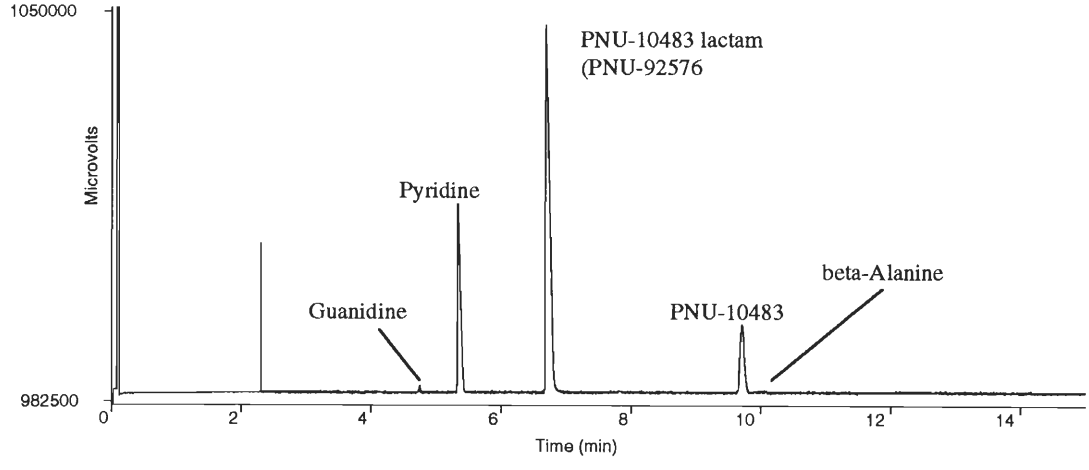


Figure 2. Separation of several impurities from pyridine (internal standard) and PNU-10483.

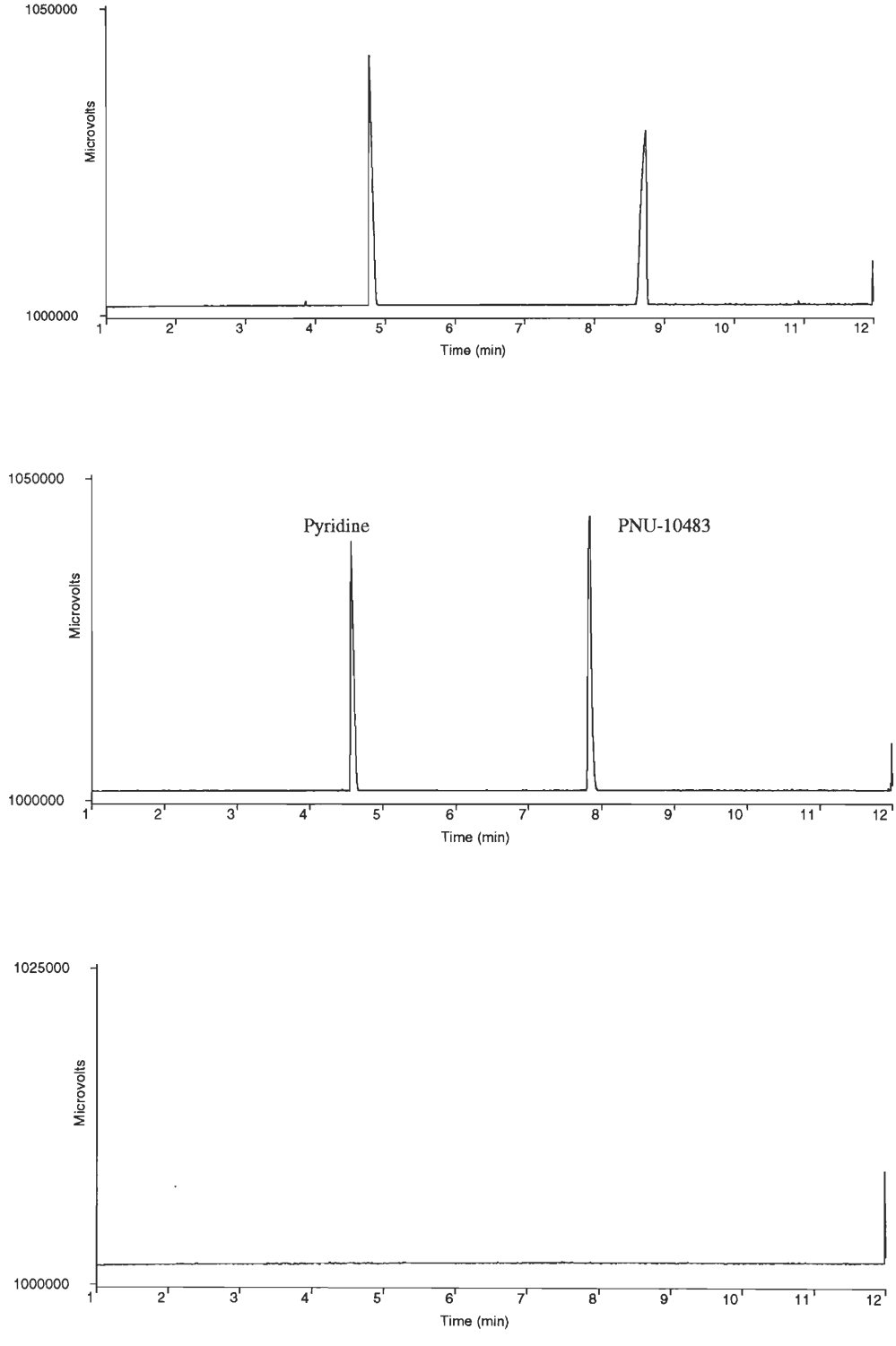


Figure 3. Electropherogram showing slightly degraded performance (top) compared with optimal performance (middle). A blank is shown at the bottom.

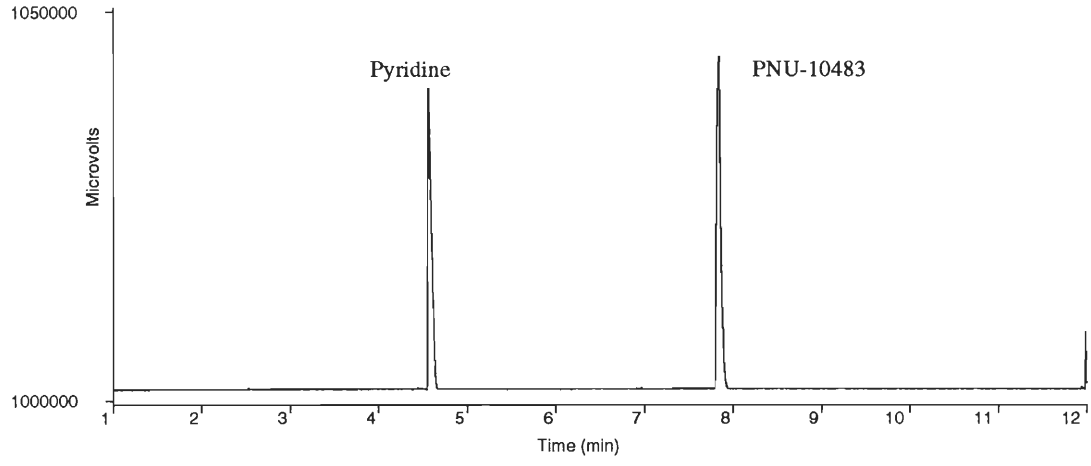


Figure 4. End product of development, full scale. Conditions: capillary, 50/72 cm, 50 μm inner diameter; run buffer, 50 mM NaH_2PO_4, pH 3.30; sample buffer, 2.5 mM NaH_2PO_4, pH 3.3; internal standard, 0.3 μl pyridine/ml sample buffer; sample concentration, 300 ppm PNU-10483; injection, vacuum, 2.5 s; temperature, 30°C; V = 25 kV; λ = 200 nm.

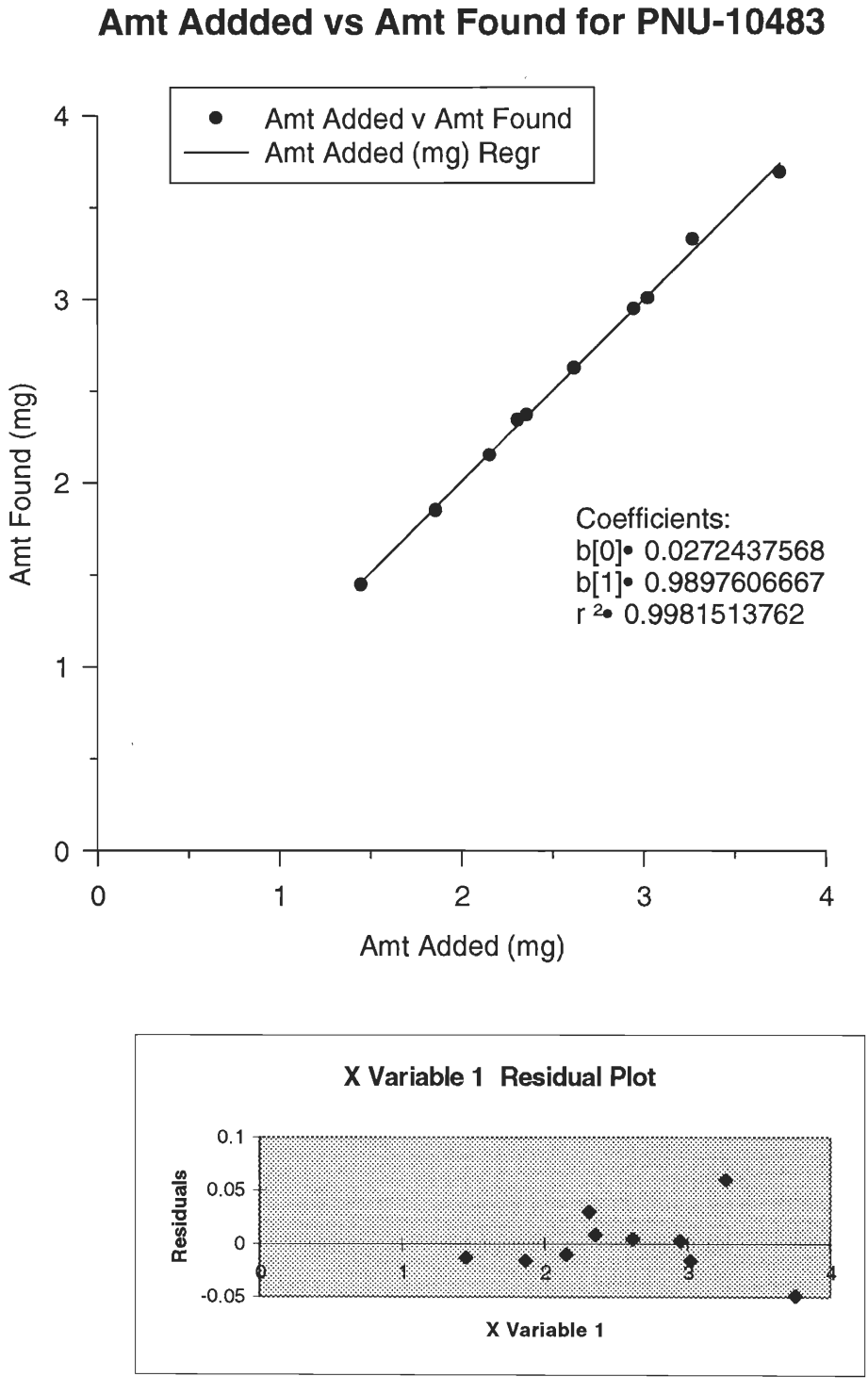


Figure 5. First-order regression curve for recovery over 50-120% of the nominal assay concentration. The corresponding residuals plot is also shown.