Detection of Cotinine in Blood Plasma by HPLC MS/MS

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Abstract

Tobacco smoking is a major killer in the United States and is attributed to approximately 434,000 deaths per year. Primary and secondary exposure to tobacco and tobacco smoke can be monitored by measuring cotinine levels in blood, urine, as well as other matrices. This article describes a HPLC MS/MS assay to detect low concentration levels of cotinine in blood plasma. The assay was developed at Children's Hospital, Boston, and thus it was specifically designed for use with young children. This assay allows for high throughput and turnaround because it does not use a column-based purification process; it is also fairly inexpensive, using common laboratory reagents. Upon completion of the study, the concentration ranges were found to be accurate from 0.l to10.0 ng/mL. The limit of quantitation was calculated to be 0.2 ng/mL (CV% < 20%, accuracy range ± 20%). The HPLC MS/MS assay is now ready for comparison tests with the ELISA test using patient plasma samples.

Introduction

About one in five U.S. deaths are a result of tobacco usage.[1] It is one of the main causes of premature death in the country. Tobacco use, mainly in the form of cigarette smoking, has been shown to increase the chances of various types of mouth and lung cancers, cardiovascular disease, and emphysema.

The harmful effects of tobacco can be seen in both active and passive smokers. Active smokers are those who smoke tobacco and tobacco products, while passive smokers are those exposed to tobacco smoke. Exposure of nonsmokers includes second-hand smoke from the air, as well as fetus exposure due to maternal smoking. Passive tobacco exposure is a major health problem in the United States, and is classified as a Group A carcinogen by the Environmental Protection Agency. Due to the magnitude of this health problem, analysis of passive tobacco smoke has increased over the years.

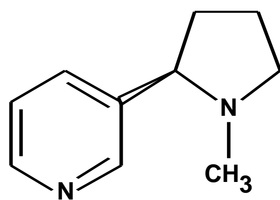


Figure 1. Chemical structure of nicotine.

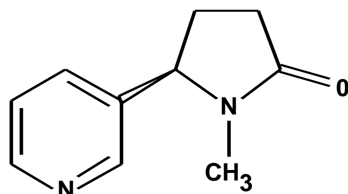


Figure 2. Chemical structure of cotinine.

Studies have shown that nicotine and cotinine are present in both active and passive smokers alike. Nicotine (Figure 1) is a natural product of tobacco, and it is present in the body with a half-life of 1–2 hours. Cotinine (Figure 2), on the other hand, is a metabolite of nicotine with a half-life of 18–20 hours.[2] The longer half-life makes cotinine a more stable and prominent compound in the human body than nicotine. This makes it a more desirable compound, as it is easier to analyze. The objective of this study was to develop an assay to detect and quantify cotinine levels in blood plasma.

The primary patient population of Children's Hospital, Boston, consists of adolescents and young teenagers. Given this patient demographic, any samples that the hospital receives would be from passive smokers. Therefore, the cotinine levels in their blood (<10 ng/mL) would be much lower than in active smokers ($\geq 10 - 15$ ng/mL).[3] Previous cotinine assays were not sufficient because the extraction procedures were too long and complicated, the limits of quantitation were too high, or the sample matrix was incompatible.[4] An inexpensive assay with a high throughput, a low limit of quantitation, and plasma as the sample matrix was designed to meet these needs.

Doctors at the hospital could analyze patients' cotinine levels quickly and accurately using the designed assay. By having the results back within a few working days, doctors could then promptly tailor treatments to the patient's needs and condition. In addition, this assay greatly enhances the ability to detect cotinine levels in young children and teenagers.

Method

A high-throughput, inexpensive, and accurate assay was developed to meet the needs of Children's Hospital, Boston. The assay utilized the PE SCIEX API 3000 with the TubroIon Spray HPLC MS/MS (high-performance liquid chromatography tandem mass spectrometer) machine owned by the hospital to detect the cotinine levels in blood plasma. Before the sample could be injected into the HPLC MS/MS, the cotinine was first purified and separated from the rest of the plasma. The assay was divided into the following five steps: sample acquisition, standards creation, sample protein precipitation, sample reconstitution, and HPLC MS/MS configuration and injection.

Sample Acquisition

A blood sample was acquired from one of the workers in the Hematology Lab at Children's Hospital, Boston. The blood sample was centrifuged at 14,000 rpms for 10 minutes to separate out the plasma. The plasma was then transferred to a clean tube and stored at -20° C for preservation. Before each use, the plasma sample was thawed at room temperature and certain amounts were transferred to other tubes used for the experiment. The remainder of the sample was placed back into a -20° C freezer for storage.

Standards Creation

Blood plasma samples were equilibrated with a D_3 – cotinine Internal Standard (ISTD). 25 µL of plasma of various cotinine concentrations were mixed with 25 µL of ISTD in 1.5mL Eppendorf tubes. The following are the cotinine concentrations that were used: 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/mL. These standards were used to measure assay and machine accuracy and recovery during HPLC MS/MS analysis.

Sample Protein Precipitation

500 µL of Acetonitrile was mixed into each tube to precipitate out heavy proteins. The mixtures were vortexed for 30 seconds

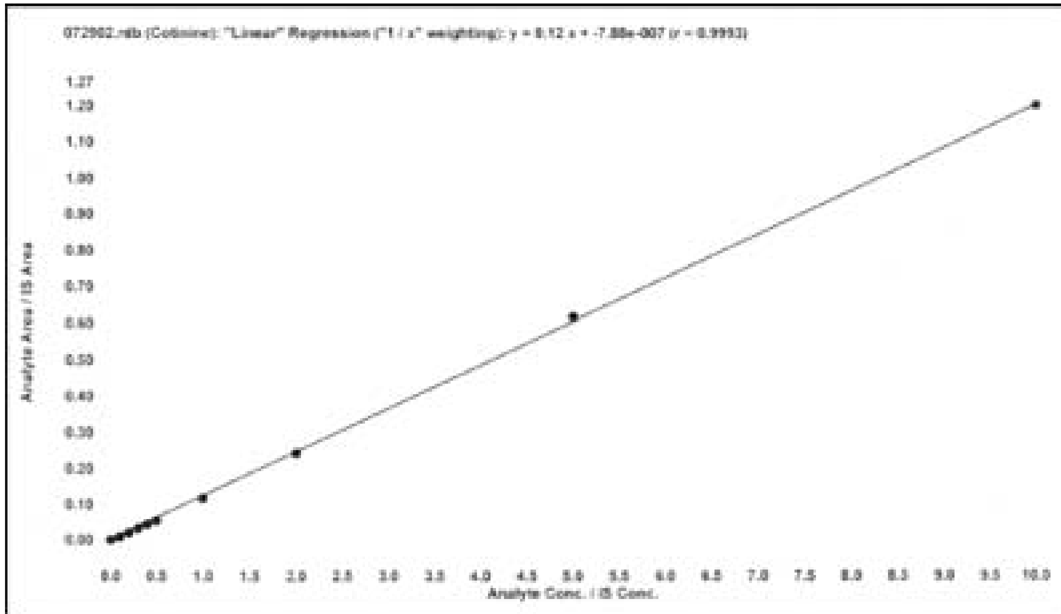


Figure 3. Calibration curve from 7-29-02. R-Value = 0.9993.

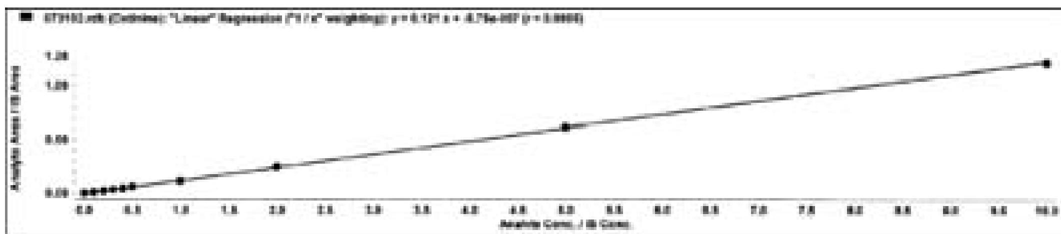


Figure 4. Calibration curve from 7-31-02. R-Value = 0.9995.

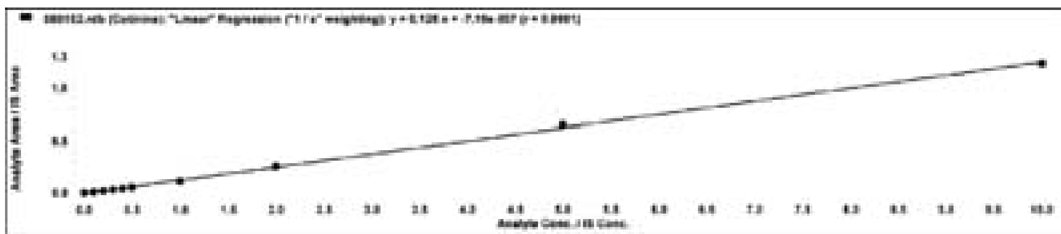


Figure 5. Calibration curve from 8-1-02. R-Value = 0.9991.

Figures 3 to 5 show the calibration curves from the three analysis days. As demonstrated, all three curves were linear from 0.0 to 10.0 ng/mL of cotinine concentration. This range covered both the high and low ends of cotinine concentrations. More importantly, the R-value from each of the days was greater than 0.9990, as desired.

and then centrifuged for 4 minutes at 14,000 rpms in order to form a cell debris pellet. 500 μL of the supernatant was then placed in clean glass test tubes and placed in a N_2 vaporizer for 15 minutes to evaporate the liquid from the supernatant.

Sample Reconstitution

Each glass test tube was reconstituted with 150 μL of MeOH to dissolve the cotinine and any remaining proteins. The tubes were again vortexed for 30 seconds to further precipitate out any remaining heavy proteins. The solution in each tube was transferred to 150 μL vials for HPLC MS/MS analysis.

HPLC MS/MS Configuration and Injection

The buffer solution used for the assay was 10 mM Ammonium Acetate in 30% MeOH.

20 μL of each sample was injected into the HPLC MS/MS at a flow rate of 200 μL/min. The analysis time was set for 2 minutes. The temperature was set to 450° C. The Q1/Q3 ratio for cotinine is 177/80, and the ratio for D_3 – cotinine is 180/80.

Results

Samples were prepared on 7-29-02 and analyzed on 7-29-02, 7-31-02, and 8-1-02 for accuracy and inter- and intra-day precision. A set of samples was created for intra-day analysis and another set was created for inter-day analysis. This enabled us to test for HPLC MS/MS accuracy as well as inter- and intra-day precision. The information obtained from these analyses helped determine the detection and quantification abilities of the newly developed assay.

Calibration Curve

Standard samples were prepared with cotinine concentrations of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/mL. These concentrations allowed us to test the accuracy and precision of both the high-end concentrations (5.0 – 10.0 ng/mL) and the low-end concentrations (0.1 – 0.5 ng/mL) as well as to identify the limit of quantitation of the assay. Each sample also contained 10.0 ng/mL of ISTD (D_3 – cotinine). This internal standard aided in determining the accuracy of both the assay and the machine.

The data from the standard samples was used to construct a calibration curve for each testing day. The calibration curve was linear, with a "1/x" weighting. A regression value (R-value) greater than R = 0.9990 was desired. Figures 3 to 5 show the calibration curves from the three days of analysis.

Inter-Day Precision

One sample each of 0.5 and 2.0 ng/mL cotinine containing 10.0 ng/mL of ISTD was prepared and run three times each in the HPLC MS/MS. The data was used to calculate the standard deviation, mean, and correlation value (CV%). The CV% was calculated by the quotient of the standard deviation and mean. A CV% of less than 20% was desired. Table 1 contains the inter-day precision data from the two types of samples.

Table 1. Inter-Day precision of 0.5 and 2.0 ng/mL of cotinine. Table 1 provides the overall values obtained from the inter-day analysis of 0.5 and 2.0 ng/mL of cotinine. As shown, the CV% is well below the 20% maximum for both samples. The 0.5 ng/mL sample has a CV% of 3.75, while the 2.0 ng/mL sample has a CV% of 11.35%.

Sample	0.5 ng/mL	2.0 ng/mL
S.D.	4.93E+05	1.25E+06
Mean	1.32E+07	1.10E+07
N	3	3
CV%	3.75	11.35

Intra-Day Precision

Samples were prepared with 0.5 and 2.0 ng/mL of cotinine containing 10.0 ng/mL ISTD. These samples were run on 7-29-02, 7-31-02, and 8-01-02 to determine the intra-day precision. The standard deviation, mean, and CV% were calculated from the data. Again, a CV% of less than 20% was desired. Table 2 contains the intra-day precision data for the two types of samples.

Accuracy

The recovery rate for the 0.5 and 2.0 ng/mL was calculated by taking the quotient of the measured cotinine concentration and prepared cotinine concentration for each of the samples. The desired recovery range was between 80% and 120%. Samples between this recovery range were deemed accurate. Table 3 provides the recovery range for each of the samples.

Limit of Quantitation

The limit of quantitation is determined as the lowest concentration of cotinine that has a CV% of less than 20% as well as a recovery rate between 80% and 120%. The analysis showed that 0.2 ng/mL of cotinine was the limit of quantitation for this assay because it met these criteria. The only lower concentration, 0.1 ng/mL, did not meet these criteria.

Discussion

The goal of this project was to develop an assay that would detect and quantify the levels of cotinine in blood plasma. Given that the assay would be used at Childern's Hospital, Boston, it also needed to be sensitive—have a low limit of quantitation as well as high throughput capabilities. We believe that an adequate assay was developed.

The developed assay uses a HPLC MS/MS without the need of a column to purify the sample. This significantly reduces the time needed per sample. If a column was to be used, the run time for each of the patient samples would easily triple or quadruple, because the sample would enter and exit the column and the column would need cleaning between sample runs. Previous assays performed in the laboratory demonstrated that a column did not improve the resolution or sensitivity of the assay. Therefore, a column was not included as part of the proposed assay.

In addition, blood plasma is the most compatible matrix for the target patients (young children and teenagers) at the hospital; it is very hard to obtain enough saliva or urine samples from infants and toddlers. Also, this assay requires the use of only 25 μL of plasma, thus only a small amount of blood needs to be drawn from the patient.

The sample matrix also needs to be purified before it can enter the HPLC MS/MS. The extraction method that was developed for the assay is sufficient in its purification abilities. It precipitates out the heavy proteins with the use of Acetonitrile, a common and easily obtainable laboratory reagent. The other steps in the extraction process further purify the sample, such as the N_2 evaporation. This assay does not damage the HPLC MS/MS machine by injecting unpurified samples, thereby safely allowing for repeated analyses.

The results showed the assay's limits of quantitative capabilities. An upper bound of 10.0 ng/mL and a lower bound of 0.2 ng/mL of cotinine were established. These were the upper and lower limits in which the assay would quantitate effectively; any concentration outside of this range will produce unreliable results using the developed assay. Because the hospital deals primarily with children, a modified upper bound of 2.0 ng/mL was created—only significant passive smokers contain over 2.0 ng/mL of cotinine in their blood, as demonstrated by previous studies. An example of a significant passive smoker is one who is constantly exposed to environmental smoke, such as a waitress. The hospital is mainly concerned with relatively low passive smokers who have plasma cotinine levels much lower than 2.0 ng/mL.

Table 2. Intra-Day precision of 0.5 and 2.0 ng/mL of cotinine. Table 2 provides the overall values obtained from the intra-day analysis of 0.5 and 2.0 ng/mL of cotinine. As shown, the CV% values for each of the samples were under the maximum level of 20%. These matched the desired results for this analysis.

Sample	0.5 ng/mL			2.0 ng/mL		
S.D	1.04E+05	1.62E+04	6.64E+05	1.05E+05	2.53E+05	5.07E+05
Mean	5.49E+05	1.92E+05	4.77E+06	2.59E+06	2.47E+06	4.81E+06
N	7	9	9	7	9	9
CV %	18.94	8.42	13.91	4.05	10.25	10.54

Table 3. Concentration of cotinine and recovery rate for 0.5 and 2.0 ng/mL of cotinine. Table 3 shows the recovery rates of the 36 analyzed samples. As shown in the table, all 18 of the 0.5 ng/mL cotinine samples had a recovery rate between 80% and 120%. Based on the criterion established before, these 18 compounds were determined to be accurate. For 2.0 ng/mL cotinine samples, 15 of the 18 compounds had a recovery rate between 80% and 120%. Therefore, 15 of 18 2.0 ng/mL cotinine samples were accurate.

0.5 ng/mL (N=18)				2.0 ng/mL (N=18)			
Conc.	Recovery	Conc.	Recovery	Conc.	Recovery	Conc.	Recovery
0.464	92.80	0.425	85.00	1.410	70.50	1.870	93.50
0.456	91.20	0.433	86.60	1.470	73.50	1.920	96.00
0.468	93.60	0.438	87.60	1.430	71.50	1.900	95.00
0.505	101.00	0.493	98.60	1.870	93.50	2.030	101.50
0.492	98.40	0.489	97.80	1.850	92.50	2.050	102.50
0.509	101.80	0.487	97.40	1.810	90.50	2.140	107.00
0.493	98.60	0.477	95.40	1.680	84.00	2.030	101.50
0.470	94.00	0.483	96.60	1.700	85.00	2.040	102.00
0.491	98.20	0.492	98.40	1.750	87.50	2.110	105.50

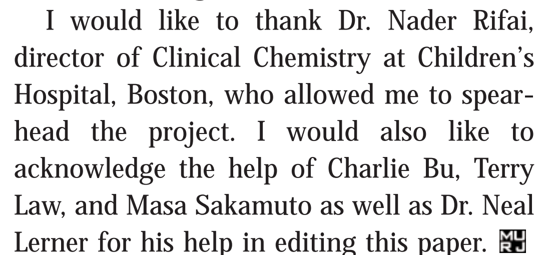
It is the hospital's goal to identify those patients who have a low, yet significant, exposure to environmental smoke. This assay allows the hospital to do exactly that.

The experimental data showed that the samples were linear within the range of 0.2 to 2.0 ng/mL of cotinine. This is substantiated by the R-value of the calibration curve being greater than 0.9990 and the CV% being less than 20%. From the data, we conclude that our assay is also accurate from 0.2 to 2.0 ng/mL.

The data gathered from the analyses indicates that we accomplished our goal of developing an assay that is inexpensive, sensitive (low limit of quantitation), and has high throughput capabilities.

However, before this can be used as a standard patient assay in the hospital, a comparison test needs to be carried out. First, samples need to be collected from hospital patients. The cotinine concentrations should then be quantitated using the developed assay and the ELISA test, the standard assay currently used. If this comparison shows that the newly developed assay is just as accurate as the ELISA test, then the hospital will be able to quantify cotinine levels in patient samples using the new assay. This study supports the outcome that the developed assay will be as accurate as the ELISA test.

Acknowledgments



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