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A systematic column performance comparison for the confirmation of opioids used in pain management by LC-MS

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A systematic column performance comparison for the confirmation of opioids used in pain management by LC-MS.

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By

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Abstract

In this study, three different chromatographic column chemistries (C18, Pentafluorophenyl (PFP), Hydrophilic Interaction Chromatography (HILIC)) were compared under optimal conditions to determine which stationary performed best in the separation and detection of a mixture of opioids using LC-MS. Furthermore, these stationary phases were examined in three different column technologies – traditional silica, porous shell, and porous polymer (PRP). The PRP column had the best peak shape for all 13 opioids and dominated for later-eluting compounds. In terms of column reproducibility, the Hamilton C18 column had the lowest %RSD values. The Kinetex HILIC produced the most theoretical plates and best resolution for polar compounds as did the Hamilton C18 for nonpolar compounds. Finally, Kinetex PFP and Hamilton PRP both demonstrated themselves as viable alternatives to the C18 column chemistry for analysis of this drug class.

1. Introduction

Opioid analgesics are a well-known class of pain management drugs geared towards relief of severe, chronic, or acute pain. Most drugs in this class are synthetic or semi-synthetic analogs of the active alkaloid of the opium poppy, morphine. Due to the nature of these drugs, opioid tolerance or the development of opioid-induced pain sensitivity (hyperalgesia) are possible cellular consequences of long-term exposure to opioids [1]. Long-term and high dose use of opioids causes an up-regulation of opioid-receptors and a down-regulation of dopamine-receptors, causing a disruption in the normal function of the mesolimbic pathway, and subsequently, opioid withdrawal symptoms when the opioids are discontinued [2]. In principle, the dosage must be monitored to provide sufficient pain relief without starting a cascade of effects related to addiction and/or dependence commonly associated with opioids. Improved drug monitoring and surveillance should help reduce some of these problems and, as a result, lower the resistance to using chronic opioid therapies [3].

Therapeutic drug monitoring is critical for the optimal use of drugs such as opioids. The aim of monitoring patient drug concentrations is to provide pain relief without the adverse effects. Opioids lie within a narrow therapeutic window in which elevated concentrations can cause toxicity, whereas, a minimal dose may result in an ineffective treatment. Currently, automated and high-output immunoassays are a standard tool for therapeutic drug monitoring, but often lack specificity for parent drugs or for differentiating among drugs in the same class [4]. A relatively new technique to monitor drug concentrations is liquid chromatography mass spectrometry (LC-MS). LC-MS is amenable to most non-volatile analytes and combines analyte separation with selective detection based on a compound's mass. In liquid chromatography, the compounds of interest are separated based on their partition between a solid stationary phase (in

a column) and a liquid mobile phase. Variations in LC column chemistry can confer differences in selectivity and compound retention order. For each peak obtained in the chromatogram, there is a corresponding mass spectrum that helps the analyst identify the compound present.

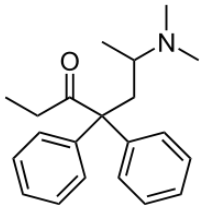
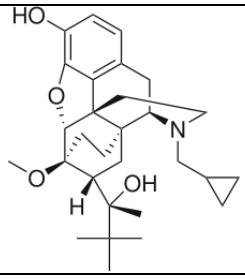
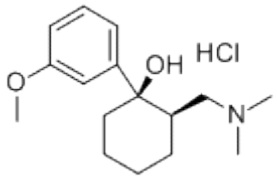
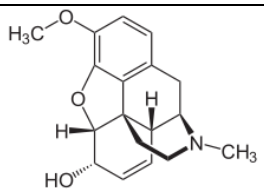
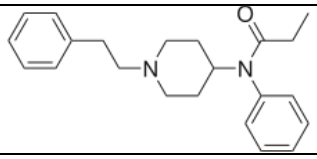
The purpose of this study is to compare three different column chemistries (C18, Pentafluorophenyl, and HILIC) under optimal conditions to find which stationary phase had the best reproducibility and performance in the separation and detection of a mixture of opioids using LC-MS. With this in mind, the retention time of the analytes used in this study will differ between the respective stationary phases due to the hydrophobic, hydrophilic, or ionic interactions. For instance, the C18 stationary phase utilizes the hydrophobic nature of the lengthy alkyl chains to retain the analytes of interest. For separations only involving hydrophobic interactions, retention tends to increase with the concentration of organic stationary phase, as long as the organic ligands are completely accessible to solutes [5]. The common bonded octadecyl stationary phases allow efficient separation of analytes within a broad range of polarity and fast column equilibration [6]. The Pentafluorophenyl (PFP) stationary phase contributes a different set of interactions to aid in difficult separations. Compounds separate based on unique dipole-dipole, π-π, charge-transfer, and ionic interactions due to the presence and reactivity of the fluorinated phenyl ring [7]. In comparison, the HILIC stationary phase serves as an alternative to C18 or PFP by employing a variant of normal phase chromatography (NPC) to aid in the retention of polar/hydrophilic compounds. In 2006, Hemstrom and Irgum [8] noted that both adsorption and the partitioning mechanism between the bulk mobile phase and a layer of mobile phase enriched with water contribute to retention. In addition, HILICs notable performance for the separation of ionizable compounds of varied polarity may serve as a

substitute to reversed-phase chromatography (RPC); however, the separation mechanism is not well understood [6].

These stationary phases will be examined in three different column technologies – traditional silica, porous shell, and porous polymer. Traditionally, totally porous silica particles present a strong advantage due to their consistent mechanical durability with water and organic solvents [5]. Furthermore, these particles possess the capability to become chemically modified with multiple bonded phases. However, traditional silica only operates within a narrow pH range due to changes in particle solubility [5]. Moreover, surface acidity may become problematic for the separation of basic compounds [5]. On the other hand, porous shell technology consists of fused-core particles with solid cores wrapped in a porous shell averaging 0.4 µm thick with reduced theoretical plates of 1.5 or lower for small molecules. This may be attributed to higher particle density and narrow particle size distribution to form homogeneous packed beds [8]. The resulting reduced backpressure allows for smaller particle size and longer column lengths to achieve better separations [9]. Furthermore, improved mass transfer kinetics have been obtained due to solutes rapidly diffusing in and out of the stationary phase-containing porous shell [9]. Lastly, these particles develop around twice the theoretical plates/bar pressure when measured at the plate height minimum, compared to sub-2-µm particles [9]. This allows for the added resolution achievable with sub-2 micron particle columns, often referred to as UPLC columns, without the added backpressure, which makes then compatible with conventional HPLC machinery. Alternatively, porous polymer chemistry represents a new breed of technology developed over the last decade. The majority of these particles for RPC are composed of divinylbenzene-cross-linked polystyrene with hydrophobic character [10]. However, the main advantage is their usability in a broad pH range from 1 to 13 and their high chemical and thermal

stability [5]. Therefore, this technology is suitable for separating highly basic, non-ionized compounds at high pH resulting in good peak shape. Furthermore, strong hydrophobic retention broadens the capabilities of this column technology.

A mixture of 13 opioids supplied by Cerilliant Analytical Reference Standards were separated using combinations of the stationary phases and column technologies previously mentioned. In Table 1 below, pertinent information regarding chemical characteristics of these opioids is listed:

Component	Log P	Molecular Weight (g/mole)	Chemical Structure	pKa
(±)-Methadone	3.93	309.45		8.3
Buprenorphine	2.83	467.65		8.42
cis-Tramadol HCl	2.32	299.84		9.41
Codeine	1.39	299.37		8.2
Fentanyl	3.68	336.48		8.4

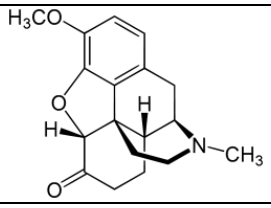
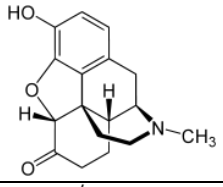
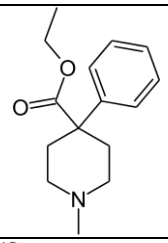
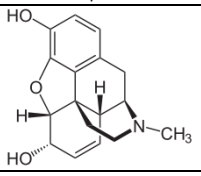
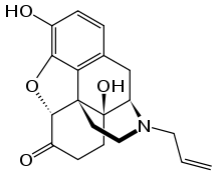
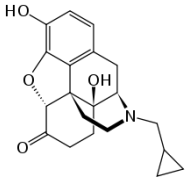
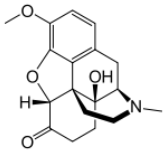
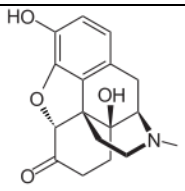
Hydrocodone	2.57	299.37		8.9
Hydromorphone	2.13	285.34		8.2
Meperidine	2.19	247.34		8.7
Morphine	0.87	285.34		8.0
Naloxone	1.78	327.38		7.9
Naltrexone	2.05	341.41		8.13
Oxycodone	1.59	315.37		8.9
Oxymorphone	1.15	301.34		8.5

Table 1: Compilation of the 13 opioids under study [11]. Relevant specifications for each drug are listed to help compare/contrast columns.

These opioids share a tertiary amine functional group that chemically complements the hydrophobicity of the surrounding carbons. With this in mind, altering the pH of the aqueous buffer creates either ionized or non-ionized forms of the drugs. Using other information, such as the partition coefficient and pKa values, allows for a deeper understanding of the interactions that take place in each stationary phase.

After the chromatographic separations, tandem mass analyzers helped identify the resulting compounds eluting from the columns. First, electrospray ionization produces the ions that filter into an ion trap mass analyzer. For our purposes, the ion trap functions to guide the ions into the time-of-flight mass analyzer. Initially, in this analyzer, the incoming ions receive the same kinetic energy and are sent through the field-free drift zone by an extraction pulse [12]. In this zone, mass separations occur due to lighter ions traveling faster which, in turn, aids in the recording of all ions and improves the sensitivity [12]. The resulting data from the chromatogram and tandem mass analyzers provide key pieces to calculate column performance. First, resolution, the degree of separation between two peaks on a chromatogram, can be calculated [13]:

$$R_S = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B} \quad (1)$$

where R_s is resolution, t_r is time of retention, and W is the base peak width. Next, the concept of theoretical plates provides another measure of column efficiency. Theoretical plates result from the equilibrium between liquid and vapor states of a substance that form this hypothetical zone in the column. Since more "plates" equals better performance, theoretical plates will be calculated for each column using Equation 2 [13]:

$$N = 16\,(\frac{t_R}{W})^2 \qquad (2)$$

where N is the number of theoretical plates, t_R is time of retention, and W is the base peak width. To account for different column lengths in this study, the Height Equivalent to Theoretical Plates (HETP) essentially normalizes the data by dividing the column length by the initial theoretical plate number. In other words, this describes the variance per unit length of the column [14].

2. Materials and Methods

2.1 Chemicals and Materials

The reference standard mixture of 13 opioids (see Table 1) was obtained from Cerilliant Analytical Reference Standards (Round Rock, Texas). All components of the reference standard were at a concentration of 100 µg/ml except for fentanyl, which was 10 µg/ml. The solvents used were methanol, water, and 0.1% v/v formic acid in acetonitrile. All of these solvents were of LC-MS optima grade (Burdick & Jackson, Muskegon, MI). The ammonium acetate and ammonium formate salts for mobile phase preparation were purchased from Fisher Scientific (Pittsburgh, PA). Glacial acetic acid (Amresco, Solon, OH; 98%+ purity) and formic acid (Acros Organics, Fair Lawn, NJ; 98%+ purity) were used to adjust pH levels.

A pH meter and microfuge 16 were purchased from Beckman Coulter (Brea, CA). Micropipettes were purchased from Rainin, a Mettler-Toledo company (Columbus, OH). Syringe filters, 13mm with 0.2µm PTFE membrane, were purchased from VWR International (Radnor, PA). Autosampler vials and closures (10-425) were purchased from Fisher Scientific (Pittsburgh, PA).

2.2 HPLC Analysis

The Shimadzu liquid chromatography system consisted of two LC-20AD pumps with UFLC-XR upgrade, SIL-20ACHT autosampler, CTO-20A column oven, DGU-20A_3 degasser, and CBM-20A communications module. This system was coupled to the Shimadzu IT-TOF mass spectrometer with an electrospray (ESI) source (Columbia, MD). The columns used were Hamilton HxSil C18, Kinetex C18 Porous Shell, Kinetex PFP, Kinetex HILIC, and Hamilton PRP-H1. Hamilton columns were manufactured and distributed by the Hamilton Company (Reno, NV), Kinetex columns were made by Phenomenex (Torrance, CA). Physical and chemical properties of these columns can be found in Table 2. In all cases, parameters such as mobile phase (type, %, pH), gradient, flow rate, and oven temperature were optimized for each column. After optimization, each column ran 25, 1 µL injections of 10x diluted, syringe-filtered (13mm with 0.2µm PTFE membrane) opioid standard.

	Hamilton C18	Kinetex C18	Kinetex PFP	Kinetex HILIC	Hamilton PRP
Bonded Phase	Octadecyl-Silane	Octadecyl-Silane	Pentaflurophenyl	Divinylbenzene cross-linked polystryene	Octadecylated Polystyrene-Divinylbenzene
Particle Platform	Spherical	Core-Shell	Core-Shell	Core-Shell	Spherical
Particle Size (µm)	5	2.6	2.6	1.7	5
Pore Size	100	100	100	100	100

(Å)					
pH Range	2.0 – 7.5	1.5 - 10	1.5 – 8	2.0 – 7.5	1 - 13
Length (mm)	150	100	100	100	150
Inner Diameter (mm)	2.1	2.1	2.1	2.1	2.1
Mobile Phase A	95:5 v/v H_2O : 0.2M Ammonium Acetate	95:5 v/v H_2O : 0.2M Ammonium Acetate	0.1% v/v Formic Acid and 5mM Ammonium Acetate in H_2O	10mM Ammonium Formate in H_2O	0.2% v/v Acetic Acid in H_2O
Mobile Phase B	Acetonitrile with 0.1% v/v formic acid	Acetonitrile with 0.1% v/v formic acid	0.1% v/v Formic Acid and 5mM Ammonium Acetate in 50-50 Acetonitrile-Methanol	10:90 v/v 10mM Ammonium Formate : Acetonitrile	Acetonitrile with 0.1% v/v formic acid
Initial %B	10	10	20	100	40
Final pH (A)	4.25	4.25	n/a	4.30	10
Flow Rate (ml/min)	0.200	0.200	0.250	0.350	0.500

Temperature (^{o}C)	40	40	40	40	80
Gradient	40-100% over 6.5 min Hold 100% for 2 min	40-100% over 6.5 min Hold 100% for 2 min	20-95% over 1 min Hold 95% for 4.5 min	65-10% over 3 min*	40-100% over 3 min Hold 100% for 2 min

Table 2: Physical and chemical properties of the Hamilton and Kinetex columns.
*The gradient as shown indicates a decreasing %B over time.

2.3 Statistical Analysis

One-way ANOVA, with a set p-value < 0.05, was performed on resolution and theoretical plate data to determine if statistical differences between the performance parameter means of the columns were due to random chance or not. Buprenorphine, morphine, oxycodone, and tramadol underwent statistical analysis using GraphPad Prism (version 5.03) software. These four compounds were chosen for analysis because they span the full retention time of the chromatographic runs (early, middle, and late elution). In addition, a post-run Bonferroni Multiple Comparison Test further analyzed the statistical differences between each set of columns in the study by comparing the columns in pairs. The resulting p-values from the Bonferroni analysis coupled with the performance parameter of interest, theoretical plates and resolution, were used to create graphs to better visualize column performance.

3. Results and Discussion

3.1 Identification of Peaks

The chromatographic peaks resulting from the separation analysis of the 13 opioids underwent peak identification by mass spectroscopy. Each peak was matched and labeled to the corresponding drug component's molecular weight with aid from the parent molecular ion ($[M+H]^+$) present under the peak. However, two sets of structural isomers existed within the opioid mixture – codeine/hydrocodone and morphine/hydromorphone. In order to differentiate these isomers, fragmentation patterns were compared to a reference produced by Imma Ferrer and E. Michael Thurman [15]. The fragmentation patterns used for identification are as follows:

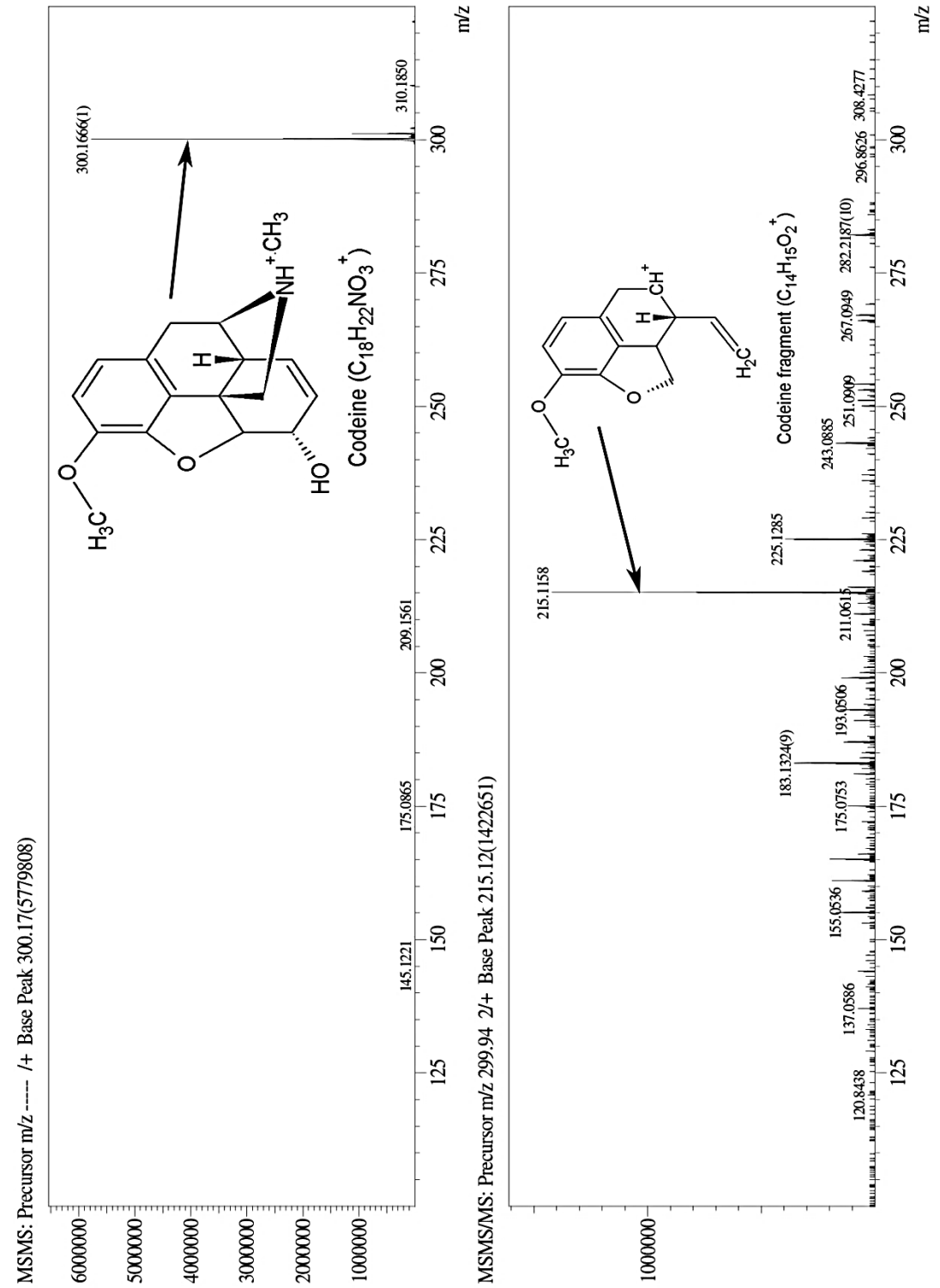


Figure 1: Codeine fragmentation pattern. The ion fragment used for identification had a m/z of 215.1158.

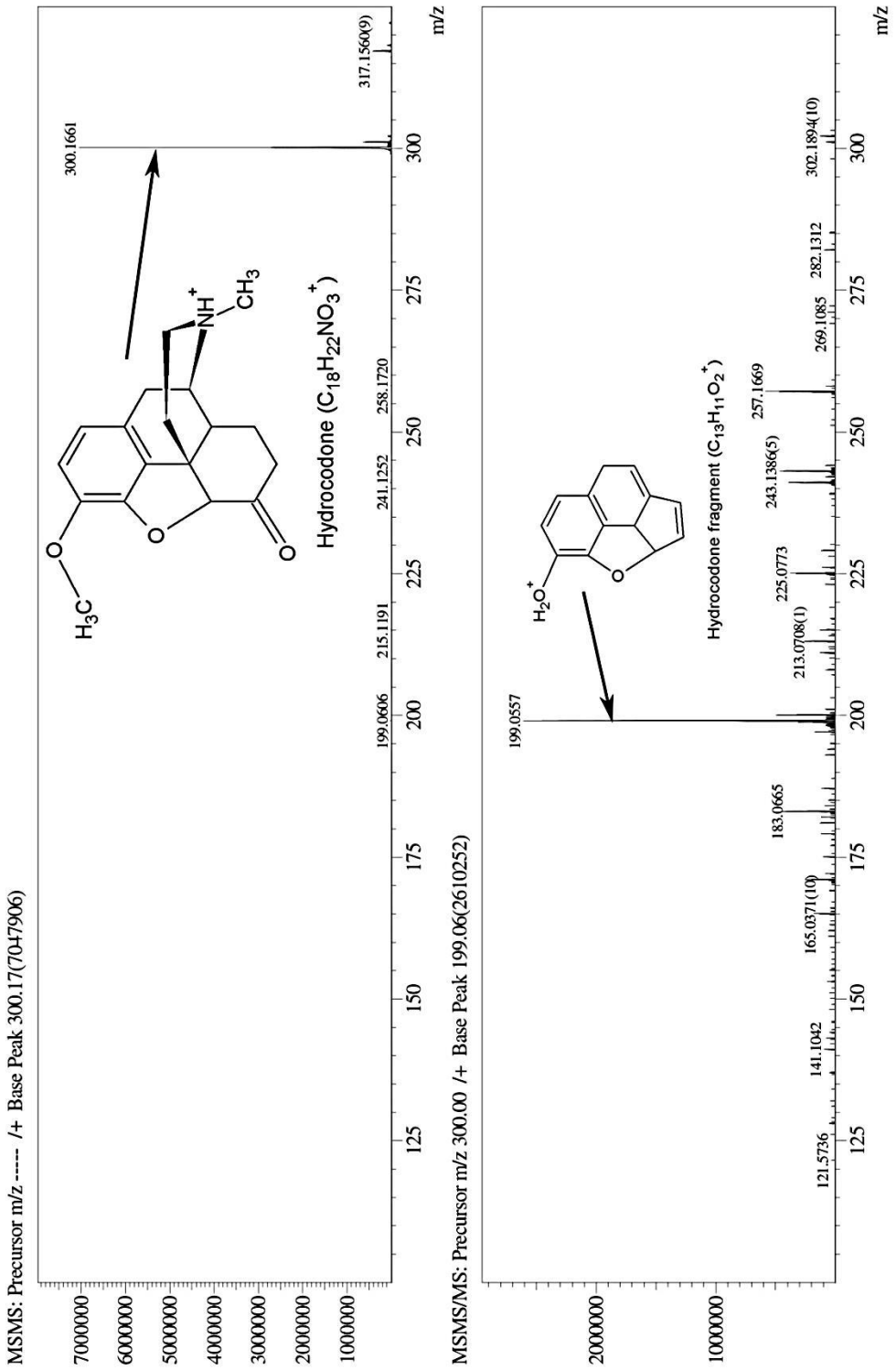


Figure 2: Hydrocodone fragmentation pattern. The ion fragment used for identification had a m/z of 199.0557.

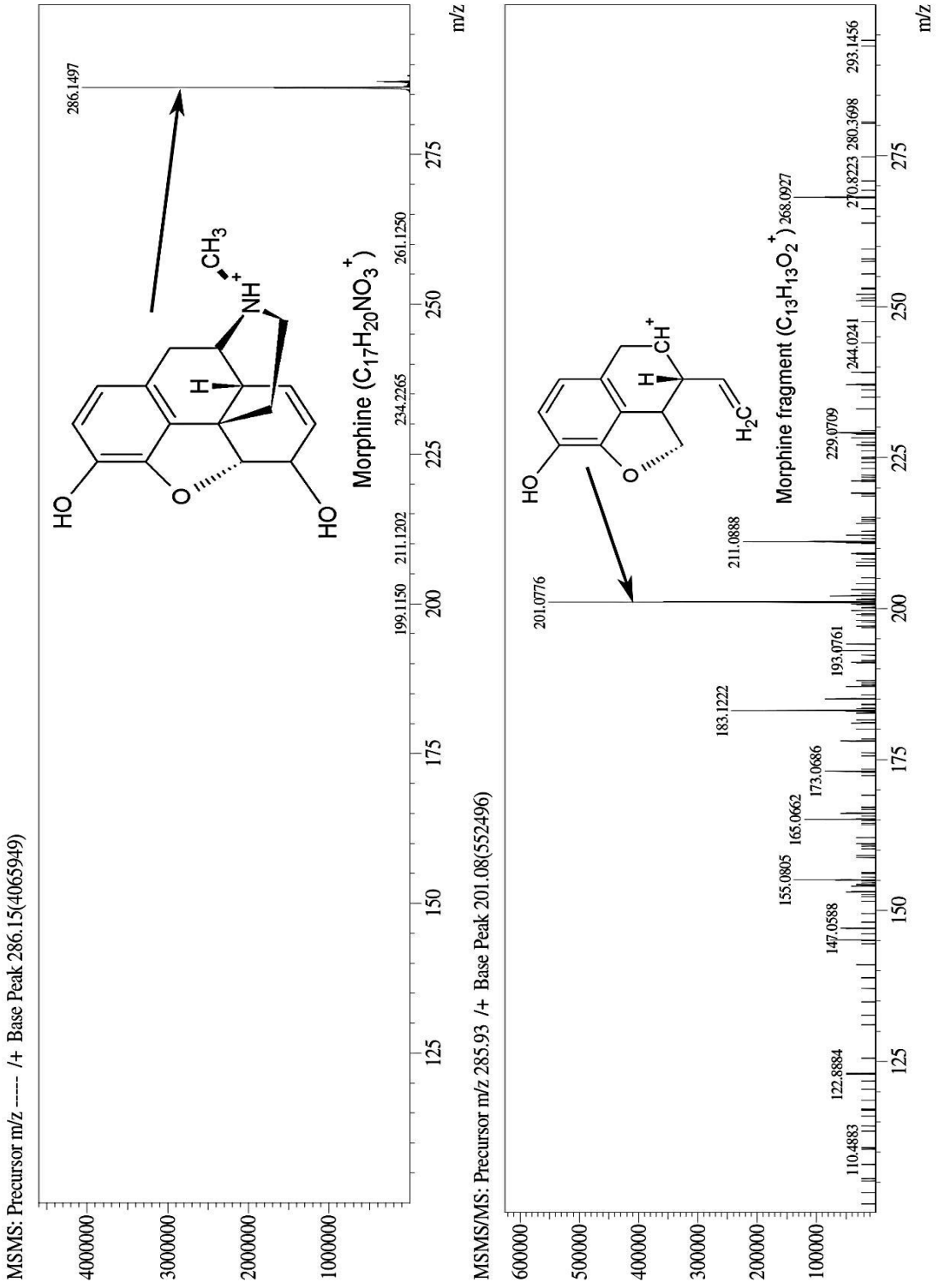


Figure 3: Morphine fragmentation pattern. The ion fragment used for identification had a m/z of 268.0927.

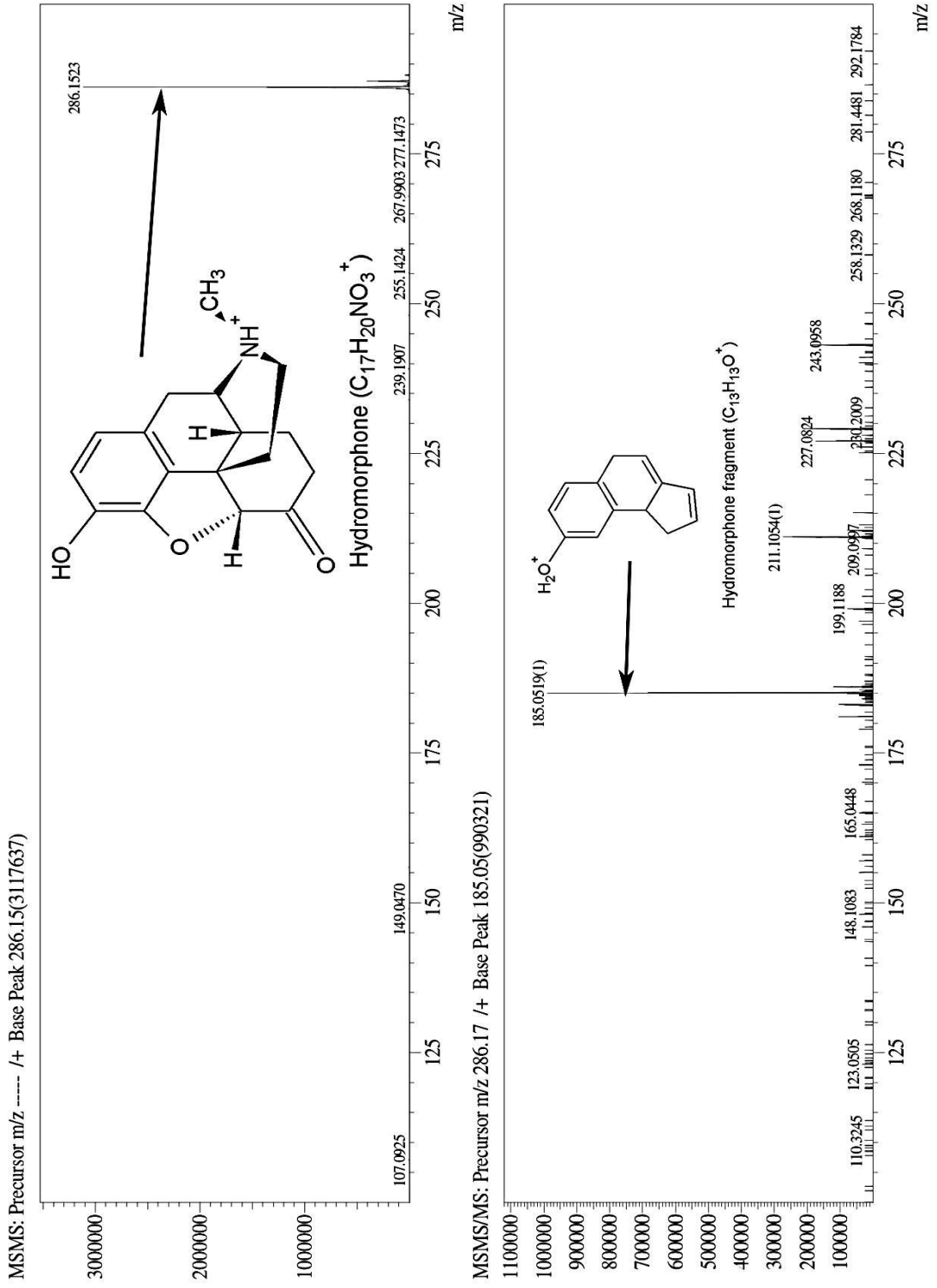


Figure 4: Hydromorphone fragmentation pattern. The ion fragment used for identification had a m/z of 185.0519.

As noted between Figures 1 and 2, the fragment ion of codeine with a m/z ratio of 215.1158 distinguishes this isomer from the 199.0557 m/z ratio of hydrocodone's fragment ion. Furthermore, Figures 3 and 4 provide the same differentiating information with the fragment ion of morphine at a m/z ratio of 201.0776 and hydromorphone at a m/z ratio of 185.0519. After identification, the software was used to automatically label these peaks after each injection.

3.2 Representative Column Chromatograms

The traditional C18 particle-packed silica column produced by Hamilton provided a baseline chromatogram for the separation of the opioids using fully porous beads. In Figure 5 below, a representative chromatogram shows the retention order as well as critical bands.

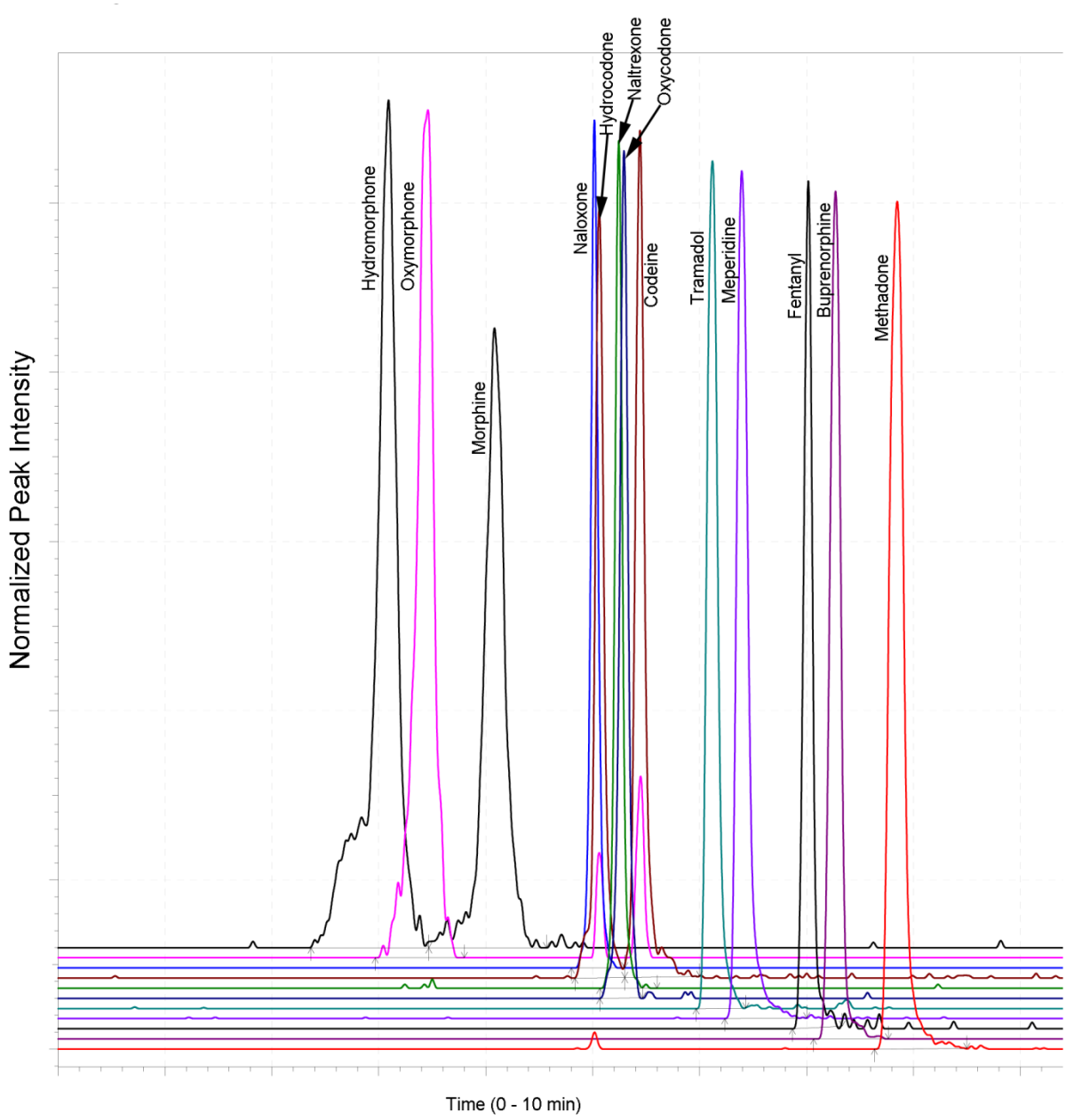


Figure 5: Hamilton C18 chromatogram. The retention order was fairly consistent with RP hydrophobic expectations. Critical bands existed for the naloxone/hydrocodone and naltrexone/oxycodone pairs.

The advanced monolithic fused core-shell technology offered by the Kinetex C18 distinguished this stationary phase from the traditional Hamilton C18 column. In Figure 6 below, a representative chromatogram shows the retention order as well as a few critical bands:

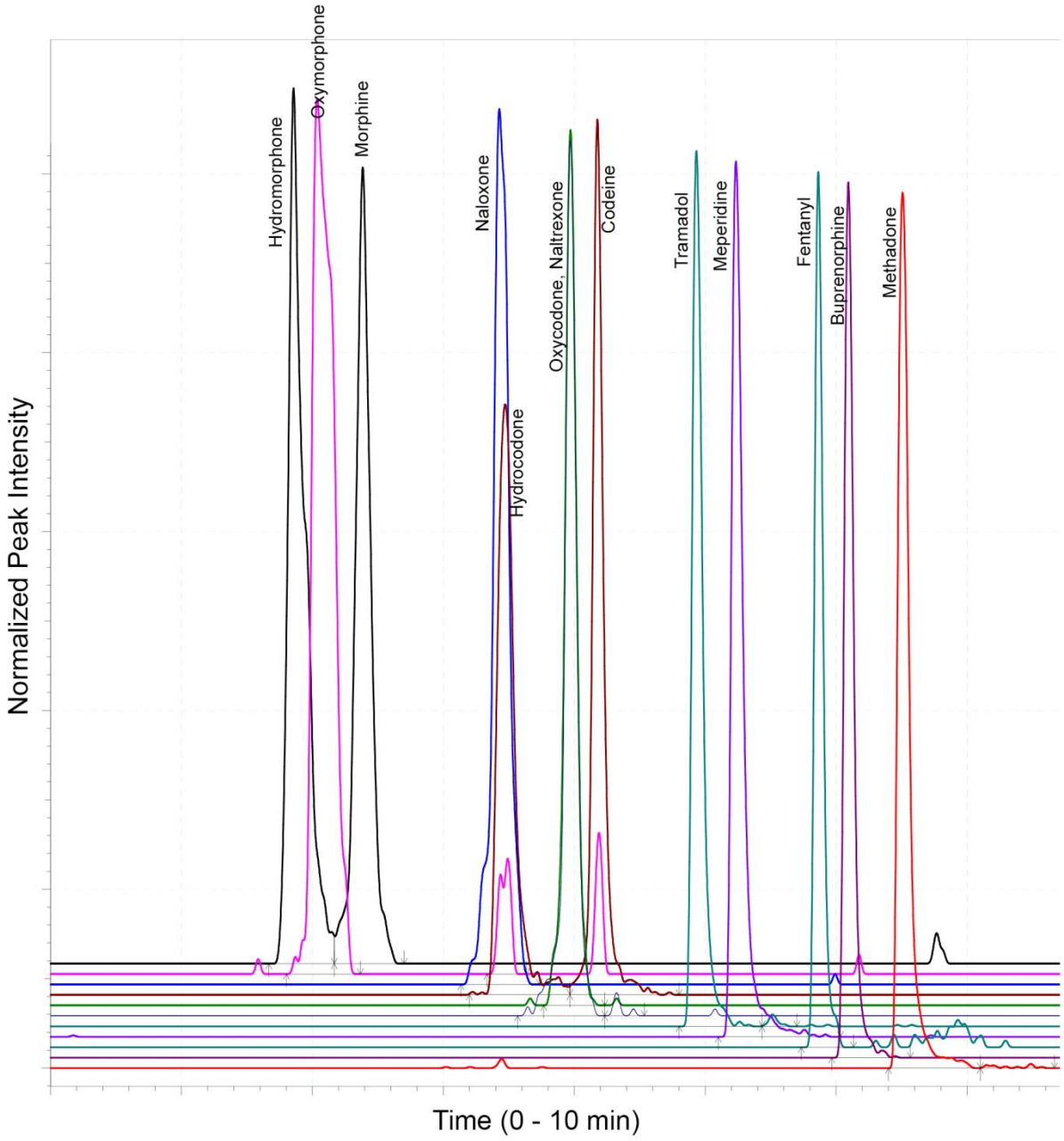


Figure 6: Kinetex C18 chromatogram. The retention order was fairly consistent with RP hydrophobic expectations. The core-shell technology allowed for better resolution between the same critical band pairs associated with the Hamilton C18.

The monolithic core-shell PFP stationary phase offered by Kinetex represented a revolutionary alternative to the traditional C18 column interactions. In Figure 7 below, an illustrative chromatogram shows the retention order as well as critical bands:

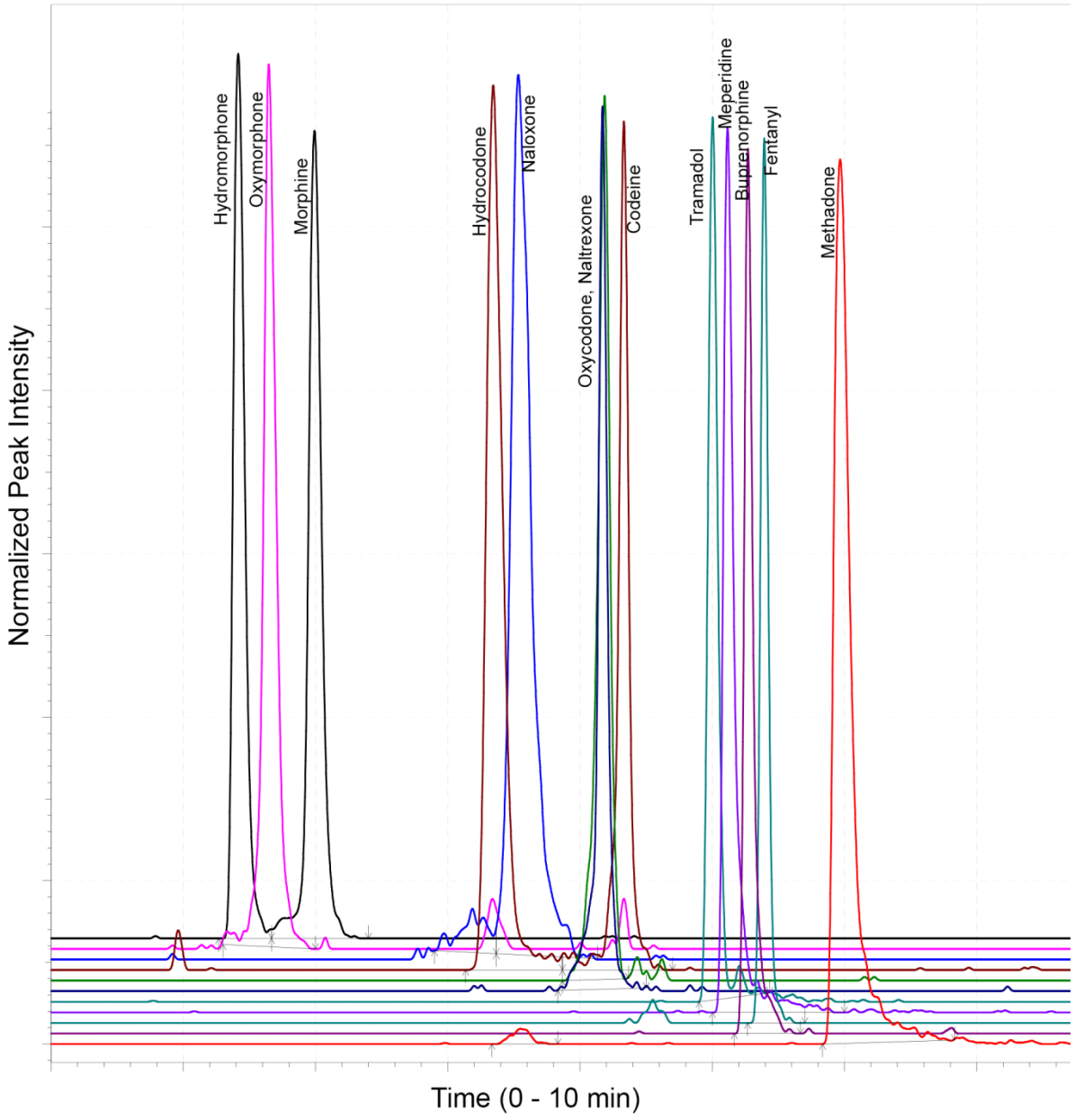


Figure 7: Kinetex PFP chromatogram. The retention order was fairly consistent with RP hydrophobic expectations. A critical band existed in the overlap of oxycodone/naltrexone peaks.

The Kinetex HILIC technology employs a NPC variant to focus retention on hydrophilic compounds. In Figure 8 below, a representative chromatogram shows the retention order and a few critical bands:

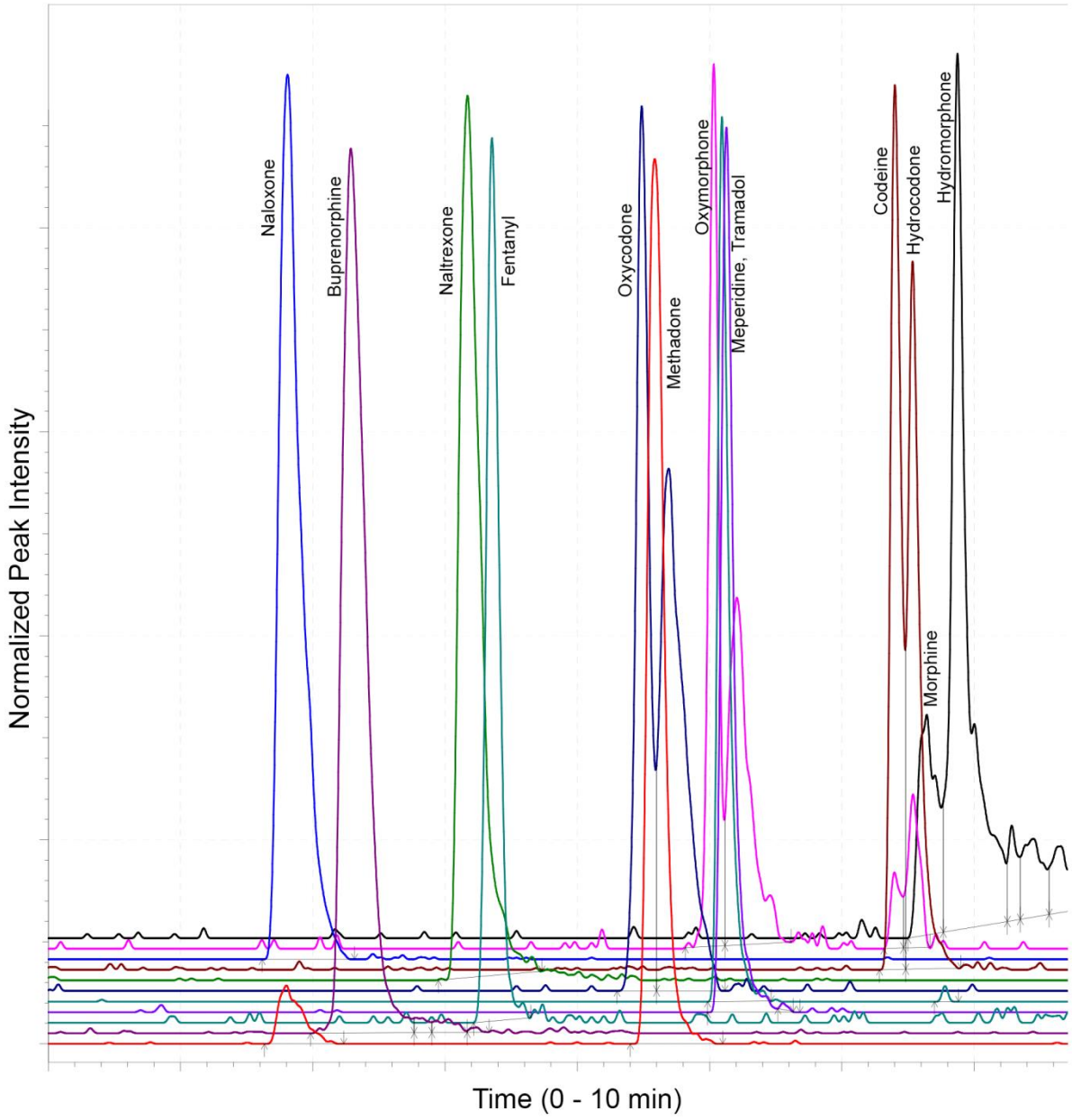


Figure 8: Kinetex HILIC chromatogram. The retention order was essentially reversed due to normal phase conditions – a few anomalies existed. A co-eluting meperidine/tramadol band was also present.

The Hamilton PRP column utilizes porous polymer technology that prefers the use of extreme conditions. In Figure 9 below, a representative chromatogram shows the retention order of the compounds under study:

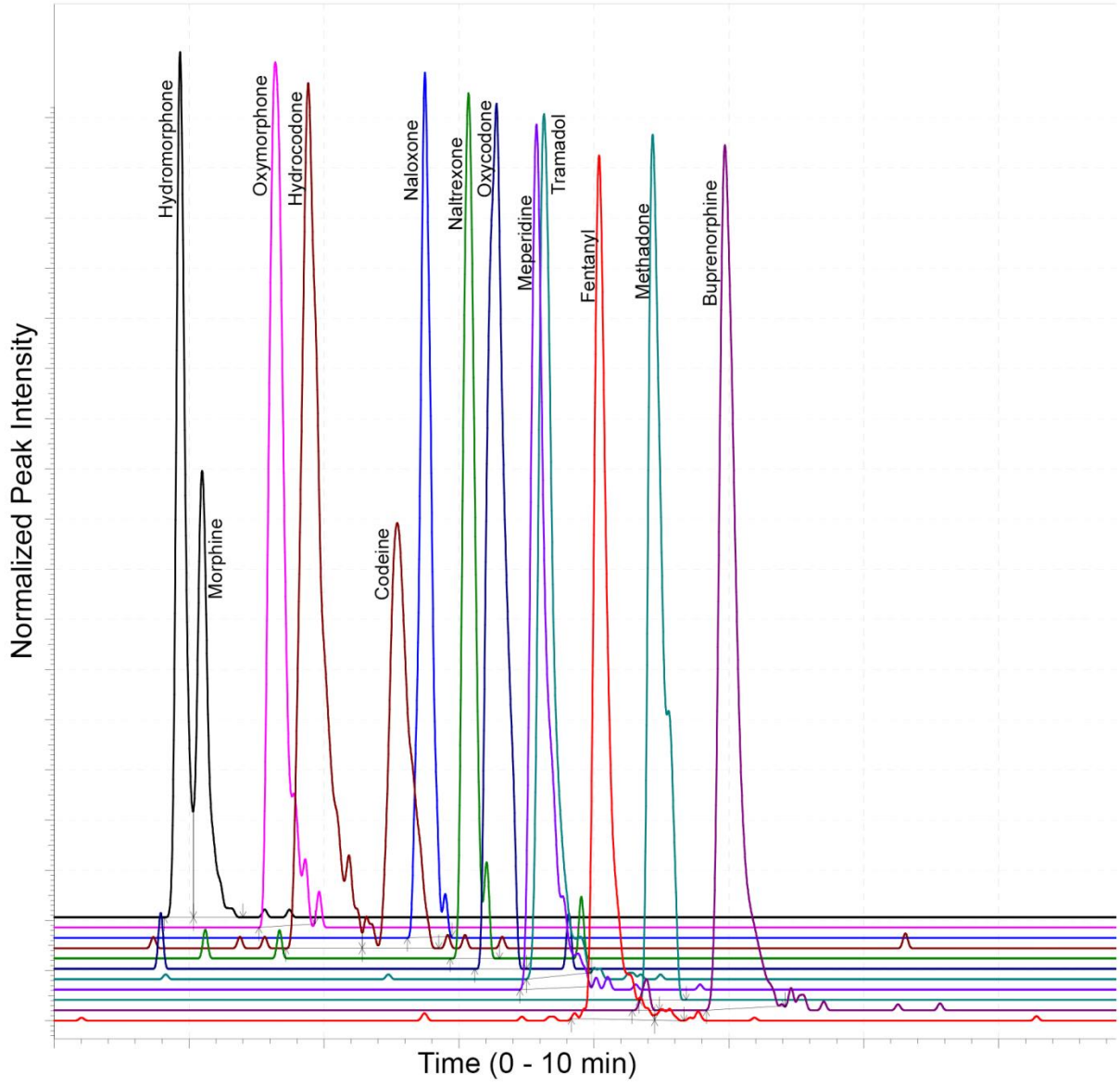


Figure 9: Hamilton PRP chromatogram. The retention order was fairly consistent with RP hydrophobic expectations. A critical band existed at the overlap of meperidine/tramadol peaks.

From these chromatograms, the retention order observed on the C-18, PFP, and PRP columns was fairly consistent with RP hydrophobic expectations for columns exhibiting this behavior. For example, compounds such as methadone and fentanyl, with higher partition coefficients, tended to retain in the column for longer periods of time. On the other hand, the Kinetex HILIC column produced a retention order essentially reversed due to the NPC conditions. For example, compounds exhibiting a low partition coefficient such as the log P = 0.87 of morphine tended to retain longer in the column than compounds such as buprenorphine with a partition coefficient of log P = 2.83. However, the results were not entirely consistent with hydrophilic interaction expectation due to some nonpolar compounds such as hydrocodone (log P = 2.57) retaining in the column for the longest time. Nevertheless, there is not a current mechanism to explain these complex interactions.

In addition, critical bands were present in each of the column chromatograms. With this in mind, the two C18 stationary phases represented in Figures 5 and 6 show overlapping naloxone/hydrocodone and naltrexone/oxycodone peaks; however, the core-shell technology of the Kinetex C18 provided better resolution between these critical bands. Additionally, the Kinetex C18 better separated the two sets of the critical bands from the closely-eluting codeine peak compared to the Hamilton C18. Comparatively, the Kinetex PFP (Figure 7) and Kinetex HILIC (Figure 8) each had one critical band with overlapping peaks of oxycodone/naltrexone and co-eluting meperidine/tramadol, respectively. Furthermore, the Kinetex HILIC was the only column not able to fully resolve the structural isomers. With the exception of the Hamilton PRP in Figure 9, the remaining RP columns each had complications resolving the oxycodone/naltrexone critical band. In continuation, the porous polymer technology must have interacted further with the perimeter methyl groups of oxycodone to resolve this problem area.

However, the Hamilton PRP column still had one critical band present, containing the meperidine/tramadol peaks. In the case of each column, mobile phase conditions were optimized to minimize the number of critical band pairs.

Furthermore, peak shape differences were evident between the column chromatograms. The two C18 stationary phases and the Hamilton PRP each formed chromatograms with better resolution for later-eluting compounds. However, the Hamilton C18 produced noteworthy peak fronting for early eluting compounds which was improved by the Kinetex C18 2.6 µm particle size. Despite the higher resolving power and peak shape (lack of peak fronting/tailing) associated with smaller particle size, the chromatograms indicate that the analyte – stationary phase interactions in a C18 column may not be sufficient for all the drugs in this class. Nevertheless, the Hamilton PRP also experienced peak shape issues as slight tailing was observed for the early-eluting structural isomers as shown in Figure 9. In comparison, the Kinetex HILIC and Kinetex PFP produced chromatograms with better peak shape for early eluting compounds. Both columns offered unique interactions such as the π-π interactions of the PFP column, but the columns generated peak tailing with the Kinetex HILIC being most profound with respect to the final eluting structural isomers as shown in Figure 8.

3.3 Column Reproducibility

Tables 3 and 4 below contain data to compare the columns in terms of retention time and peak area reproducibility. A lower percent relative standard deviation (RSD%) corresponds to a more reproducible column for the variable calculated.

	Column Type				
Compound	Hamilton C18	Kinetex C18	Kinetex PFP	Kinetex HILIC	Hamilton PRP
Morphine	4.098 (1.50%)	2.402 (4.40%)	2.006 (0.23%)	6.888 (1.89%)	1.103 (0.24%)
Oxymorphone	3.463 (1.48%)	2.136 (4.80%)	1.666 (0.21%)	4.840 (5.42%)	1.650 (0.49%)
Codeine	5.453 (0.08%)	4.178 (0.53%)	4.340 (0.10%)	6.339 (1.31%)	2.542 (0.36%)
Oxycodone	5.303 (0.06%)	3.972 (1.03%)	4.183 (0.15%)	4.293 (6.07%)	3.268 (0.21%)
Naloxone	5.027 (0.10%)	3.440 (3.38%)	3.539 (0.42%)	1.690 (9.85%)	2.749 (0.21%)
Naltrexone	5.254 (0.09%)	3.970 (1.00%)	4.195 (0.08%)	2.991 (7.71%)	3.079 (0.15%)
Hydromorphone	3.102 (1.31%)	1.902 (3.88%)	1.431 (0.20%)	6.620 (1.04%)	0.940 (0.23%)
Meperidine	6.409 (0.05%)	5.236 (0.14%)	5.132 (0.10%)	4.990 (2.94%)	3.578 (0.11%)
Tramadol	6.129 (0.05%)	4.933 (0.14%)	5.014 (0.12%)	5.037 (2.53%)	3.639 (0.08%)
Hydrocodone	5.071 (0.08%)	3.480 (3.17%)	3.347 (0.38%)	6.501 (0.73%)	1.890 (0.51%)

Buprenorphine	7.283 (0.05%)	6.097 (0.11%)	5.285 (0.14%)	2.131 (10.70%)	4.984 (0.09%)
Fentanyl	7.027 (0.04%)	5.866 (0.10%)	5.404 (0.12%)	3.180 (7.48%)	4.449 (0.11%)
Methadone	7.856 (0.05%)	6.520 (0.10%)	5.979 (0.16%)	4.427 (4.82%)	4.045 (0.08%)

Table 3: Retention times (minutes) with variations represented by % Relative Standard Deviation (%RSD) in parenthesis for each compound and each column. The compounds are listed in order of increasing log P. Shaded compounds reflect those used for additional column performance analysis.

	Column Type				
Compound	Hamilton C18	Kinetex C18	Kinetex PFP	Kinetex HILIC	Hamilton PRP
Morphine	9961716.2 (5.88%)	8588159.7 (3.92%)	15778197.1 (2.58%)	14162301.7 (69.20%)	3516868.9 (25.26%)
Oxymorphone	4692246.4 (7.22%)	4401604.9 (6.70%)	11171337.4 (3.84%)	11391551.2 (73.87%)	2043429.4 (29.20%)
Codeine	16064593.3 (2.66%)	13462995.9 (3.93%)	16822753.5 (4.73%)	22507150.8 (37.24%)	3849347 (28.98%)
Oxycodone	6092190.8 (2.42%)	5250541.3 (4.17%)	7238709.3 (3.65%)	10583590.2 (26.01%)	1170435.7 (29.56%)

Naloxone	5237537.9 (3.04%)	4976167.6 (5.92%)	16297688.4 (3.74%)	39343356.7 (11.40%)	1295680.4 (30.64%)
Naltrexone	6881812.4 (3.39%)	5455296.9 (4.05%)	11142902.5 (3.97%)	37495836.8 (12.10%)	1897460.9 (29.62%)
Hydromorphone	13222320.8 (11.22%)	10202624.7 (5.22%)	15832224 (2.36%)	6269698.2 (131.09%)	5238957.4 (20.44%)
Meperidine	34035388.7 (2.55%)	26657964.2 (2.61%)	30045847.8 (3.19%)	18922786.7 (19.33%)	13223637.3 (21.92%)
Tramadol	27669472.8 (2.04%)	20959759.3 (2.88%)	25423393.9 (2.98%)	19137140.3 (17.38%)	11575245.2 (23.18%)
Hydrocodone	13769322.6 (2.47%)	13635053.5 (3.93%)	25440049.8 (2.53%)	17550909.9 (20.07%)	7613801.8 (24.28%)
Buprenorphine	23629742.9 (4.52%)	20020996.3 (2.96%)	12489041.7 (7.78%)	51337385.4 (4.23%)	13333380.5 (24.35%)
Fentanyl	4878628.6 (4.96%)	4069698.2 (4.82%)	5667056.4 (3.26%)	6102219 (6.73%)	1174644.6 (29.68%)
Methadone	47317440.4 (2.82%)	31684613.9 (3.33%)	49734398.7 (2.89%)	36909196.6 (5.52%)	18620757.3 (24.44%)

Table 4: Peak areas with variations represented by % Relative Standard Deviation (%RSD) in parenthesis for each compound and each column. The compounds are listed in order of increasing log P. Shaded compounds reflect those used for additional column performance analysis.

From the collated data, the Hamilton C18 column had the largest number of low RSD% values for both retention time and peak area. However, the Kinetex PFP generated the lowest RSD% values for the two earliest eluting compounds – morphine (0.23%) and oxymorphone (0.21%). In addition, the PFP column notably reproduced compounds of average log P such as retention time of naltrexone (0.08%) and retention time/peak area of hydromorphone (0.20%/2.36%). Since these compounds share similar chemical structures, the Kinetex PFP must have interacted with these compounds more effectively. With this in mind, the maximum reproducibility with the exception of morphine, oxymorphone, and hydromorphone occurred with the Hamilton C18 column for this compilation of opioids. Furthermore, a trend existed concerning the C18 stationary phases and the value of log P. Besides the low RSD% peak area values for buprenorphine and fentanyl attributed to the Kinetex C18, the Hamilton C18 produced the lowest RSD percentages across the board for partition coefficients upwards of 2.19; however, the C18 columns dominated this region in terms of reproducibility. The consistency of the Hamilton C18 column can be attributed to the mechanical durability associated with totally porous silica particles. Alternatively, two separate columns, one concerning retention time and one for peak area, produced the highest RSD% values on average. Firstly, the Kinetex HILIC column consecutively had higher RSD percentages for retention time (up to 10.70%). This is not surprising as the interactions in this column cater towards a more hydrophilic class of drugs. Lastly, the Hamilton PRP column had similar elevated RSD% values for peak area (up to 30.64%). Due to this column technology's reliability on hydrophobic interactions, these values prove the lengthy alkyl chains of the C18 stationary phases are superior for reproducibility.

3.4 Column Performance

The column performance parameters, theoretical plates and resolution, were measured for each column using the representative drugs morphine, oxycodone, tramadol, and buprenorphine in order to statistically determine the "best" performing column over a wide range of polarities. For example, the graph displaying each column's average theoretical plates with morphine is shown below in Figure 10:

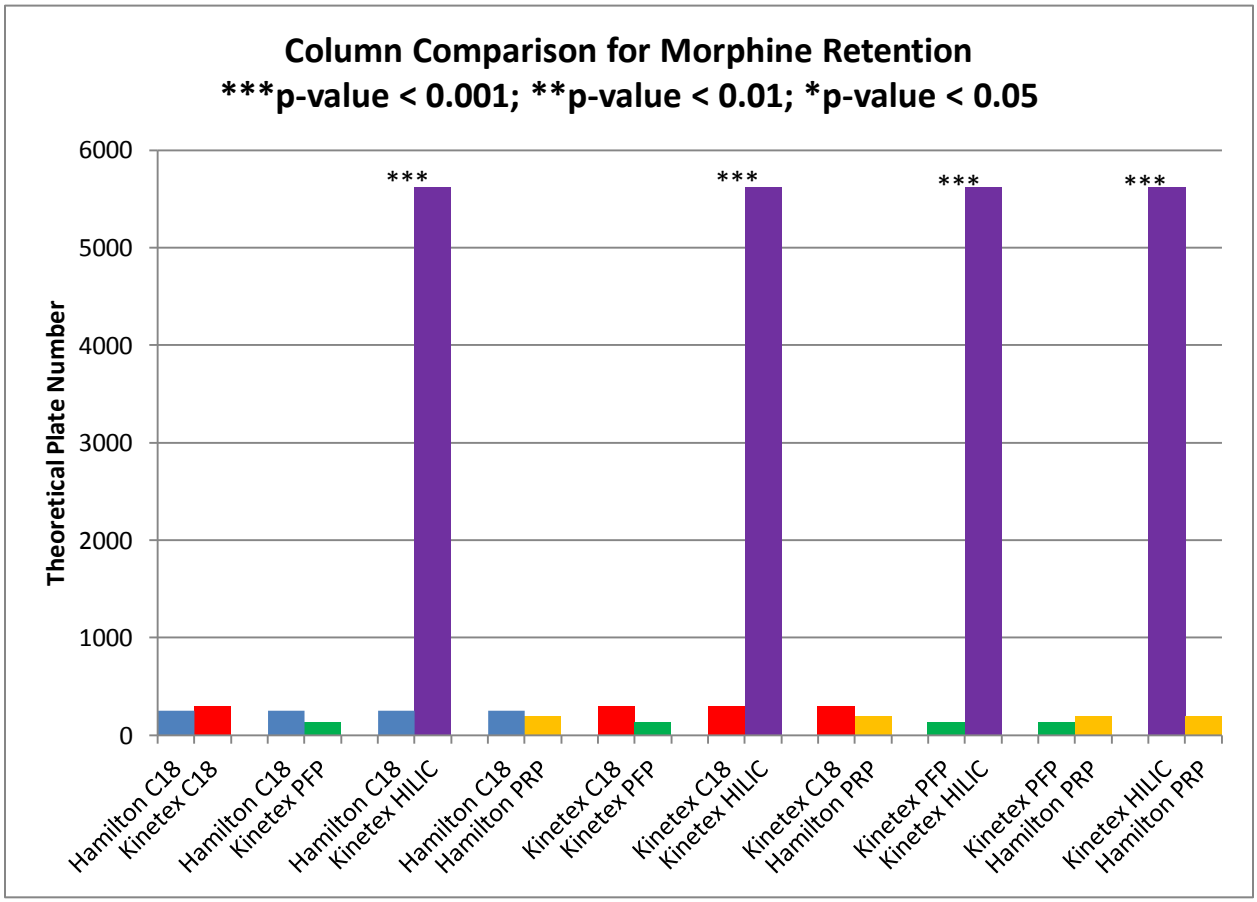


Figure 10: Column comparison of average theoretical plate number for morphine. The Kinetex HILIC column formed the highest number of theoretical plates on average for morphine.

As shown above, the Kinetex HILIC column forms the most theoretical plates (5614.92) on average when morphine is the drug under study. In addition, the p-value of 0.001 indicates the differences between the Kinetex HILIC and four other columns were significant and unlikely due to random sampling. This astounding performance can be attributed to the hydrophilic (Log P = 0.87) nature of morphine compared to the other opioids, as the Kinetex HILIC column performs best with polar molecules. Furthermore, a column comparison concerning average resolution with morphine was also executed as shown in Figure 11:

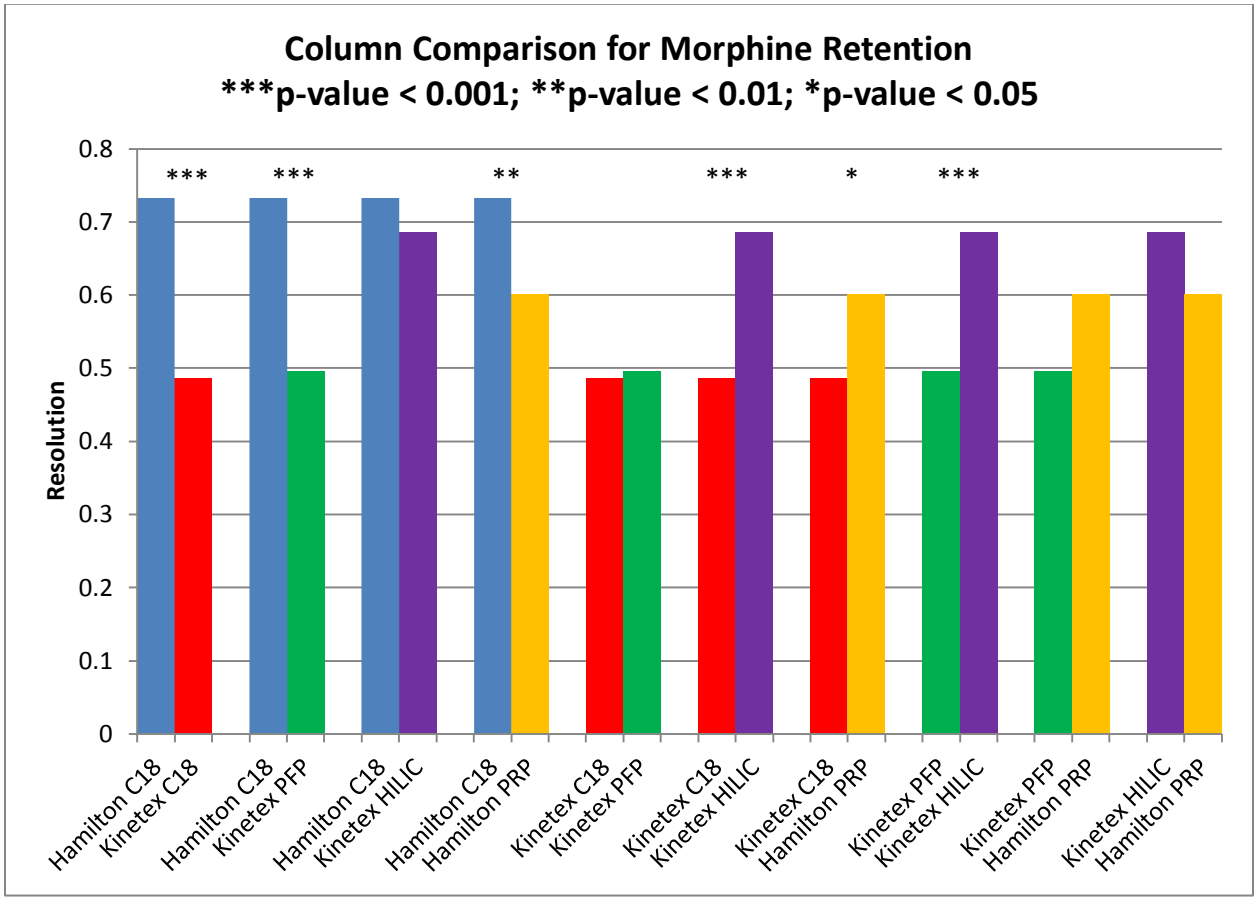


Figure 11: Column comparison of average resolution for morphine. The Hamilton C18 and Kinetex HILIC both produced notable resolutions for morphine.

In comparison to the theoretical plate data, the Kinetex HILIC column fell just short to the performance of the Hamilton C18 in terms of resolution (0.68556 versus 0.73252); however, the difference was not statistically significant. The Hamilton C18 outperformed the Kinetex C18 and PFP (p-value < 0.001), as well as the Hamilton PRP (p-value < 0.001) in terms of morphine resolution.

Next, the same performance parameters were calculated and compared across the columns with oxycodone. The column comparison graph showing the average number of theoretical plates is shown in Figure 12:

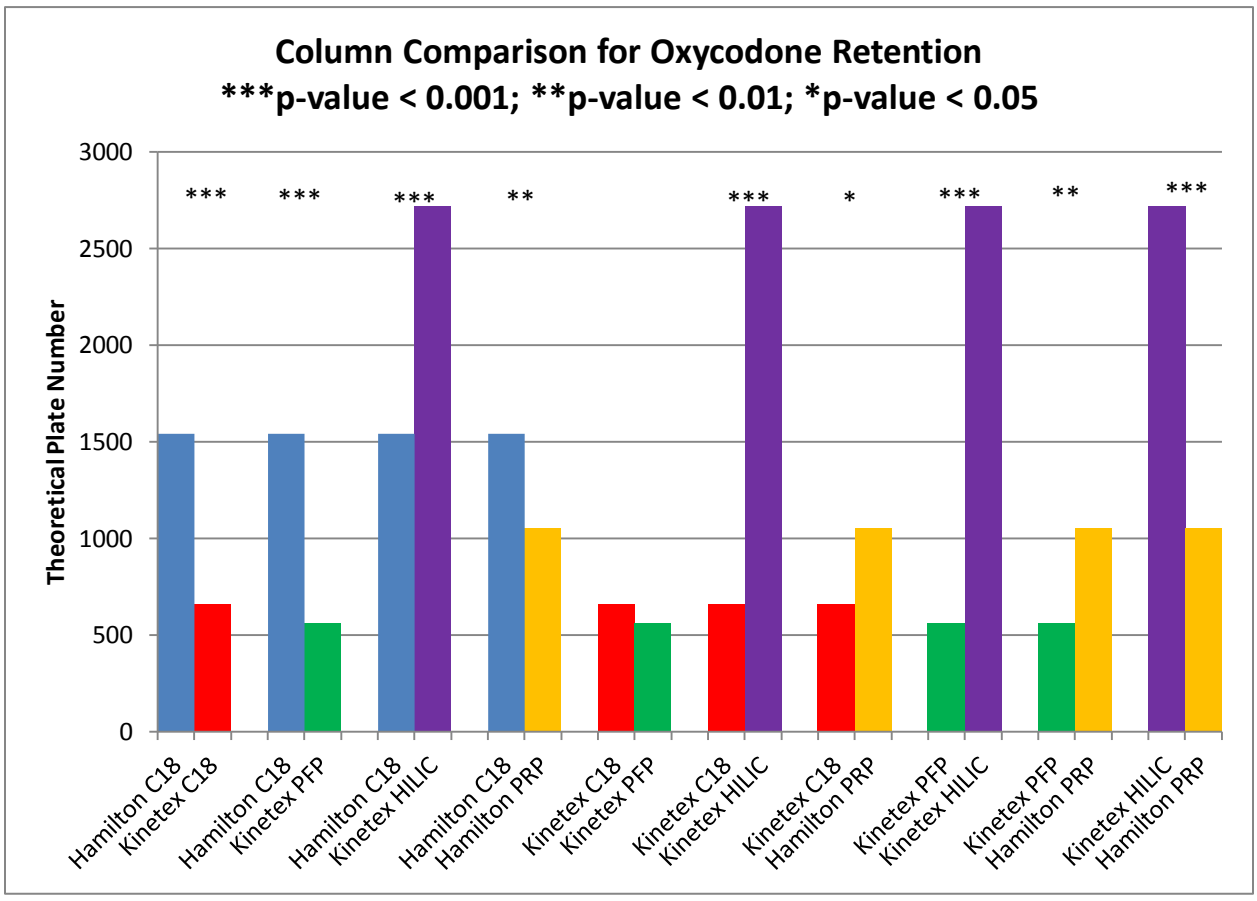


Figure 12: Column comparison of average theoretical plate number for oxycodone. The Kinetex HILIC formed the highest number of theoretical plates on average for oxycodone.

The Kinetex HILIC column proved to create the largest number of theoretical plates for oxycodone with the Hamilton C18 at a distant second (2716.71 versus 1542.05). These theoretical plates for oxycodone are statistically higher than the other four columns (p-value < 0.001). Like morphine, oxycodone is one of the more polar compounds under study (log P = 1.59), which would entail hydrophilic interactions with this column, resulting in a higher average plate number. Additionally, a column comparison for resolution with oxycodone was accomplished as shown in Figure 13:

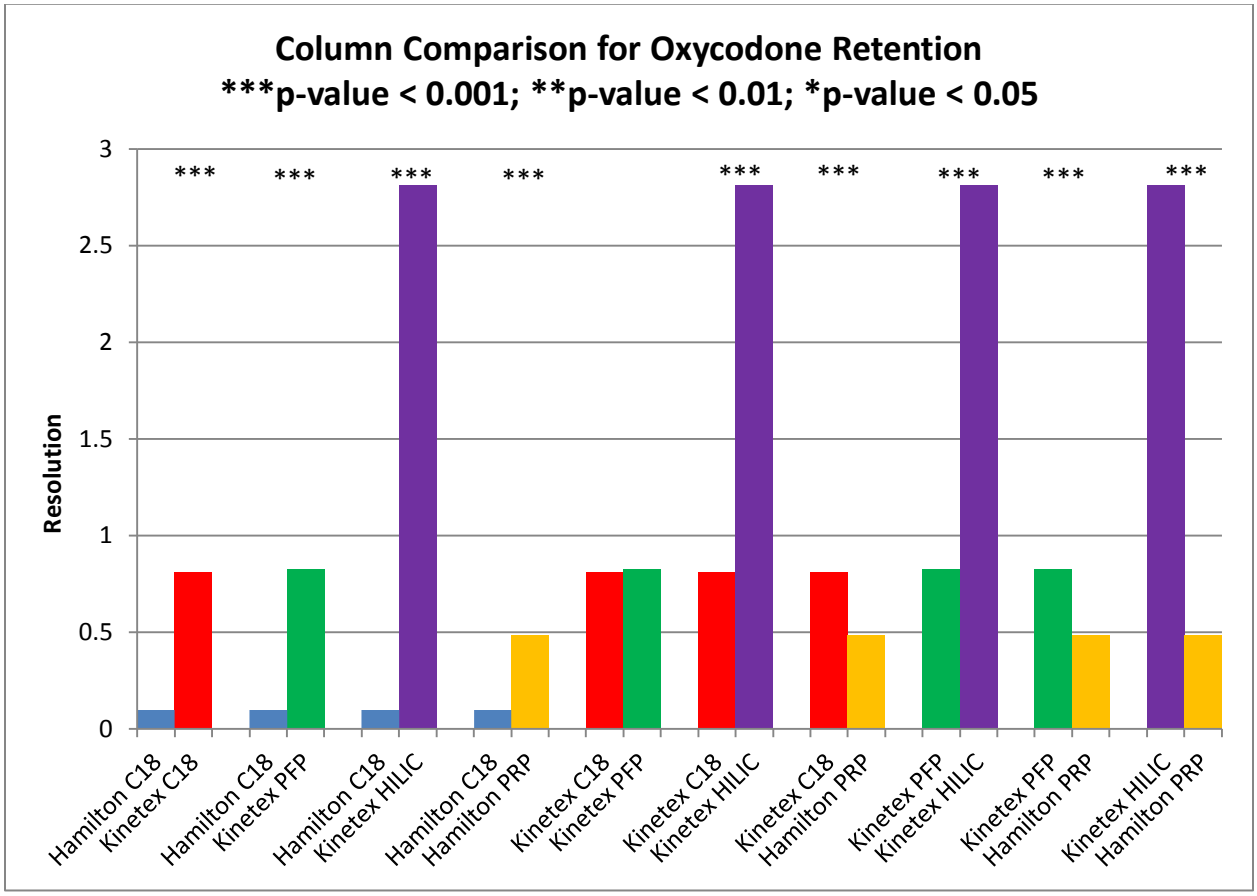


Figure 13: Column comparison of average resolution for oxycodone. The Kinetex HILIC had the highest resolution on average for oxycodone.

The Kinetex HILIC obtained resolutions consistently over 2.5 for oxycodone with the Kinetex C18 and PFP as the next closest not breaking a resolution value of 1. As a point of reference, baseline resolution is defined as a value of 1.5 [5]. The HILIC column significantly outperformed each column in oxycodone resolution as shown by the p-value of 0.001.

Thirdly, a column comparison was performed with tramadol consisting of the same variables -theoretical plates and resolution. In Figure 14, a column comparison graph illustrating the average theoretical plate number and ANOVA is shown:

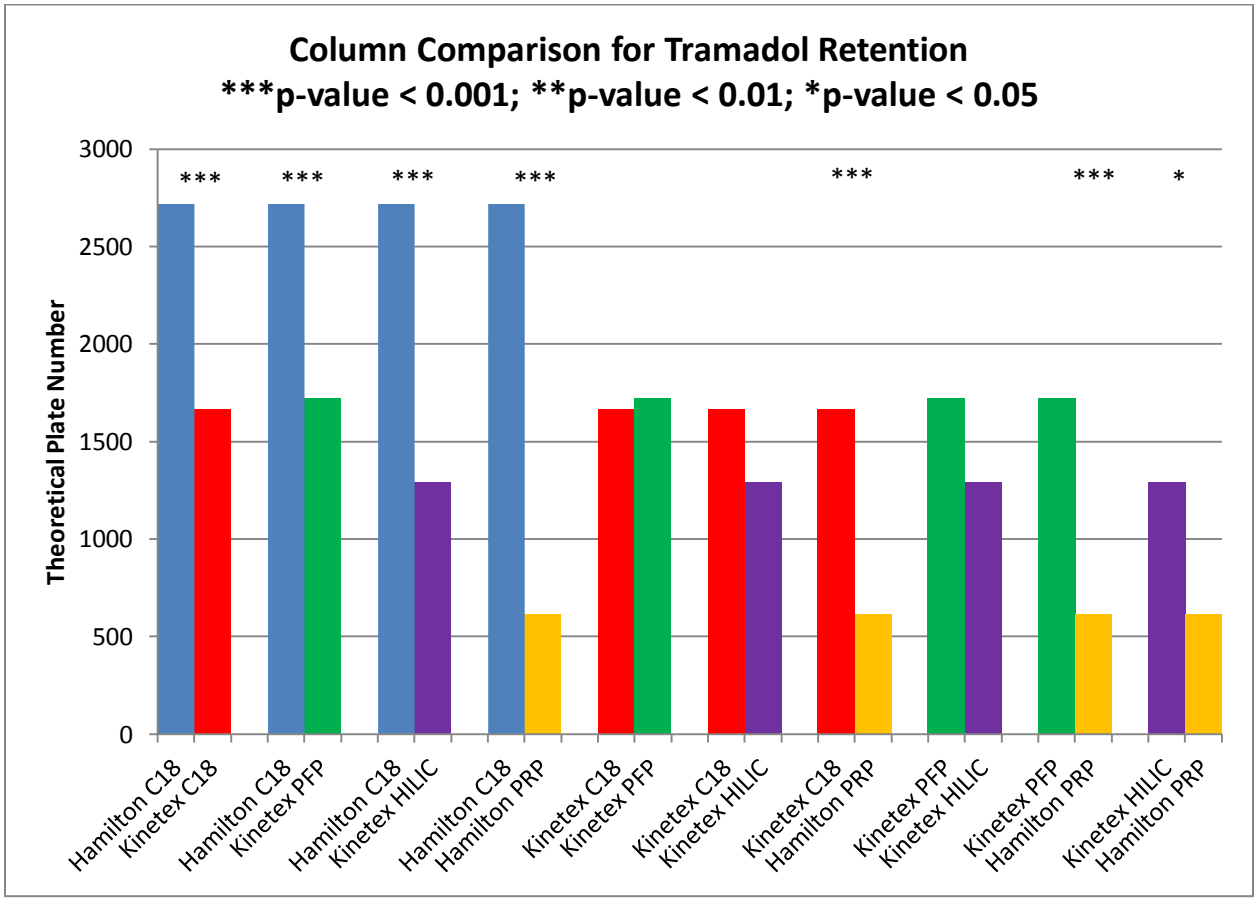


Figure 14: Column comparison of average theoretical plate number for tramadol. The Hamilton C18 formed the highest number of theoretical plates on average for tramadol.

The Hamilton C18 column significantly outperformed the remaining columns with regards to theoretical plates associated with the separation of tramadol (p-value < 0.001). The Kinetex C18 and PFP formed relatively half the average theoretical plates of the Hamilton C18 (~ 1700). With respect to tramadol's size, the 5 µm spherical particle size allowed better interaction with the stationary phase and hence beat the Kinetex C18 with the smaller 2.6 µm monolithic form. Also, a column comparison concerning resolution of the peaks with tramadol was carried out and is presented in Figure 15:

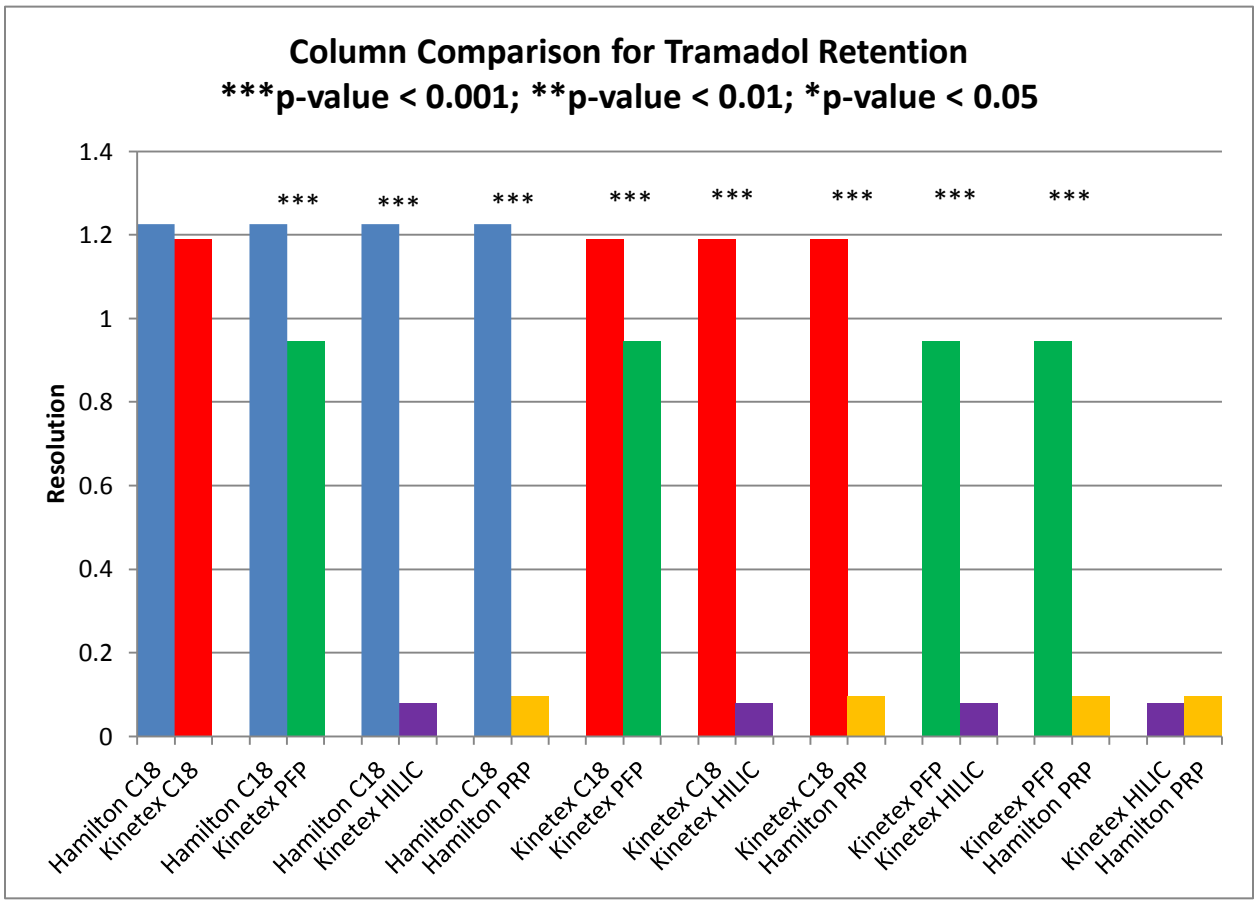


Figure 15: Column comparison graph of average resolution for tramadol. The Hamilton C18 and Kinetex C18 both had comparable resolutions for tramadol.

The Hamilton C18 provided the best average resolution for tramadol (1.22633); however, the Kinetex C18 demonstrated comparable resolutions at 1.18841. These data indicate that tramadol achieves higher resolutions when interacting with octadecyl stationary phases, although no statistically significant difference could be found between the porous shell phase and the traditional silica phase.

Finally, buprenorphine was the last drug used for the column comparison of performance parameters theoretical plates and resolution. In Figure 16, a column comparison graph of the average theoretical plate number with buprenorphine is illustrated:

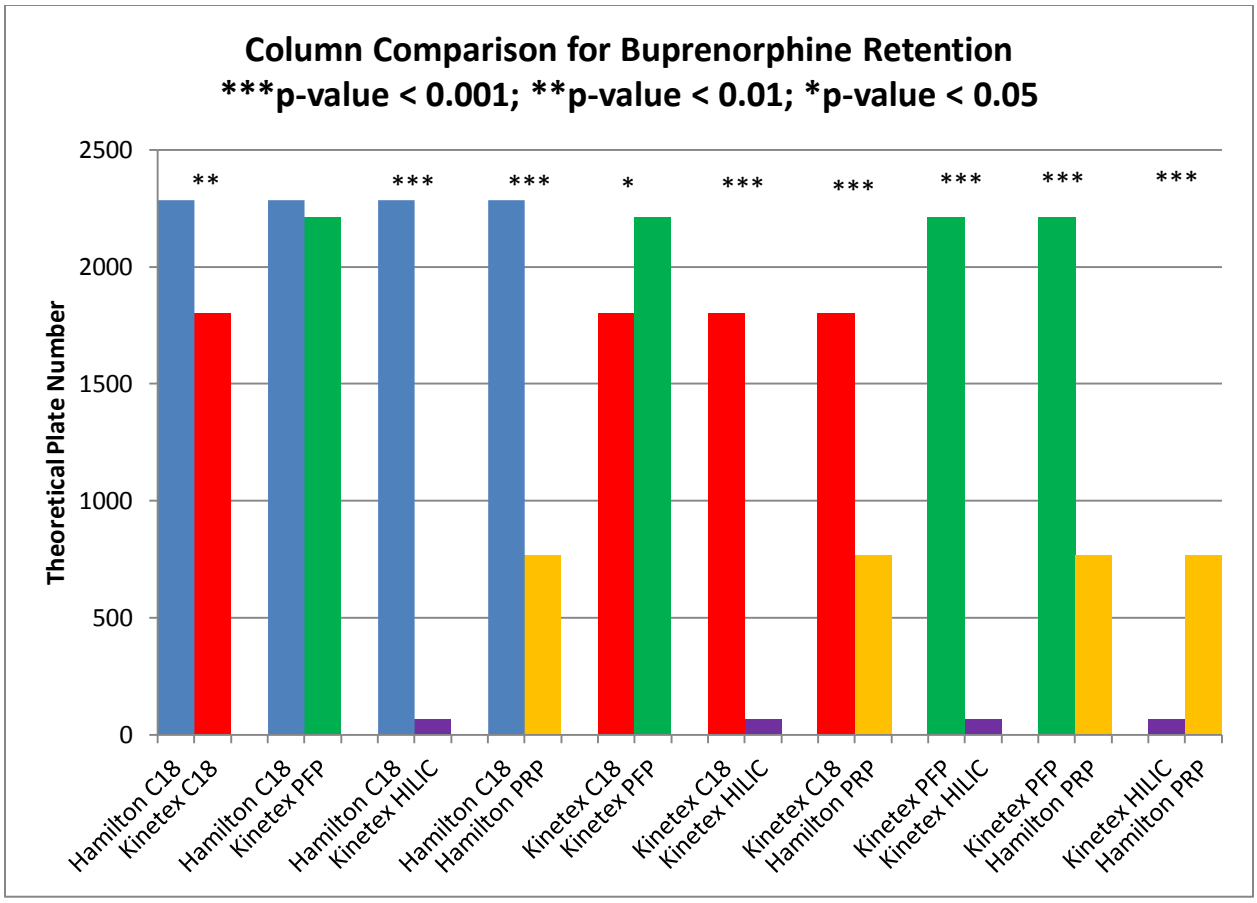


Figure 16: Column comparison of average theoretical plate number for buprenorphine. The Hamilton C18 and Kinetex PFP both formed a high number of theoretical plates for buprenorphine.

The Hamilton C18 and Kinetex PFP significantly outperformed the remaining columns when buprenorphine is the drug under study with p-values ranging from < 0.001 to < 0.05. The variation between Hamilton C18 and Kinetex PFP for buprenorphine theoretical plates was not significant (2283.91 versus 2214.27) . In terms of comparison, the 150 mm length of the Hamilton C18 compared to the 100 mm PFP must have been the deciding factor for the slightly increased resolution of the C18 column. In addition, a column comparison concerning resolution of the peaks with buprenorphine was carried out and is presented in Figure 17:

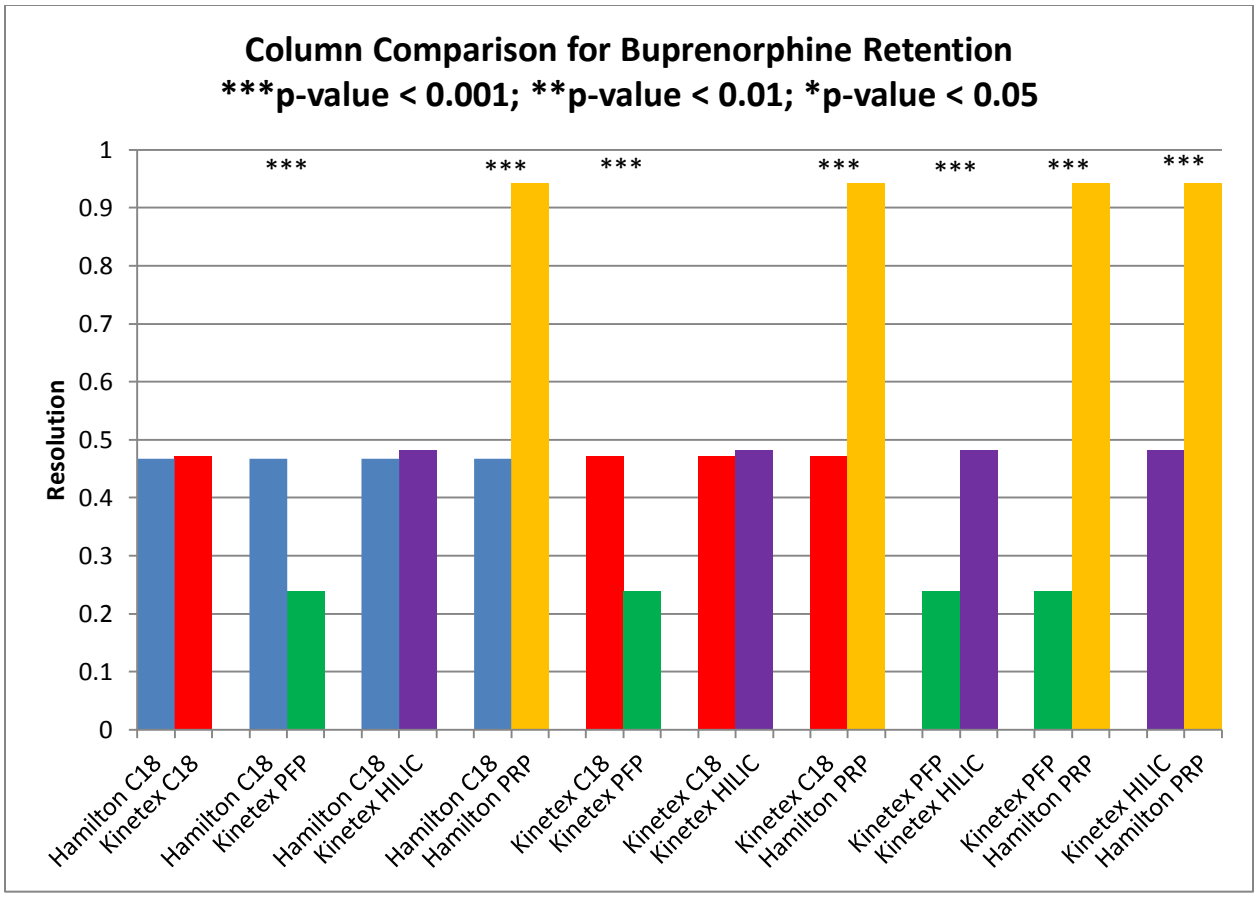


Figure 17: Column comparison of average resolution for buprenorphine. The Hamilton PRP had the highest average resolution for buprenorphine.

The Hamilton PRP had significantly better resolved peaks with buprenorphine than the remaining columns as shown with the consistent p-value < 0.001. With buprenorphine as one of the more nonpolar compounds, the PRP column achieves great resolution (0.9426) with highly basic, nonionized compounds with hydrophobic character. Since buprenorphine fits these qualifications at the pH suited to the PRP mobile phase, it is no surprise the PRP column performs better under these conditions.

3.5 Concluding Remarks

In terms of overall performance, the Kinetex HILIC column generated excellent theoretical plate and resolution data for hydrophilic compounds such as morphine and oxycodone. However, the less than average reproducibility of this column could lead to unreliable data if the method was used for quantitative purposes; nonetheless, the most suitable compounds for this column are of hydrophilic nature. For more nonpolar opioids, such as tramadol and buprenorphine, the Hamilton C18 traditional silica column consistently achieves high theoretical plate numbers and resolution. Interestingly, both the Hamilton C18 and the Kinetex C18 were operated under the same mobile phase conditions, and yet the traditional column scaffolding with larger particle size typically outperformed its competitor, although the difference in performance was not always statistically significant. Besides falling short to the Hamilton PRP in buprenorphine resolution, the Hamilton C18 would make a great choice for studying more nonpolar opioids of varying sizes even more so than the Kinetex C18, especially given their price difference ($433 for the Hamilton column versus $708 for the Kinetex column). Furthermore, this column had consistent reproducibility for retention time and peak area as well as strong mechanical durability across the board, which proved to be advantageous.

On another note, the Hamilton PRP column produced the best chromatogram concerning peak shapes and critical bands as compared to the other four columns and would better suit qualitative studies with multiple opioids. Also, the majority of the compounds eluted faster from this column, thus showing potential for higher throughput. Lastly, the Kinetex PFP column generated average data for all performance and reproducibility characteristics in this study, indicating that it would be a viable alternative to the C18 chemistries, especially considering that it had fewer co-eluting peaks than either C18 column. However, the Hamilton PRP column costs

$457 while the Kinetex PFP column costs $708, likely due to the added complexity of the stationary phase scaffolding. Overall, the Hamilton C18 and Kinetex HILIC would best suit quantitative studies for nonpolar and polar opioids, respectively.

4. References

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