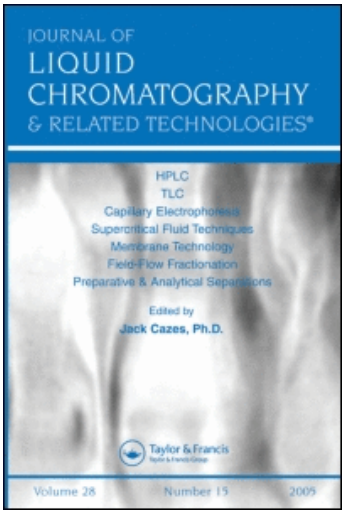

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Measurement of Nicotine in Plasma by High-Performance Liquid Chromatography

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MEASUREMENT OF NICOTINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A new procedure has been developed to measure nicotine in blood plasma by high-performance liquid chromatography (HPLC). Nicotine is extracted from plasma by elution with cholorform. Final determination is achieved by isocratic HPLC with ultraviolet detection. Twenty microliters of plasma extract is deluted over a silica column at a flow rate of 1.0 ml/min with a dioxane:isopropanol:NH_4OH (80:3.0:0.4) mobile phase. The procedure is sensitive to 0.05 ug of nicotine per ml of plasma and is linear within the range of 0.05 to 10.0 ug/ml of plasma. When a known amount of nicotine was added to plasma, the concentration of nicotine measured averaged 99.9 $\pm$ 3.9 (S.D.)% of the known concentration. The within-sample coefficient of variation was 3.9%

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INTRODUCTION

The measurement of nicotine in body fluids has become an important pharmacological assay to both clinical and research laboratories. Since nicotine is a major addictive component of tobacco smoke and thus plays a significant role in maintaining the smoking habit, there is increasing clinical interest in monitoring plasma nicotine concentrations of smokers[1,2]. Nicotine, a potent nicotinic receptor agonist, is utilized in research laboratories investigating the autonomic nervous system[3]. Studies of the pharmacokinetics of nicotine and its physiological actions require an accurate, sensitive, and rapid method for the quantitative actions of nicotine in blood plasma.

A variety of analyses have been developed to quantitate nicotine in biological fluids, but these analyses lack sensitivity and specificity[4,5] or are too tedious for routine applications in many clinical and research laboratories[6]. Previously, gas chromatography has been the principal analytical technique for measuring nicotine, but the instrumentation is expensive, and the assay is tedious and complicated[7-9]. Radioimmunoassays have been described[10,11], but these methods are laborious and require frequent checking of standard curves[2]. Recently, HPLC procedures to measure nicotine in urine have been reported[2,12,13]. Of these, the report of Maskarinec et al.[14] also demonstrated that HPLC could be utilized to measure nicotine

in plasma; however, their method required a complex pre-separation step. Futhermore, Maskarinec et al. did not evaluate the accuracy and precision of their procedure as applied to blood plasma.

Thus, we developed and evaluated a new, sensitive, rapid, accurate, and reproducible HPLC assay for measuring small amounts of nicotine in blood plasma.

EXPERIMENTAL

Materials

Nicotine was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Other HPLC-grade reagents were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). When preliminary measurements of nicotine in human and canine plasma demonstrated no differences due to the source of plasma, we evaluated the accuracy and precision of this procedure with canine plasma because of its availability in our laboratory.

Chromatographic Conditions

A Rainin Rabbit HP/HPX solvent delivery system (Rainin Instrument Inc., Wolburn, MA, U.S.A.) and a fixed wavelength ultraviolet absorbance detector (UV Monitor III, Milton Roy Co., Riviera Beach, FL, U.S.A.) were used. Output from the detector was recorded on a Kipp and Zonen recorder (Kipp and Zonen Inc., Bohemia, NY, U.S.A.).

The alkaloids were separated by a Microsorb-Si column (15 x 4.6 mm I.D.) obtained from Rainin. The mobile phase consisted of a dioxane:isopropanol:NH_4OH (80:0.3:0.4) solution. The operating conditions were as follows: temperature, ambient; pressure, 2000 p.s.i.; flow-rate, 1 ml/min; wavelength, 254 nm; chart speed, 10 mm/min.

Extraction Procedure

Two milliliters of plasma were mixed with an equal volume of chloroform. Nicotine in the plasma was extracted by vortex mixing for 5-6 min. The samples were then centrifuged for 10 min at 0^{o} C. The aqueous upper layer was discarded and the lower chloroform layer was again centrifuged for an additional 5-6 min if not clear. One milliliter of the clear chloroform layer was removed and gently evaporated to dryness under nitrogen. The residue was redissolved in 200 ul of the mobile phase solution, and a 20 ul sample was then injected onto the column.

RESULTS

Sensitivity

This method was sufficiently sensitive to detect nicotine concentrations $\geq$ 0.05 ug/ml of plasma. We evaluated the procedure for concentrations of 0.05 to 10.0 ug/ml plasma. No interference was encountered from substances normally present in plasma. A representative chromatogram is shown in Fig. 1. The alkaloids, nicotine and cotinine, were well separated with no

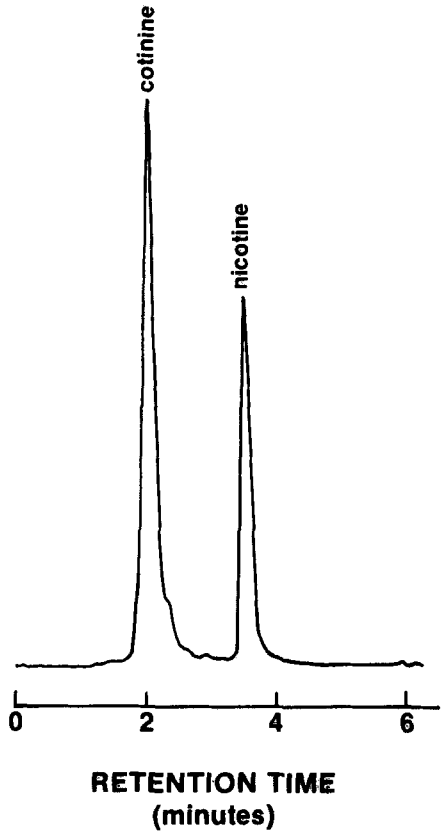

FIGURE 1. HPLC chromatogram of nicotine alkaloids in dog plasma. Absorbance range, 0.0 - 0.1.

interfering peaks; the baseline was stable and noise-free. As shown in Fig. 2, the calibration curve of the peak height response was linear for nicotine concentrations of 0.5 to 10.0 ug/ml plasma. As shown in Figure 3, the calibration curve of the peak height response was also linear for concentrations of 0.05 to 1.0 ug/ml. Cotinine is extracted and separated by our

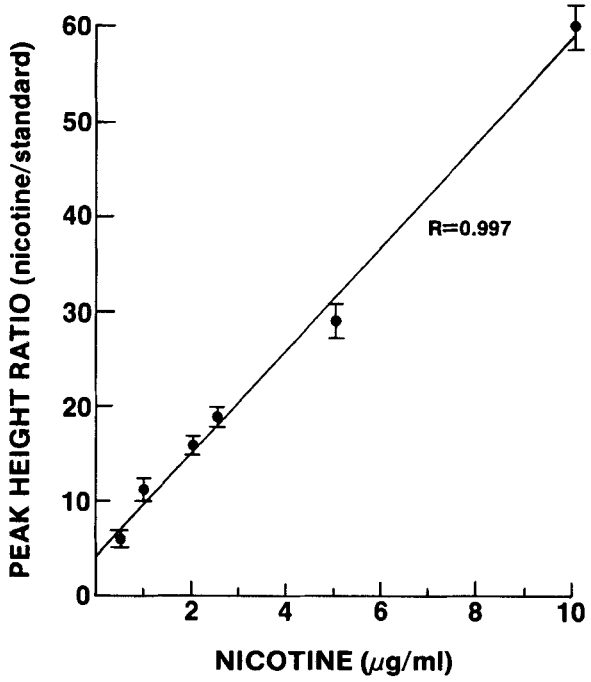

FIGURE 2. Calibration curve of the peak height response in the range 0.50 - 10.0 ug nicotine per ml plasma. Values are means ± S.D. for 30 determinations at each concentration.

procedure (Fig. 1), but we did not evaluate the accuracy of the procedure for cotinine determinations.

Accuracy

We added nicotine to plasma in vitro to yield concentrations of 0.05, 0.10, 0.50, 1.00, 2.50, 5.00, and 10.0 ug/ml. On ten separate occasions, each concentration was prepared and assayed in triplicate. Mean ± S.D. values for 30 determinations at each concentration from 0.5 to 10.0 ug/ml are

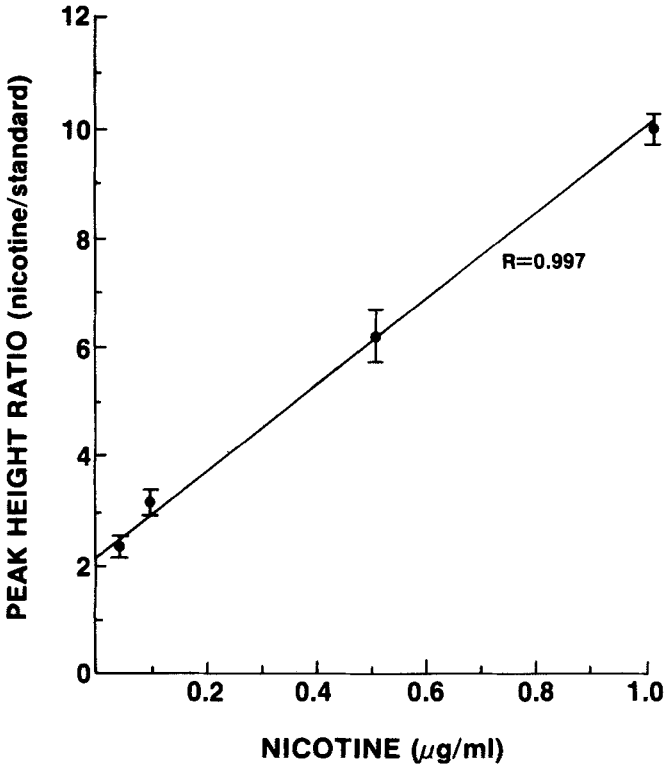

FIGURE 3. Calibration curve of the peak height response in the range 0.05 - 1.0 ug nicotine per ml plasma. Values are means ± S.D. for 30 determinations at each concentration.

presented in Fig. 4. Mean ± S.D. values for 30 determinations at each concentration from 0.05 to 1.0 ug/ml are presented in Fig. 5. Linear regression analysis of this data demonstrated that the relationship between nicotine measured and nicotine added was linear ($P < 0.01$) from 0.05 to 10.0 ug/ml. The slope of this relationship was 0.997, which was not statistically different

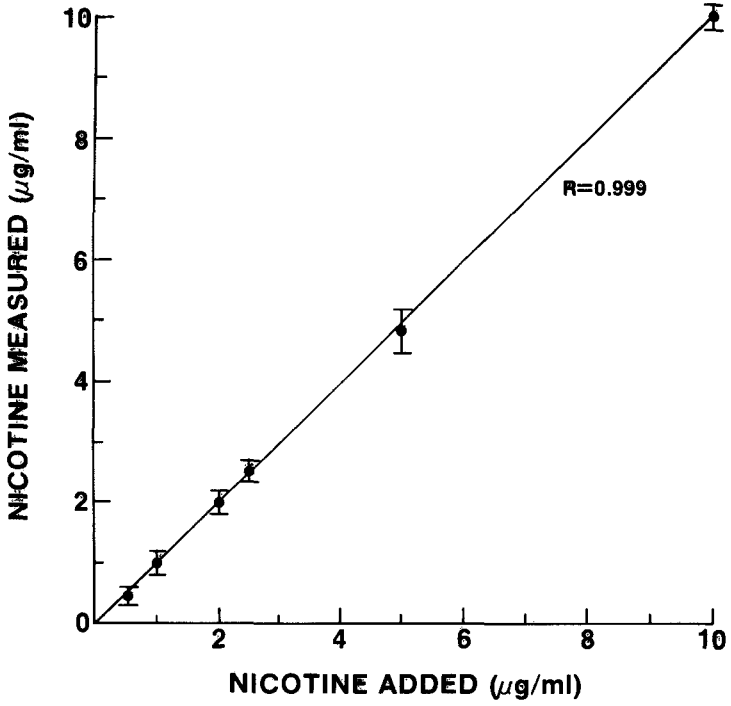

FIGURE 4. Standard addition curve of nicotine in dog plasma over the range of 0.5 - 10.0 ug nicotine per ml plasma. Values are means ± S.D. for 30 determinations at each concentration.

from 1.0000 ($P > 0.200$). The correlation coefficient was 0.999. The concentration of nicotine measured in these samples averaged 99.9 ± 3.9% (S.D.)% of the known concentration.

To determine the accuracy of the procedure for measuring nicotine in whole blood, we added nicotine to blood in vitro to yield concentrations of 0.05, 0.10, 0.50, 1.00, 2.50, 5.00, and 10.0 ug/ml of blood. Six milliliter blood samples were

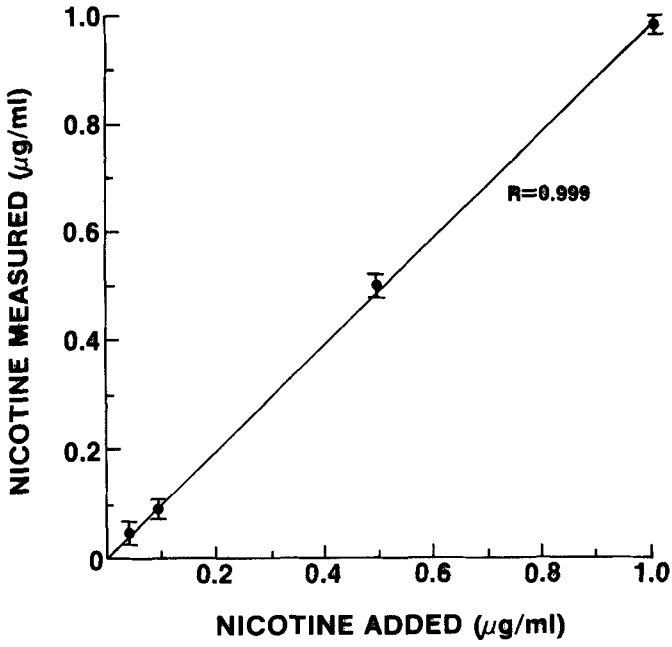

FIGURE 5. Standard addition curve of nicotine in dog plasma over the range of 0.05 - 1.0 ug nicotine per ml plasma. Values are means ± S.D. for 30 determinations at each concentration.

centrifuged at 0° C for 10 mins at 1500 G. The plasma was then pipetted into chilled tubes and assayed. On four separate occasions, each concentration was prepared and assayed in triplicate. Mean ± S.D. values for 12 determinations at each of the seven concentrations in plasma are presented in Table I. The concentration of nicotine in plasma for these samples averaged 94.0 ± 1.2% of the known concentration. The relationship between nicotine measured and nicotine added was linear ($P < 0.01$) from 0.05 to 10.0 ug/ml. The slope of the relationship was

TABLE 1

Distribution Of Nicotine After Introduction To Whole Blood

Known Concentration of Nicotine in in Blood (μg/ml)	Time Interval*	Measured Concentration of Nictoine in Plasma (μg/ml)	$\frac{[\text{Nicotine}]_{\text{Blood}}}{[\text{Nicotine}]_{\text{Plasma}}}$	$\frac{[\text{Nicotine}]_{\text{Erythrocytes}}}{[\text{Nicotine}]_{\text{Plasma}}}$
0.05	1	0.04±0.004	ND	ND
0.10	1	0.09±0.003	ND	ND
0.50	5	0.47±0.03	0.94	1.21
1.00	15	0.93±0.04	0.93	1.23
2.00	30	1.91±0.06	0.96	1.15
5.00	45	4.72±0.21	0.94	1.19
10.00	60	9.19±0.45	0.92	1.27

Values are means ± S.D. for 12 determinations at each concentration.

*Interval from addition of nicotine to initiation of centrifugation.

#Calculated by formula in text.

ND Not determined.

0.921, which was significantly less than 1.0000 ($P < 0.01$). The correlation coefficient was 0.999.

Precision

Multiple determinations of nicotine in plasma were performed for the accuracy study described above and for plasma - erythrocyte equilibrium study described below. The mean coefficient of variation for all multiple determinations was 3.9% $\pm$ 0.8 (S.E.)%.

Plasma - Erythrocyte Equilibrium

To examine the distribution of nicotine between plasma and erythrocytes, and to determine how rapidly this distribution reaches equilibrium, nicotine was added *in vitro* to whole blood to yield concentrations of 0.50, 1.00, 2.00, 5.00, and 10.0 ug/ml. Blood cells were then separated from plasma by centrifugation at various intervals after addition of nicotine. The 5 ug/ml sample was analyzed at 5 mins, whereas the other samples were waffled for 15, 30, 45, or 60 mins before analysis. The results are presented in Table I. Nicotine was distributed between plasma and whole blood with an average ratio of 0.94 $\pm$ 0.01. There was no statistical difference between the ratios of distribution between plasma and whole blood as a function of time. Thus, the distribution reaches equilibrium within five minutes after the introduction of nicotine into whole blood, and this distribution remains constant for at least one hour.

Since we found the concentration of nicotine in plasma to be consistently lower than that of whole blood (Table I), the intracellular concentration of nicotine was apparently greater than that of plasma. After calculating plasma and cell volumes from the blood hematocrit, we calculated the nicotine concentration in erythrocytes by the following formula:

$$[\text{Nicotine}]_E = \frac{[\text{Nicotine}]_B \times BV - [\text{Nicotine}]_P \times PV}{\text{Erythrocyte Volume}}$$

Where $[\text{Nicotine}]_E$ is the concentration of nicotine in erythrocytes, $[\text{Nicotine}]_B$ is the concentration of nicotine in whole blood, $[\text{Nicotine}]_P$ is the concentration of nicotine in plasma separated from the whole blood, BV is blood volume, and PV is the plasma volume. Nicotine was distributed between erythrocytes and plasma with an average ratio of 1.21 $\pm$ 0.04 (S.D.).

DISCUSSION

The measurement of nicotine in body fluids has become of significant importance to both research and clinical laboratories. Of techniques previously described, HPLC has proven to be the most useful technique for the separation of the tobacco alkaloids. HPLC is simpler and faster than gas chromatography[7-9] or radioimmunoassay[10,11], both of which may be too expensive and laborious for routine applications.

Although HPLC has become state-of-the-art in measuring tobacco alkaloids, the literature has focused on the quantitation of nicotine in urine[2,4-6,12,13]. Maskarinec et al.[14] demonstrated that their procedure for measuring nicotine in urine could be applied to plasma. However, they did not evaluate the accuracy and precision of this procedure for nicotine in plasma. The procedure of Maskarinec et al. required a complex pre-separation step which appreciately lengthens the analysis time. Thus, we developed and evaluated a new HPLC procedure to measure small amounts of nicotine in plasma.

This procedure is advantagous in several aspects. The analysis time is short; retention times for nicotine and cotinine are 2.1 and 3.7 mins, respectively. The sample size is small, with only 6 ml of whole blood needed to make triplicate within-sample determinations. The extraction procedure is simpler and shorter than that described by Maskarinec et al. This procedure is sensitive to 0.05 ug of nicotine per ml of plasma and is linear within the range of 0.05 to 10.0 ug/ml. The excellent accuracy, precision, and sensitivity of the procedure should enable its routine use in clinical and research laboratories. It has been reported that plasma nicotine levels of smokers range between 0.06-0.10 ug/ml of blood [9,10,15]. The method presented here has sufficient sensitivity to detect nicotine at these levels.

When a known amount of nicotine was added to plasma, the concentration of nicotine measured 99.9 ± 3.9 (S.D.)% of the known concentration. However, when a known amount of nicotine was added to whole blood, the concentration of nicotine in the plasma measured only 94.0 ± 1.2 (S.D.)% of the known concentration. The difference in accuracy between plasma and whole blood measurements caused us to further investigate the possibility of selective partitioning of nicotine in erythrocytes. We calculated the concentration of nicotine in erythrocytes and found a consistently higher nicotine content in the erythrocytes at each of the five concentrations studied. Further investigation of mechanisms which determine the plasma-erythrocyte distribution of nicotine appears warranted.

ACKNOWLEDGMENT

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