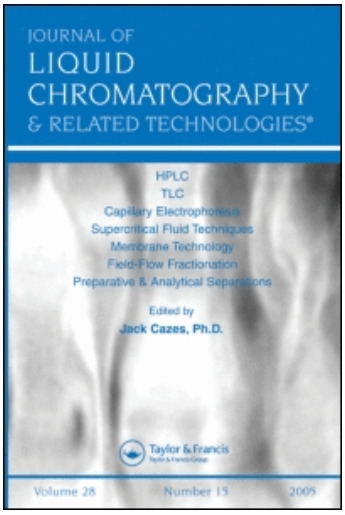

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Sample Preparation for the Analysis of Catecholamines and their Metabolites in Human Urine

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SAMPLE PREPARATION FOR THE ANALYSIS OF CATECHOLAMINES AND THEIR METABOLITES IN HUMAN URINE

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ABSTRACT

A specific, sensitive, qualitative and quantitative extraction procedure followed by an high pressure liquid chromatography equipped with electrochemical detector assay of catecholamines (CATs) and their metabolites from human urine has been developed. Using an unique multiple interaction bonded silica gel disposable solid phase extraction (SPE) column, various analytes were selectively isolated from the urine components. After following three different extraction procedures, the presence of free CATs (epinephrine, norepinephrine and dopamine) and their basic (normetanephrine, metanephrine, and 3-methoxydopamine) and acidic (vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid) metabolites was confirmed and quantitated by electrochemical detector.

INTRODUCTION

Quantification of 3,4-dihydroxyphenolic amines, i.e., catecholamines and their metabolites, is one of the most important ways to evaluate the activity of the sympathetic nervous system. Catecholamines are synthesized in the sympathetic nervous system, the brain and in the cells of neutral crest origin. These amines are primarily neurotransmitters which exhibit a wide range of physiological effects. Clinically, the quantification of catecholamines has been mainly used for the diagnosis of pheochromocytoma (1-6), a tumor of the neutral crest. The most important endogenously produced compounds of this group are epinephrine (adrenalin), norepinephrine (noradrenalin), and dopamine (3,4-dihydroxyphenylethyl amine). The chemical properties of catecholamines are presented in great detail in a review article (7). Epinephrine is quantitatively produced by the adrenal medulla, where as norepinephrine is mainly generated by sympathetic nervous system. Dopamine and norepinephrine are important neurotransmitters in

the central nervous system (the brain and the spinal cord) which are responsible for co-ordination and movement of fine muscles and body movement, the control of memory, alertness and emotion, as well as hunger, temperature regulation, thirst, blood pressure, reproduction, behavior, etc. The properties of catecholamines as neurotransmitters have been discussed in a review by Bleich and Moore (8).

Each of these three amines also have characteristic physiological functions and pharmacological actions (9). Dopamine and norepinephrine influence the vascular system, whereas epinephrine influences metabolic processes such as carbohydrate metabolism. In vivo and in vitro studies have shown that aromatic L-amino acid tyrosine is the precursor of catecholamines (10). For the release of these amines nerve stimulation is necessary. After release from storage vesicles these amines act on effector sites. They are transported in blood and have very short plasma half life of about 2 minutes. They are inactivated by o-methylation and oxidative

deamination to form various basic and acidic metabolites.

Since catecholamines play an important role in health and disease, detection of these amines has been an analytical challenge. In the last few years a great number of different methods have been reported for the detection and analysis of catecholamines and their metabolites in urine (11-23), plasma (14-18, 21, 24-27) and tissue samples (15).

This paper describes sensitive, specific, qualitative and quantitative extraction procedures and hplc/electrochemical detection assay analyzed for free CATs and their metabolites in human urine using a newly developed bonded silica gel solid phase extraction column. AccuCAT is a chemically modified silica gel exhibiting multiple interactions (a combination of polar, nonpolar, anion exchange and cation exchange properties). Due to mixed mode properties and selective nature of the phase it is possible to extract free CATs and their metabolites on a single column, by following a specific procedure for each class, to provide clean extracts with high recovery.

EXPERIMENTAL

Materials

AccuCAT extraction columns, AccuCAT HPLC column and a Vac Elut^{R} vacuum manifold (AI 6000) were provided by Varian Sample Preparation Products (Harbor City, CA). A Vortex mixer was obtained from Scientific Industries Inc. (Bohemia, NY).

Reagents and Chemicals

Epinephrine, norepinephrine, dopamine, dihydroxy benzylamine, normetanephrine, metanephrine, 3-methoxydopamine, 4-methoxydopamine, vanillylmandelic acid, homovanillic acid, 5-hydroxyindoleacetic acid and iso-vanilmandelic acid were purchased from Aldrich (Milwaukee, WI). HPLC grade methanol, acetonitrile, hexane, and ethyl acetate were purchased from EM Science (Cherry Hill, NJ).

Preparation of Standards

Twenty four hours urine samples were collected from healthy individuals over 10 mL of 6 N HCl

solution. Total volumes were measured, the samples were divided into 50 mL aliquotes and stored at $0^{0}C$-$4^{0}C$. A portion of the specimen was spiked with known amounts of CATs or their metabolites separately.

Instrumentation

The chromatographic separation was developed and performed on a Varian Star LC 9010 solvent delivery system with Varian 9095 autosampler, and ESA Coulochem Model 5100A electrochemical detector.

Extraction of Free catecholamines

Epinephrine (2, 25, 125, 200, and 400 ug/L), norepinephrine (50, 100, 150, 500, and 800 ug/L) and dopamine (100, 200, 600, 800, 4000 and ug/L) were spiked into 3 mL of blank urine sample (collected over a period of 24 h in a 10 mL of 6 N HCl, final pH 1-3). Two milliliters of 20 mM ammonium phosphate buffer containing 0.1 g EDTA/L (pH=7.0) and dihydroxy benzylamine (500 ug/L) as an internal standard were added to the sample. The mixture was vortexed and pH was adjusted to

2.0-4.0 with 0.5 N potassium hydroxide solution. AccuCAT column was connected to an Analytichem Vac Elut^{R} vacuum manifold and conditioned with 2 mL of 1% potassium hydroxide solution in methanol followed by 2 mL of deionized water. The urine specimen containing catecholamines and internal standard was applied to the column and passed through the bed at a slow flow rate by applying vacuum (2-3 inch Hg). The column was washed sequentially with 5.0 mL of 10 mM ammonium phosphate buffer (pH=7.0) containing 0.05 g EDTA/L and 5 mL of 10 mM acetic acid solution.

A rack with labelled collection tubes was placed in the Vac Elut and the tips of the delivery needles were wiped. The CATs were eluted with 4 mL of 1 N HCl. One hundred microliters of the eluent was diluted in 1 mL of mobile phase and injected into HPLC. The detection was performed by ESA electrochemical detector using a 15 cm AccuCAT HPLC column and a mixture of 12.6 g citric acid, 11.35 g sodium hydrogen phosphate, 120 mg sodium octane sulfonic acid, 75 mL methanol and 925 mL deionized water as mobile phase at a flow rate of 1 mL/minute.

Basic Metabolites Extraction

The twenty four hour urine specimen was collected in a clean container over 10 mL of 6 N HCl and stored in the refrigerator (pH=1-3). Normetanephrine (NMN), metanephrine (MN) and 3-methoxydopamine concentrations of 20, 500, 1000, 2000, and 4000 ug/L were spiked into 2 mL of the above specimen. Each urine sample was spiked with 4-methoxydopamine as the internal standard at a concentration of 1000 ug/L and each specimen was diluted with 2 mL of deionized water. The pH of each specimen was adjusted to 0.8-1.0 with 2 N HCl. The samples were capped and hydrolyzed at 90°C for 30 minutes.

Following the hydrolysis, 2 mL of 20 mM ammonium phosphate buffer (pH=10.0) containing 0.1 g/L EDTA was added. The pH of the specimen should be between 2-3, if not pH was adjusted accordingly. AccuCAT columns were connected to an Analytichem Vac Elut vacuum manifold and conditioned with 2 mL of 1% potassium hydroxide solution in methanol, followed by 2 mL of deionized water. The hydrolyzed urine specimen was

added to the column and passed through the bed at a slow flow rate by applying vacuum (2-3 inch Hg). After application, the bed was rinsed with 5 mL of 10 mM acetic acid/methanol (9:1) and the column was dried for 2 minutes under full vacuum (15 inch Hg). The column was further washed with 10 mM ammonium phosphate buffer (pH=10.0) and all washes were discarded. The tips of the Vac Elut delivery needles were wiped and a rack containing labelled collection tubes was placed in the Vac Elut. The metabolites were eluted with 4 mL of (1:1) 5% ammonium hydroxide/methanol and vortexed for two minutes. One hundred microliters of the eluent was taken, mixed with 1 mL of mobile phase and injected into HPLC for separation. The detection was performed by ESA electrochemical detector using a 15 cm AccuCAT HPLC column and a mixture of 900 mL buffer (prepared from 27.6 g sodium hydrogen phosphate, 2.02 g sodium heptane sulfonic acid and 0.1 g EDTA dissolved in 1 L of deionized water) and 100 mL acetonitrile as the mobile phase at a flow rate of 1.0 mL/minute.

Acidic Metabolites Extraction

Vanillylmandelic acid (VMA), and homovanillic acid (HVA) (0.5, 2.0, 5.0, 10.0, 20.0, and 50.0 mg/L), and 5-hydroxyindoleacetic acid (1.5, 3.0, 4.5, 6.0, 8.0, and 10.0 mg/L) were spiked into 1 mL of blank urine sample (collected over a period of 24 h in 10 mL of 6 N HCl, final pH 1-3).The urine sample was spiked with iso-vanilmandelic acid (iso-VMA) as the internal standard at a concentration of 10 mg/L and each specimen was diluted with 1 mL of deionized water. The pH of the specimen was adjusted to 5.0-6.0 with 0.5 N sodium hydroxide solution, 100 uL of aqueous 0.1 M EDTA was added and vortexed. AccuCAT column was connected to an Analytichem Vac ELut vacuum manifold and conditioned with 10 mL of 100 mM sodium acetate buffer (pH=6.0) followed by 4 mL of methanol and 4 mL of deionized water. The specimen containing acidic metabolites and internal standard was applied to the column and passed through the bed at a slow rate by applying vacuum (2-3 inch Hg). The column was washed with 5.0 mL of acetone and dried under full vacuum (15

inch Hg) for 2 minutes. All washes were discarded. The tips of the Vac Elut delivery needles were wiped and a rack containing labelled collection tubes was placed in the Vac Elut. The acidic metabolites were eluted with 4 mL of 100 mM aqueous citric acid buffer and vortexed. One hundred microliters of the eluent was taken, mixed with 1 mL of mobile phase and injected into HPLC. The detection again was performed as usual using a 15 cm AccuCAT HPLC column and a mixture of 1 L buffer (prepared from 6.8 g potassium hydrogen phosphate in 800 mL deionized water, adjusting the pH to 3.0 with 1 M phosphoric acid and bringing the volume to 1 L with deionized water) and 16 mL methanol as the mobile phase at a flow rate of T_0=1 mL/minute, T_7=1 mL/minute, T_{12}=2 mL/minute, T_{31}=2 ml/minute and T_{35}=1 mL/minute.

RESULTS AND DISCUSSIONS

The solid phase extraction procedures described here using AccuCAT disposable columns, provide rapid, realiable and reproducible isolation of free catecholamines and their basic and acidic

TABLE 1
ESA Detector Setting for the Analysis of Various Components of Free Catecholamines and their Metabolites

	Detector Setting		
Analyte	Det 1 (Gain)	Det 2 (Gain)	C or G Cell
CATs	+0.10(0x10)	-0.20(60x10)	+0.25
Basic	+0.10(0x10)	+0.50(30x10)	-0.35
Acidic	+0.10(1x10)	+0.65(40x10)	+0.70

metabolites from human urine. Due to multiple properties of the bonded phase, by following specific extraction process, three different types of components (CATs, basic and acidic metabolites) have been retained and eluted by hydrophobic, polar, anion, and cation exchange interaction mechanisms. Experimental procedures for the analysis of these compounds by HPLC/electrochemical detector show the cleanliness and reproducibility of the extraction processes and the bonded phase. Table 1 represents the setting of ESA detector for each type of the analyte. Figure 1 is the HPLC chromatogram of free

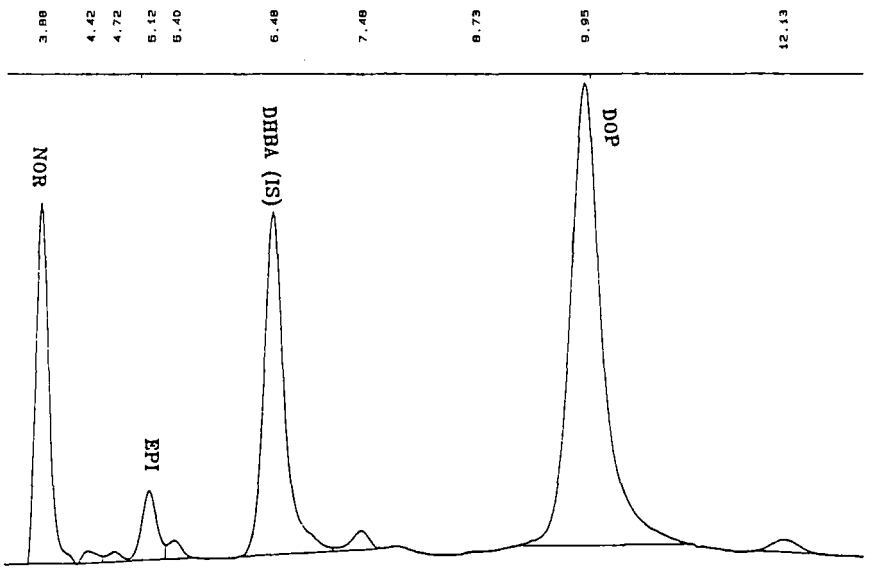

FIGURE 1. HPLC Chromatogram of CATs Extracted at Concentrations of 200 ug/L of Epinephrine, 500 ug/L of Norepinephrine and 800ug/L of Dopamine.

CATs extracted at concentrations of 200 ug/L of epinephrine, 500 ug/L of norepinephrine and 800 ug/L of dopamine. Figure 2 shows the linearity curve generated from the analysis of the urine spiked with CATs. Linear plots demonstrate the quantitative response over concentration range of 2-400 ug/L, 50-800 ug/L, and 100-4000 ug/L for epinephrine, norepinephrine and dopamine respectively. Figure 3 is an HPLC chromatogram of

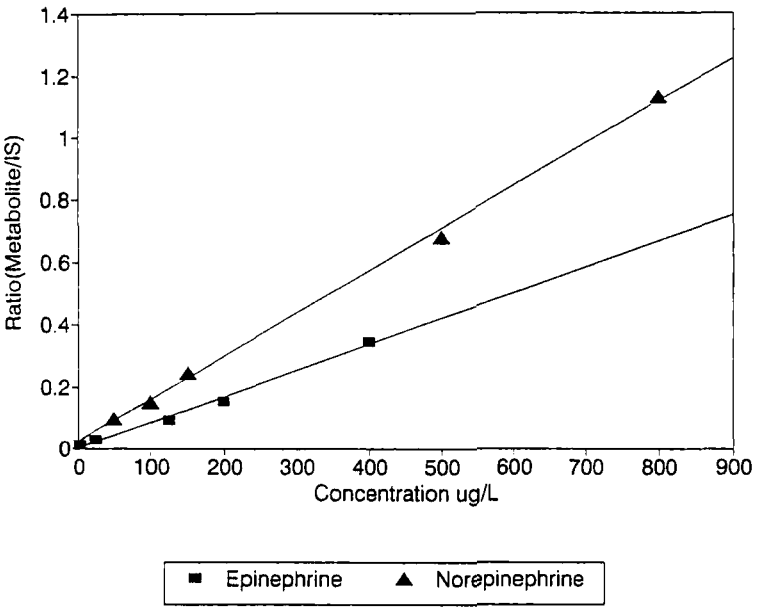

FIGURE 2. Standard Curve Generated from the Analysis of the Spiked Urine CATs Over Concentration Range of 2-400, 50-800, and 100-4000 ug/L for Epinephrine, and Norepinephrine Respectively.

basic metabolites extracted at concentrations of 1000 ug/L of NMN, MN and 3-methoxydopamine. Figure 4 represents the linear regression curves for normetanephrine, metanephrine and 3-methoxydopamine over the concentration range of 20-4000 ug/L. Similarly, Figure 5 is the HPLC chromatogram of the various acidic metabolites at

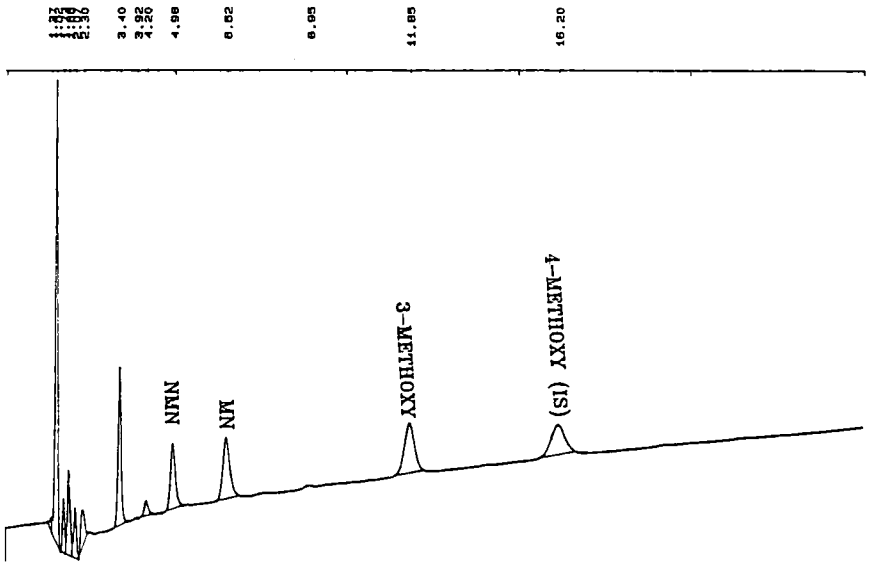

FIGURE 3. HPLC Chromatogram of Basic Metabolites Extracted at Concentrations of 1000 ug/L of Normetanephrine, Metanephrine and 3-Methoxydopamine.

concentrations of 10 mg/L for VMA and HVA and 6 mg/L for HIAA. Figure 6 represents the linear response curves for HVA and VMA over a concentration range of 0.5-50 mg/L.

The recoveries and precision data for spiked urine samples are listed in Table 2. A good linear relationship between the peak-height ratio and

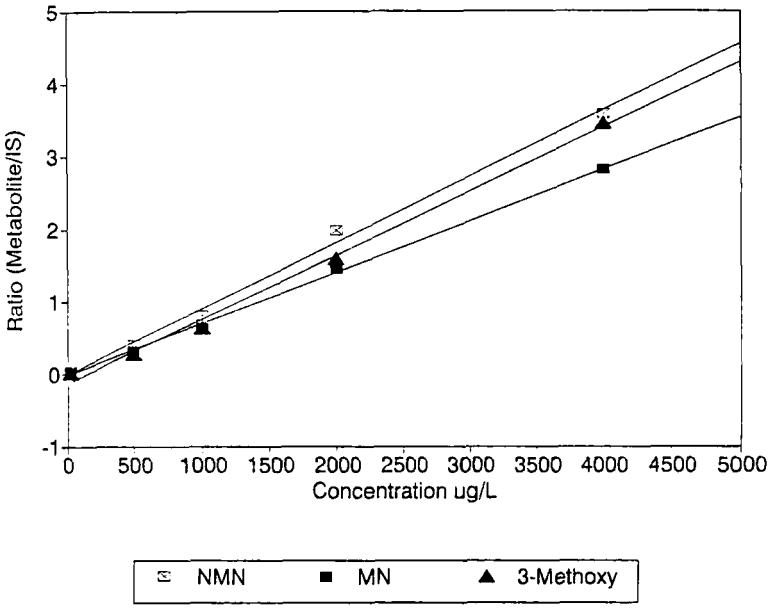

FIGURE 4. Standard Curve Generated from the Analysis of the Spiked Urine Basic Metabolites Over Concentrations Range of 20-4000 ug/L of Normetanephrine, Metanephrine and 3-Methoxydopamine.

CATs concentration was calculated. The regression parameters are listed in Table 3.

In summary the AccuCAT solid phase extraction column is designed for the extraction and analysis of catecholamines and their metabolites. Unlike other procedures AccuCAT takes less time for

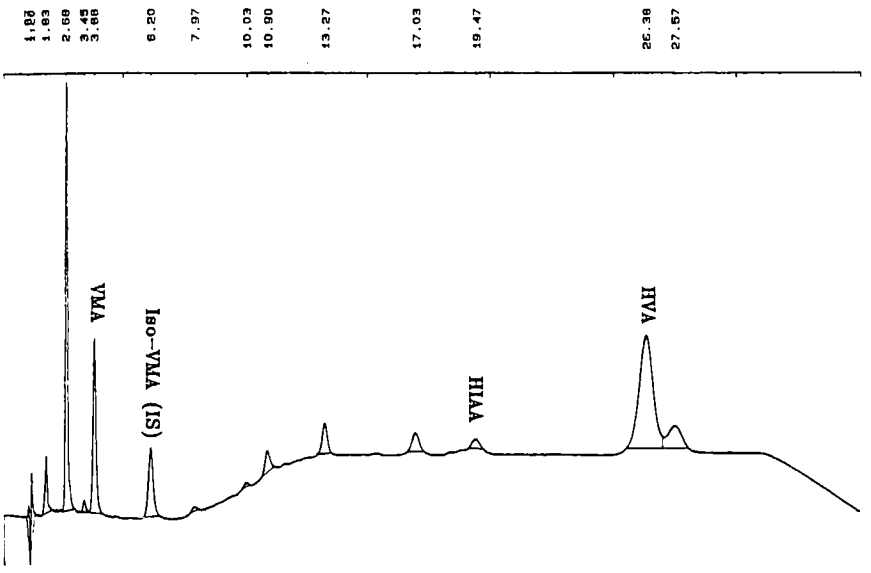

FIGURE 5. HPLC Chromatogram of Acidic Metabolites Extracted at Concentrations of 10 mg/L of VMA and HVA and 6 mg/L of HIAA.

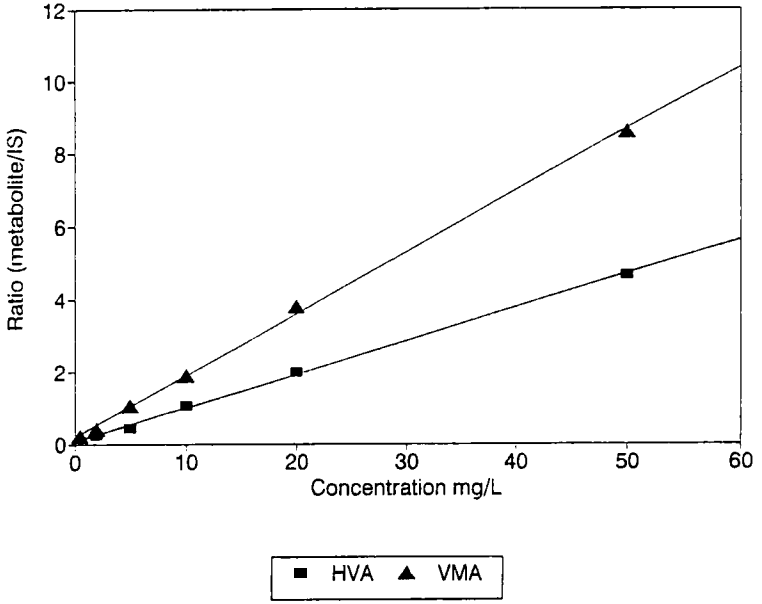

FIGURE 6. Standard Curve Generated from the Analysis of the Spiked Urine Acidic Metabolites Over Concentrations Range of 0.5-50 mg/L of VMA and HVA.

TABLE 2
Recoveries and Precision Data of the Various Extracts Extracted on AccuCAT and Analyzed by HPLC/ECD Method

Compound	Mean Recovery (n=12) (%)	Coefficient of variation (%)
Dopamine (1)	90	8.2
Epinephrine (2)	102	8.4
Norepinephrine (3)	96	9.5
3-Methoxydopamine (4)	105	12.36
Metanephrine (5)	104	10.7
Normetanephrine (6)	84	11.2
HIAA (7)	93	12.8
HVA (8)	86	4.34
VMA (9)	88	6.19

TABLE 3
Linear Regression parameters for the Various Calibration Curves

Values are for a and b in the equation Y=ax+b

Compound	a	b	Correlation Coefficient (r^2)
1	0.0011	0.692	0.993
2	0.0008	0.002	0.992
3	0.0014	0.024	0.998
4	0.0009	-0.132	0.995
5	0.0007	-0.019	0.999
6	0.0009	-0.009	0.995
7	0.0310	0.109	0.992
8	0.0924	0.075	0.998
9	0.1698	0.187	0.999

sample clean up and produces more reliable results.

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