EVALUATION OF A REDUCTIVE AMINATION DERIVATIZATION STRATEGY USING ALDEHYDES TO IMPROVE SENSITIVITY IN A PLASMA CATECHOLAMINES LC-MS/MS ASSAY

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

The catecholamines: dopamine, norepinephrine, and epinephrine, are naturally occurring amines that function as hormones and neurotransmitters. Excess concentrations of catecholamines have been observed in patients with rare neuroendocrine tumors and are associated with chronic hypertension, stroke, myocardial infarction, and cardiovascular disease. These potential consequences of high catecholamine concentrations emphasize the need for a rapid and accurate analytical measurement method. Catecholamines are often measured using high performance liquid chromatography (HPLC) with electrochemical detection; however, the liquid chromatography tandem mass spectrometry (LC-MS/MS) technique is attractive due to its selectivity and throughput. Derivatization of catecholamines prior to LC-MS/MS analysis of plasma specimens may be used to enhance assay sensitivity. The goal of this study was to evaluate reductive amination derivatization of catecholamines using straight-chain and branched-chain aldehydes as a means to improve the sensitivity of the assay. Derivatization was performed on each catecholamine in triplicate using a series of straight-chain and branched-chain aldehydes. Aqueous catecholamine standards were reacted with an aldehyde in the presence of a buffer (ammonium acetate) and reducing agent (sodium cyanoborohydride) at 37 °C for 30 minutes. Samples were quenched with formic acid at room temperature and injected onto an LC-MS/MS system for analysis. Catecholamine derivatives were identified by individual retention times and mass

transitions. Peak area counts were determined for three mass transitions for each derivative. The six-carbon straight-chain aldehyde, hexanal, and the branched-chain aldehyde, hydrocinnamaldehyde, proved to be the most effective derivatizing agents for the catecholamines in plasma assay. A derivatization protocol using hydrocinnamaldehyde was optimized for aldehyde and reducing agent concentrations, and incubation time and temperature. Derivatization with hydrocinnamaldehyde produced single alkylated products for all three catecholamines. Comparison of the derivatization agents showed higher peak area counts for norepinephrine and epinephrine derivatized with hydrocinnamaldehyde; hexanal provided greater sensitivity for dopamine. A method application experiment on patient samples using hydrocinnamaldehyde demonstrated its significant effect on assay sensitivity and supports use in a clinical setting. However, sensitivity for dopamine was inadequate and overall accuracy and precision were unsatisfactory. Further optimization of the derivatization protocol using hydrocinnamaldehyde is required to meet acceptable analytical criteria for this assay.

I would like to dedicate this thesis to my amazing mom. Thank you for your words of wisdom and encouragement the last few years. Without you, this would not be possible. I love you!

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INTRODUCTION

Catecholamines

The catecholamines dopamine (DA), norepinephrine (NOR), and epinephrine (EPI) are naturally occurring amines that function as hormones and neurotransmitters (Figure 1) (1). Catecholamines are composed of a catechol (phenyl ring with vicinal hydroxyl groups) with an ethylamine chain on the first carbon. Dopamine has the basic structure while norepinephrine and epinephrine are distinguished by additional functional groups on the ethylamine chain. Norepinephrine contains a hydroxyl group on the beta carbon of the ethylamine while epinephrine is further distinguished by a methyl group on the nitrogen atom.

These compounds are synthesized from tyrosine via a series of enzymatic reactions in the brain, sympathetic nerve fibers, and chromaffin cells of the adrenal medulla. Synthesis of different catecholamines depends on the enzymes present (see Figure 2).

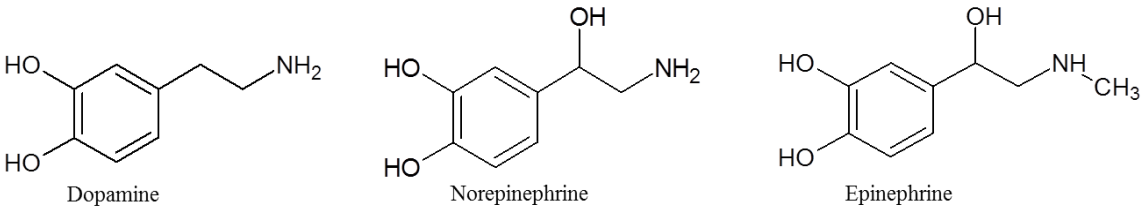

Figure 1. Structure of catecholamines
Catecholamines are composed of a catechol (phenyl with vicinal hydroxyl groups) and an ethyl amine chain on the first carbon. Norepinephrine has a hydroxyl group on the ethyl amine and epinephrine has a hydroxyl group and methyl group on the ethyl amine.

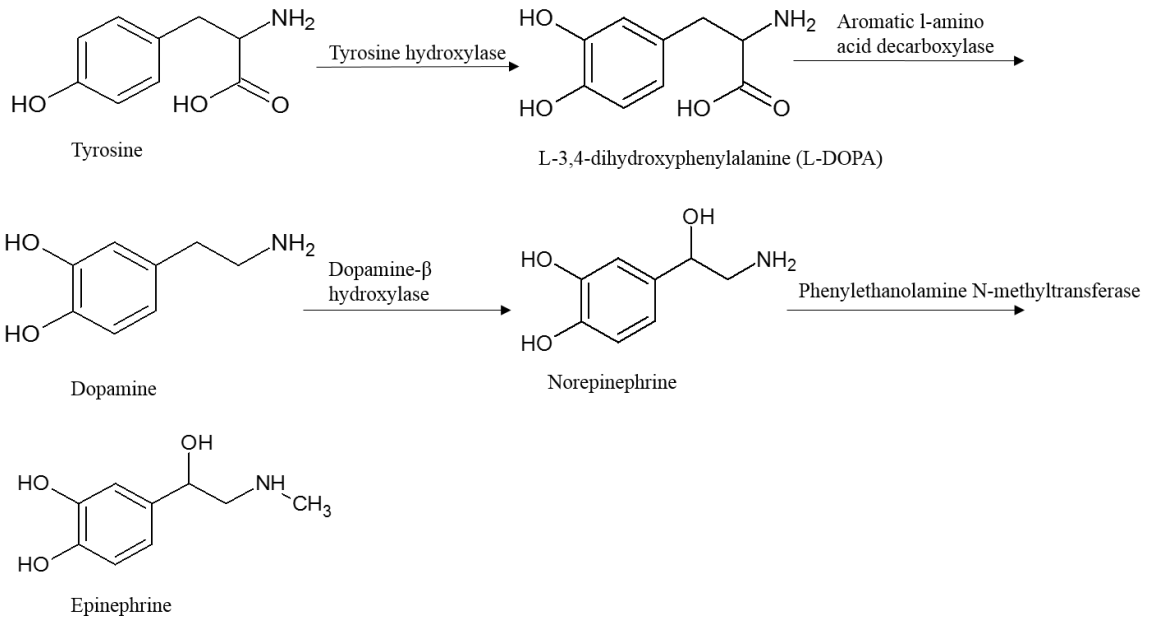

Figure 2. Synthesis of catecholamines
Catecholamines are synthesized in a series of enzymatic reactions; tyrosine is hydroxylysed to 3, 4-dihydroxyphenylalanine (l-DOPA) by tyrosine hydroxylase, l-DOPA is decarboxylated to dopamine by aromatic l-amino acid decarboxylase, dopamine is hydroxylated to norepinephrine by dopamine-β hydroxylase, and norepinephrine is converted to epinephrine by phenylethanolamine N-methyltransferase.

During synthesis, tyrosine is converted to 3, 4-dihydroxyphenylalanine (L-DOPA) in a reaction catalyzed by the enzyme tyrosine hydroxylase, L-DOPA is converted to dopamine by aromatic L-amino acid decarboxylase, dopamine is converted to norepinephrine by dopamine β-hydroxylase, and norepinephrine is converted to epinephrine by phenylethanolamine N-methyltransferase (2).

Due to their structure, catecholamines are categorized as amphoteric (react as an acid or base) compounds. In acidic conditions, the amine groups are protonated while the catechol group remains un-charged. In alkaline conditions, the catechol groups are oxidized to quinones making them electroactive. Catecholamines are also polar and hydrophilic.

Dopamine has been recognized as an intermediate for the formation of norepinephrine and epinephrine (3). It is abundant in the brain and is mainly responsible for initiation and maintenance of movement, alertness, anxiety, vision, and smell. Dopamine controls the pleasure centers in the brain, which can have an effect on drug addiction. Dopamine also plays a role in regulation of sodium excretion (4). Physiological disorders affecting release and transport of dopamine in the brain have been linked to Parkinson disease (4). It has been suggested that the degeneration of dopaminergic neurons in the brain affects communication across the brain, which leads to symptoms of Parkinson's disease (5).

In some cases, genetic disorders can produce excess dopamine. For example, deficiency of dopamine β-hydroxylase, an enzyme that oxidizes dopamine to norepinephrine, leads to excess dopamine and consequently low concentrations or lack of norepinephrine and epinephrine. This condition has been linked to schizophrenia and other psychiatric disorders (6).

Norepinephrine is mainly produced in the sympathetic nervous system and helps regulate the physiological functions of several organs in the body. It plays a role in cardiovascular regulation during stress by increasing heart rate, constricting peripheral arterioles, dilating skeletal arterioles, and elevating blood pressure. Norepinephrine concentration in plasma depends on the physiological and pathological states of the human body. For example, norepinephrine is produced in large quantities during exercise, hypertension, cardiac failure, depression, and during mental stress (4).

Epinephrine is mainly produced in the chromaffin cells in the adrenal medulla. It is released in stressful situations and plays similar roles as norepinephrine. Epinephrine

is released in larger concentrations in response events including hypoglycemia, hypotension, asphyxiation, circulatory response, and distress compared to norepinephrine. This indicates that the adrenal medulla system is greatly activated in such events compared to the sympathetic nervous system responsible for norepinephrine (7). It also plays a role in lipolysis (breakdown of fat to generate energy), ketogenesis (release of ketones when fat is break down for energy), thermogenesis (burning of calories to produce heat), and glycolysis (breakdown of glucose to extract energy), and can activate the pulmonary system by dilating the veins (4). Excess concentrations of epinephrine are also observed in Addison's disease, which is an autoimmune disease characterized by an impaired adrenal cortex in the adrenal glands (8).

Like other hormones, catecholamines are usually metabolized to terminate their actions. Numerous enzymes are responsible for their metabolism, which leads to a variety of metabolites. The primary enzymes involved are monoamine oxidase (MAO), catechol-O-methyltransferase (COMT), and alcohol dehydrogenase (ADH). Dopamine is first O-methylated by COMT and later deaminated by MAO to form its final metabolite, homovanillic acid (HVA) . Norepinephrine is O-methylated by COMT to form normetanephrine, which is later oxidized by alcohol dehydrogenase (ADH) to form its final metabolite vanillylmandelic acid (VMA). Epinephrine is O-methylated by COMT to form metanephrine, which is later metabolized by MAO and ADH to also form VMA (see Figure 3) (1, 2, 9).

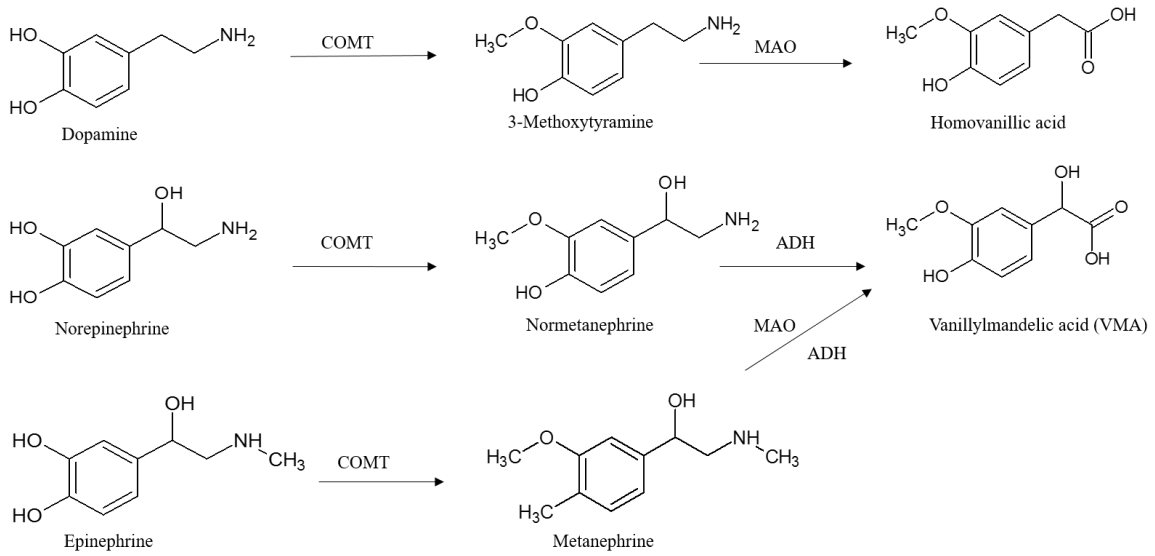

Figure 3 Metabolism of catecholamines
COMT and MAO convert dopamine to 3-Methoxytyramine and its final metabolite homovanillic acid (HVA), respectively. COMT and ADH convert norepinephrine to normetanephrine and to its final metabolite VMA. COMT, MAO, and ADH convert epinephrine to metanephrine its final metabolite VMA.

Clinical significance

Excess concentrations of catecholamines have been observed in patients suffering from rare neuroendocrine tumors such as adrenal pheochromocytomas that form in the chromaffin cells and extra-adrenal sympathetic paragangliomas (PPGLs), which form in the extra adrenal tissues of the abdomen, pelvis, and chest. Pheochromocytomas and PPGLs occur in 1-4 patients per 100,000 (2). Only 10-15% of the tumors are diagnosed as malignant while most are benign. Symptoms associated with excess concentrations of catecholamines include headaches, excess sweating, palpitations, and hypertension. These tumors are extremely difficult to diagnose as the signs and symptoms are similar to those observed in other diseases. High concentrations of catecholamines over a prolonged period can lead to severe health conditions such as chronic hypertension,

myocardial infarction, stroke, and cardiovascular disease (2).

Although measurement of catecholamines in plasma has been used as a screening test for pheochromocytomas and PPGLs, this testing is not recommended for initial evaluation of a potential tumor. Catecholamine metabolites such as metanephrine and normetanephrine are found in higher concentrations and have a longer half-life (are more stable). Testing of these metabolites is preferred for higher sensitivity and accuracy (1, 2).

However, catecholamines assays are still important for the following reasons: as a screen for PPGLs that only excrete dopamine (10) which is not metabolized into metanephrine or normetanephrine, to evaluate the role of dopamine in development of Parkinson's disease, to screen for dopamine β-hydroxylase deficiency which is associated with excess dopamine and low epinephrine and norepinephrine concentrations (4, 5), and to provide supplementary information in evaluation of clinical symptoms of excess catecholamines (11). In addition, catecholamines play an important role in neuroscience. Impaired synthesis, metabolism, and transportation of neurotransmitters, including catecholamines, can affect the body's homeostasis (12). Catecholamines norepinephrine and epinephrine have been explored as possible biomarkers in diseases such as diabetes, heart disease, pain, and anxiety (13). Therefore, the study of catecholamines and their metabolites can help in disease diagnosis, neurophysiology, understanding behavioral effects, pathology of diseases, and treatment therapies. These reasons as well as the fatal consequences associated with excess concentrations of catecholamines emphasize the need for rapid, accurate, and precise biochemical assays (2).

Literature review

Measurement of catecholamines

Measurement of catecholamines (dopamine, norepinephrine, and epinephrine) in plasma was previously performed using various analytical methods, including fluorometric, spectrophotometric, radioenzymatic, gas chromatography (GC), and high performance liquid chromatography (HPLC) techniques. The method used in most clinical laboratories is HPLC with electrochemical detection (HPLC-EC) due to its high sensitivity (14). However, one popular HPLC-EC method presents multiple challenges including large sample volume requirements (> 2 mL), long sample preparation time, and a lengthy chromatographic separation time (20-minutes injection-to-injection). In addition, the assay uses alumina at a basic pH (~ 8.5) for optimum sample extraction. Catecholamines are readily oxidized at this pH and results may be compromised (14). Matrix interferences seen in poorly resolved chromatograms are also a concern. Due to these issues, a method with higher selectivity and throughput is desired for the assay.

Multiple scientists have suggested that liquid chromatography with tandem mass spectrometry (LC-MS/MS) meets the desired criteria for this assay. Compared to electrochemical detection, LC-MS/MS provides more options for chemical analysis in terms of mass resolution (selectivity) and throughput. High selectivity is achieved in the mass spectrometer by pairing the retention time, precursor-product mass transitions to selectively identify compounds. Multiple reaction monitoring (MRM) is also used in LC-MS/MS to monitor specific precursor-product mass transitions therefore increasing selectivity and accuracy by reducing back ground noise in the detector (10, 11, 14, 15, 16). In cases where multiple compounds share similar precursor masses, LC-MS/MS is

able to distinguish them bases on their fragmentation patterns (4).

High throughput catecholamine assays have successfully been developed using ultra-performance liquid chromatography (UPLC-MS/MS) instruments. Chengjie et al. (2011) reported on a norepinephrine and epinephrine assay using UPLC-MS/MS with an (injection-to-injection) time of 3.5 minutes (11).

In the MRM technique, the sample is injected into the LC column where the analytes are separated based on their interaction with the stationery phase. Once the analytes are eluted from the LC column, they are ionized using electrospray ionization (ESI) before entering the first quadrupole of the mass spectrometer. The precursor ion (analyte of interest) is identified and directed into the quadrupole collision cell where it is fragmented into product ions. The product ions are directed into the third quadrupole filter where specific product ions are selected for detection (see Figure 4) (4).

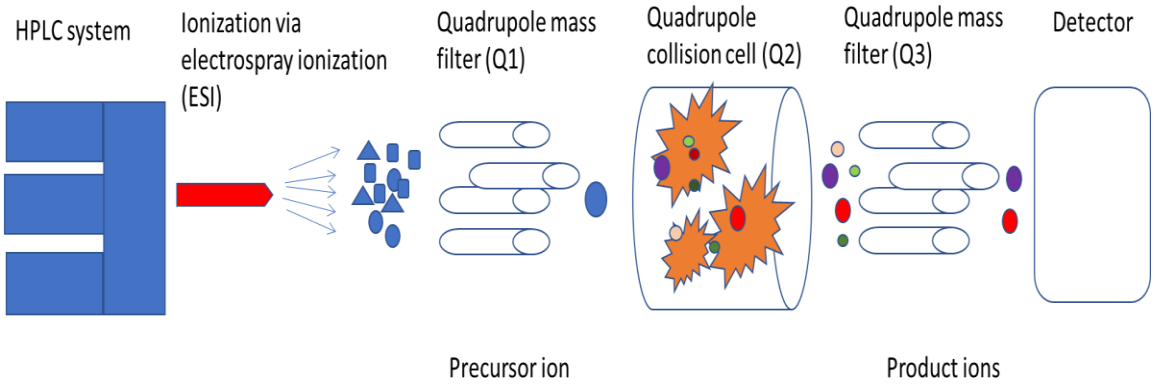

Figure 4 – Liquid chromatography mass spectrometry (LC-MS/MS) instrumentation
Samples are injected into the HPLC pump and separated in the column. The analytes are eluted at different retention times (RT) into the ionization chamber. Using MRM the precursor ion is selected in Q1 and fragmented in Q2. Specific product ions are selected in Q3 for analysis in the detector.

Despite the advantages LC-MS/MS offers, developing a plasma catecholamines assay using this technique poses some challenges, specifically the low concentrations of catecholamines in human plasma. Ji et al. (2010) reported that detection of the catecholamines (epinephrine and norepinephrine) could not be achieved at the required sensitivity because of two major reasons: the assay uses small plasma volumes and catecholamines are small, polar, and not easily ionized at the conditions compatible with chromatography (14). In addition, matrix effects due to co-eluting substances in biological samples can suppress the ionization of amine-containing compounds including catecholamines (15). For these reasons, it was suggested that derivatization of catecholamine samples would be necessary for LC-MS/MS analysis (14).

Derivatization

Derivatization is the chemical transformation of molecules to improve detection and measurement in instruments including LC-MS/MS. Derivatization of amine-containing compounds can increase their size, molecular weight, hydrophobicity (non-polar surface area), and can transform the primary/secondary amine functional groups into tertiary amines which are easily protonated (17). These effects are responsible for increased ionization efficiency (IE) and sensitivity. IE is the ease with which molecules in the liquid phase in the MS/MS are transformed to gas phase ions and reach the detector. The increase in IE and sensitivity can be explained as follows: larger molecules are more stable and therefore can more easily reach the detector once ionized; increase in hydrophobicity allows the derivatives to migrate to the surface of the liquid droplets in the ionization chamber, which therefore increases the rate of ionization and the tertiary

amines formed are easily protonated due to the increased electronegative induction on the nitrogen by the added substituents (17).

Finding suitable reagents for derivatization can be challenging. Derivatizing agents should encompass some of the following features: ability to introduce or increase a permanent charged moiety on the derivative, to yield a derivative that will fragment, and to react under mild reaction conditions. Derivatizing agents should have low molecular weight (some mass spectrometers have molecular weight detection limits) and be of reasonable cost (18).

Some of the earliest reported derivatization experiments on biogenic amines were performed on amino acids in the 1970s. A common technique was esterification (reaction of a carboxylic acid and an alcohol to produce an ester and water) using dimethylformamide dimethyl-acetal (DMF-DMA) to produce formamidene esters. The formamidene esters had increased spectral sensitivity in GC-MS (16, 19). This technique was later replaced by silyl and N-fluoroacyl ester derivatives, which provided greater mass spectral sensitivity enhancement (20). Recently, derivatization using DMF-DMA has been resurrected for use in amino acid analysis using electrospray ionization tandem mass spectrometry (ESI-MS/MS) (16). David et al. (2001) reported that the esterification of amino acids using DMF-DMA and *n*-butanol to produce formamidene butyl esters increased the mass spectral sensitivity 20 fold. Some major strengths of this derivatization technique included a short reaction time and high specificity since there was no evidence of derivatization of the other functional groups such as hydroxyl, thiol, or amides that are found on certain amino acids. However, the derivatization required two steps and some of the reagents were costly (16).

Another derivatization technique used on amino acids was acylation. Acylation is an organic reaction used to add an acyl group (RCO) to a compound. Wen et al. (2006) reported acylation of amino acids using N-hydroxysuccinimide-activated N-alkylnicotinic acid esters (C_n-NA-NHS). This derivatization technique using C_n-NA-NHS, which contains a quaternary amine (R_4N^+), aimed to further improve sensitivity by increasing the charge on the amine group of the hydrophobic chain. Sensitivity was greatly improved by the combined effects of increased charge on the derivative (quaternary amine) and introduction of a larger/longer hydrophobic alkyl chain (hydrophobicity). The same study also observed that increasing the chain length of the derivatizing agent C_n-NA-NHS led to increased sensitivity. Despite the significant increase in sensitivity, the technique presented a number of challenges including the time required to synthesize the derivatizing reagent C_n-NA-NHS from diclyclohexyl caarbodimide, dimethyl formamide, nicotinic acid, and N-hydroxysuccinimide), and the cost of the reagents (15).

In the early 2000s, several scientists reported on stable isotope dimethyl labelling for use in proteomics analysis (21, 22, 23). Guo's (2007) study on dimethylation via reductive amination was one of the most promising reports. The study described a simple, fast, specific, and mild derivatization technique that could be used to improve quantification of amine-containing metabolites (23). This technique involves chemically labelling amino acids with isotopically labeled formaldehyde using reductive amination to create inexpensive internal standards (IS). Formaldehyde and isotopically labeled formaldehyde (formaldehyde-^{13}C, d_2- formaldehyde) were used as derivatizing agents in the presence of a reducing agent (sodium borohydride) to add stable isotope tags to the

amine groups in the metabolites. The labelled isotopes were stable for two weeks at room temperature and longer at –20 ^{o}C and no byproducts were observed (23).

Although this labelling technique was used in the preparation of stable isotopically labeled amino acids, the same approach could be applied in derivatization techniques used to enhance catecholamine sensitivity in LC-MS/MS. Catecholamines, like amino acids, are small, polar, and contain primary and secondary amine groups and are therefore expected to react similarly.

Multiple scientists adopted the Guo et al. (2007) technique in development of plasma catecholamine assays (11, 14). Chengjie et al. (2008) reported the successful quantification of norepinephrine, dopamine, and neurotransmitters including serotonin, and normetanephrine in rat prefrontal cortex micro dialysates using reductive amination. The neurotransmitters were derivatized using acetalydehyde-d_4 in the presence of sodium cyanoborohydride (a reducing agent) in a mild reaction (37 ºC, 25 minutes). This derivatization technique combined with ultra-performance liquid chromatography mass spectrometry (UPLC/MS/MS) yielded 20-100 times increased sensitivity (11). Chengjie et al. (2010) also reported ~16-80 fold increased sensitivity for epinephrine and norepinephrine derivatized using acetalydehyde-d_4 (14).

Measurement of catecholamines in plasma using LC-MS/MS is still a new technique in most clinical laboratories as most assays are still performed using HPLC-EC. Some clinical laboratories prefer HPLC-EC analysis of catecholamines because sample preparation is more straightforward (no derivatization required) and the oxidation vulnerability of catecholamines aids in their measurement using electrochemical detectors (24). Although LC-MS/MS is preferred for its higher specificity and

throughput, the main challenge in the development of catecholamines assays using LC-MS/MS remains their low plasma concentrations and consequent required sensitivity (11, 14). With the significant improvement of sensitivity via a reductive amination derivatization strategy, the future looks promising for development of assays using this technique.

Current research

The literature suggests that measurement of catecholamines using LC-MS/MS is feasible and has multiple advantages compared to HPLC-EC. Development of a catecholamines assay in plasma using LC-MS/MS is currently ongoing at ARUP Laboratories. As mentioned earlier, increasing the sensitivity of catecholamines in the mass spectrometer has been the main challenge facing scientists (11, 14). Derivatization using straight-chain aldehydes (acetaldehyde-C2, butyraldehyde-C4, hexanal-C6, and octanal-C8) via reductive amination has previously been evaluated to determine the most effective aldehydes on sensitivity. However, a few questions remain on the development of this assay: what effect would other types of aldehydes have on sensitivity and what other reaction conditions could be evaluated to further increase the sensitivity? In this research, a series of straight-chain and branched-chain aldehydes were evaluated as derivatizing agents via reductive amination to analyze their effect on sensitivity. Reaction conditions including reagent concentrations and incubation time and temperature were also optimized for further sensitivity enhancement. Method application on patient samples using the most effective straight-chain (hexanal) and branch-chain

(hydrocinnamaldehyde) aldehydes were conducted to determine their performance in a clinical setting.

MATERIALS AND METHODS

Chemicals and reagents

Dopamine hydrochloride, DL-Norepinephrine hydrochloride, (+/-) Epinephrine hydrochloride, sodium cyanoborohydride, ammonium acetate (NH_4OAc), and sodium phosphate (Na_2HPO_4) were purchased from Sigma Aldrich. Straight-chain aldehydes: propionaldehyde, butyraldehyde, valeraldehyde, hexanal, heptaladehyde, octanal, and branched-chain aldehydes: 3, 3- dimethylbutyraldehyde, 2-methylpentanal, 2-ethylhexanal, hydrocinnamaldehyde, and 3-phenylbutyraldehyde, were also purchased from Sigma Aldrich. Formic acid (98%) was obtained from EMD Millipore Corporation and HPLC grade methanol from JT Baker. Clinical reagent water (CLRW) was produced in the laboratory using a ThermoScientific pump.

Individual standard catecholamine samples were prepared as follows: 5 mmol/L of dopamine was prepared by dissolving 9.5 mg of dopamine hydrochloride in 10 ml of CLRW, 5 mmol/l of norepinephrine was prepared by dissolving 10.2 mg of norepinephrine hydrochloride in 10 ml of CLRW, and 5 mmol/L of epinephrine was prepared by dissolving 10.9 mg of epinephrine chloride in 10 ml CLRW. The stock solution of each catecholamine was serial diluted to various concentrations used in the study and stored in a -70^{o} C for use. The catecholamines standard was prepared by mixing 1 ml of the dopamine, norepinephrine, and epinephrine stocks prepared above. The stock solution was serial diluted to different concentrations used in the study and

stored in a -70º C for use.

NH_4OAc (200 mmol/L) was prepared by first dissolving 1542 mg NH_4OAc in 50 ml CLRW. The pH was adjusted using acetic acid to ~ 5.3. The aqueous solution was diluted to 100 ml and stored at room temperature. Na_2HPO_4 (20 mmol/L) was prepared by dissolving 283.9 mg Na_2HPO_4 in 100 ml of CLRW and adjusting the pH to 7.2 using hydrochloric acid.

Catecholamines calibrators (standards) for analysis of patient samples were prepared in 0.5% acetic acid with 1 mg/ml sodium metabisulfite (AASM). The stock solution was prepared by pipetting AASM (760 µL), dopamine stock (40 µL), epinephrine (40 µL), and norepinephrine (160 µL) into a microcentrifuge tube. The stock solution concentration was 200/800 µmol/L (200 µmol/L dopamine/epinephrine and 800 µmol/L norepinephrine). The calibrator catecholamines concentrations reflect their reference intervals in the clinical laboratory. The stock was serial diluted to the other calibrator's concentrations (CAL 2 -50/200, CAL 3 - 200/800, CAL 4 - 500/2000, CAL 5 - 5000/20000) pmol/L. Plain water was used for the blank (calibrator preparation protocol adopted from Melissa Hughs PhD., ARUP laboratories). Low and high quality controls were acquired from Bio-Rad laboratories. Plasma patient samples were acquired from the analytic biochemistry laboratory.

Derivatization via reductive amination

Derivatization was performed by reacting catecholamines with aldehydes in the presence of a reducing agent, sodium cyanoborohydride ($NaBH_3CN$). The samples were incubated for 30 minutes at 37ºC and later quenched with formic acid. In this study,

derivatization was accomplished using a generic sample preparation protocol adopted from Guo et al. (2007) and modified by Melissa Hughs, Ph.D., ARUP laboratories. Ammonium acetate (100 µL, 0.2 M, pH 5.82), dopamine/norepinephrine/epinephrine stock (20 µL, 50 nm), sodium cyanoborohydride (25 µL, 0.4 M) prepared fresh daily, and aldehyde (20 µL, 1 M in methanol) were pipetted into a 96 well plate format. The aqueous samples were incubated at 37°C for 30 minutes and quenched with formic acid (20 µL, 1 M) for 5 minutes at room temperature before injection in the LC-MS/MS instrument.

In the beginning of the study, epinephrine was derivatized using 5 µL neat aldehyde per the method adopted from Guo et al. (2007). Pipetting small volumes was challenging and compromised the accuracy and precision of our data. Therefore, we decided to use an aldehyde solution in methanol (20 µL, 1 M in methanol) for the dopamine and norepinephrine derivatization reactions.

Straight-chain aldehydes: propionaldehyde (C3), butyraldehyde (C4), valeraldehyde (C5), hexanal (C6), heptaladehyde (C7), octanal (C8), and branched-chain aldehydes: 3, 3- dimethylbutyraldehyde (3DB), 2-methylpentanal (2MP), 2-ethylhexanal (2EH), Hydrocinnamaldehyde (HC), and 3-phenylbutyraldehyde (3PB), were used as derivatizing agents (see Figure 5).

The catecholamines standards (dopamine, norepinephrine, and epinephrine) were derivatized individually in triplicate with each aldehyde on three separate days. The derivatization was carried out in sets using straight or branched-chain aldehydes.

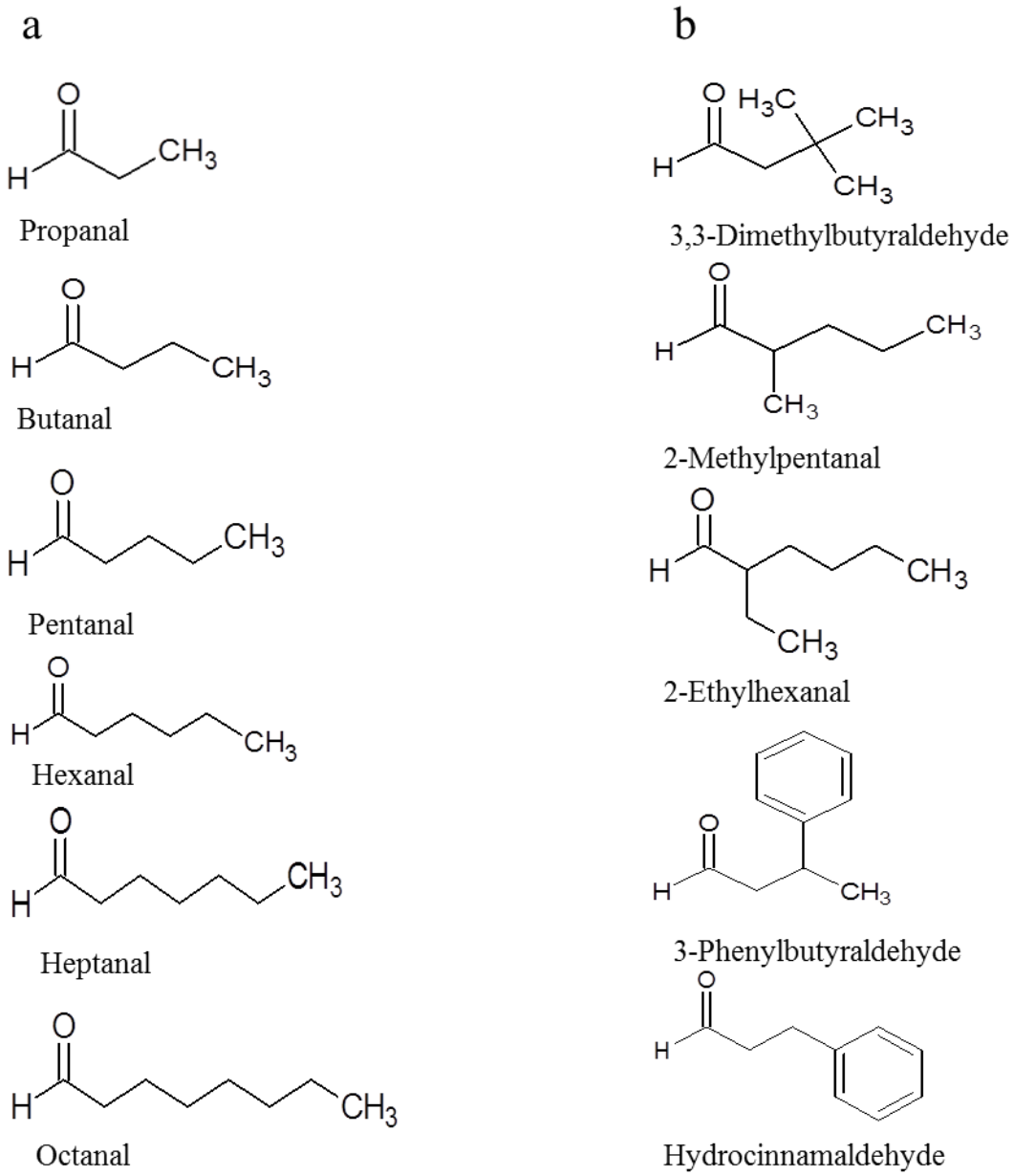

Figure 5. Straight-chain and branched-chain aldehydes

Aldehydes are organic compounds composed of a carbonyl bonded to a hydrogen atom and an R group (alkyl or side chain). a) Straight-chain aldehydes are composed of a carbonyl with a straight alkyl chain. b) Branched-chain aldehydes are composed of a carbonyl with a branched-alkyl chain.

Optimization of a hydrocinnamaldehyde derivatization protocol

Experiments were carried out to optimize a derivatization protocol for hydrocinnamaldehyde, the branched-chain aldehyde that provided the best sensitivity (derivatization for hexanal had been completed). Optimization experiments were performed to establish $NaBH_3CN$ concentration, hydrocinnamaldehyde concentration in methanol, and on incubation time and temperature in that order.

$NaBH_3CN$ concentration

Derivatization of the three catecholamines (in one reaction) was performed at different $NaBH_3CN$ concentrations. The other reagent concentrations were similar to the generic protocol described above. Ammonium acetate (100 µL, 200 mmol/L, pH 5.82) and catecholamines stock (20 µL, 50 nmol/L) were added to eight different wells of a 96 well plate format. $NaBH_3CN$, 25µ L of each concentration (25 mmol/L, 50 mmol/L, 100 mmol/L, and 400 mmol/L), was added to two of the eight wells (each concentration was analyzed in duplicate). Hydrocinnamaldehyde dissolved in methanol (20 µL of 1000 mmol/L) was pipetted into the wells. The aqueous samples were incubated at 37 °C for 30 minutes and quenched with formic acid (20 µL, 1000 mmol/L) for 5 minutes at room temperature. The samples (20 µL) were injected in an LC-MS/MS system for analysis.

Hydrocinnamaldehyde concentration

Derivatization of the three catecholamines (in one reaction) was performed at different hydrocinnamaldehyde (in methanol) concentrations. Ammonium acetate (100 µL, 0.2 mmol/L, pH 5.82), catecholamines stock (20 µL, 50 nm), and $NaBH_3CN$ optimal

concentration (25 µL, 50 mmol/L) were added to eight different wells in a 96 well plate format. Hydrocinnamaldehyde dissolved in methanol (20 µL) of each concentration (250 mmol/L, 500 mmol/L, 1000 mmol/L, and 2000 mmol/L) was added to two of the eight wells (each concentration was analyzed in duplicate). The aqueous samples were incubated at 37°C for 30 minutes and quenched with formic acid (20 µL, 1000 mmol/L) for 5 minutes at room temperature. The samples (20 µL) were injected in an LC-MS/MS system for analysis.

Reaction conditions – time and temperature

Derivatization of the three catecholamines (in one reaction) was performed at different incubation times and temperatures: 25 ºC for 10, 15, and 20 minutes, and 37 ºC for 15, 20, and 30 minutes. Ammonium acetate (100 µL, 200 mmol/L, pH 5.82), catecholamines stock (20 µL, 50 nmol/L), $NaBH_3CN$ (25 µL, 50 mmol/L), and hydrocinnamaldehyde dissolved in methanol (20 µL of 500 mmol/L) were pipetted into vials. A set of two vials were incubated at 25 ºC for each time, 10, 15, or 20 minutes, and at 37 ºC for 15, 20, or 30 minutes. The samples were quenched with formic acid (20 µL, 1000 mmol/L) for 5 minutes at room temperature. The samples (20 µL) were injected into the LC-MS/MS instrument for analysis.

Comparison aldehydes – hexanal versus hydrocinnamaldehyde

Catecholamines were derivatized in triplicate using the respective optimized protocols for hexanal and hydrocinnamaldehyde. The hexanal derivatization protocol was adopted from Melissa Hughs Ph.D. ARUP Laboratories; ammonium acetate (100

µL, 200 mmol/L, pH 5.82), catecholamines stock (20 µL, 50 nmol/L), $NaBH_3CN$ (25 µL, 100 mmol/L), and hexanal dissolved in methanol (20 µL of 1000 mmol/L) were pipetted into three wells in a 96 well plate format. The optimized hydrocinnamaldehyde derivatization was performed using the following protocol (from my study); ammonium acetate (100 µL, 200 mmol/L, pH 5.82), catecholamines stock (20 µL, 50 nmol/L), $NaBH_3CN$ (25 µL, 50 mmol/L), and hydrocinnamaldehyde dissolved in methanol (20 µL of 500 mmol/L) were pipetted into three wells of the same 96 well plate format. The samples were incubated at 37°C for 30 minutes, and quenched with formic acid (20 µL, 1000 mmol/L) for 5 minutes at room temperature. The samples (20 µL) were injected into the LC-MS/MS instrument for analysis.

Method application using hydrocinnamaldehyde

Five repeat plasma patient samples previously analyzed using HPLC-EC and one plasma patient pool were derivatized using HC. A 5 point calibration system 0 pmol/L, 50/200 pmol/L, 200/800 pmol/L, 500/2000 pmol/L, and 5000/20000 pmol/L (first concentration for dopamine and epinephrine, second concentration for norepinephrine) was used for quantification. A 96 weak cation exchange phenomenex plate was first conditioned with methanol (1 ml) and later with Na_2HPO_4 (1 ml, 20 mmol/L). Calibrators, controls, and patient samples (500 µL) were pipetted into the plates followed by Na_2HPO_4 (500 µL). The plates were washed with NH_4OAC (1 ml, 10 mmol/L) and methanol (1 ml) then dried with nitrogen for 60 seconds. The catecholamines were eluted with 5% formic acid in methanol (2x300 µL) into a 96 well plate. The eluent was dried with nitrogen gas at 37ºC for 20-30 minutes.

The precipitated catecholamines were derivatized using an optimized hydrocinnamaldehyde derivatization protocol; ammonium acetate (100 µL, 200 mmol/L, pH 5.82), catecholamines stock (20 µL, 50 nmol/L), $NaBH_3CN$ (25 µL, 50 mmol/L), and hydrocinnamaldehyde dissolved in methanol (20 µL of 500 mmol/l) were pipetted into the 96 well plate. The samples were incubated at 37ºC for 30 minutes and later quenched with formic acid (20 µL, 1000 mmol/L) for 5 minutes at room temperature. The samples (20 µL) were injected into the LC-MS/MS instrument for analysis.

LC-MS/MS

Chromatography conditions

The LC-MS/MS system consisted of an Agilent Technologies HPLC 1260 Infinity binary system coupled to an ABSCIEX Triple Quad 5500 mass spectrometer. Samples (20 µL) were injected into a reversed phase (RP) Phenomenex C18 column (50 x 2.1 mm, 2.6 µm particle size, 100-Å pore size). The RP mobile phase A (aqueous) was 0.1% formic acid in CLRW and mobile phase B (organic) was 0.1% formic acid in methanol. The formic acid, CLRW, and methanol used were LC-MS grade. Quantitation was carried out using ABSCIEX MultiQuant 3.0 software.

A 17-minute binary gradient elution profile was used for this research project. The gradient was set up as follows: t = 0 - 1 minute (99% A, 1% B), t = 1 - 11 minutes (5% A, 95% B), t = 11 - 14 minutes (5 A, 95% B), t = 14 - 17 minutes (99% A, 1% B). Other chromatography settings were as follows: flow rate = 0.3 ml/min, temperature = 40 ˚C and equilibration time = 0 minutes (gradient adopted from Melissa Hughs, PhD. ARUP Laboratories).

Mass spectrometry methods

A MRM mass spectrometry method was determined for each dopamine, norepinephrine, and epinephrine derivative before analysis. The LC-MS/MS was infused with a high concentration (250 µmol/L of dopamine, norepinephrine, or epinephrine) derivatization solution to optimize the mass spectrometer parameters. The precursor ion for each aldehyde derivative was first identified in quadruple mass spectrometer 1 (Q1 MS1), then the following parameters were optimized: voltage required to ionize the precursor ion in aqueous solution from the HPLC (declustering potential/DP), voltage required to move the precursor ion into the collision cell (entrance potential/EP), voltage required for fragmentation of the precursor ion into each product ion (this was performed for at least five transitions for each catecholamines derivative), and the voltage required to move the product ions to the detector (collision exit potential/CEX). An optimized method for each individual catecholamine derivative using the aldehydes was created in the mass spectrometer analyst software (see Appendix Tables 12 and 13).

A MRM mass spectrometry method was developed for hydrocinnamaldehyde derivatization optimization and method application experiments (a hexanal catecholamines MRM method was adopted from Melissa Hughs, Ph.D. ARUP laboratories). The method was developed using the three catecholamines in one reaction. The LC-MS/MS was infused with a high concentration (250 µmol/L) of hydrocinnamaldehyde catecholamine derivative solution to optimize the mass spectrometer parameters (DP, EP, CE, and CEX) (see Appendix Table 14).

RESULTS

Straight-chain aldehydes

Dopamine, norepinephrine, and epinephrine were derivatized using C3, C4, C5, C6, C7, and C8. Each derivative was identified by retention time and mass fragmentation pattern; peak area counts were determined for the three transitions leading to the most abundant ions.

Dopamine

Formation of mono-alkylated and di-alkylated derivatives was observed for C3 and C4. C5, C6, and C7 produced only the di-alkylated dopamine derivative. No derivative peaks were observed for C8. The straight-chain dopamine derivatives had similar mass fragmentation patterns to each other. The three most abundant product ions for all the derivatives were 137, 91, and 65 (see Table 1 and Figure 6).

Norepinephrine

All the aldehydes transformed norepinephrine into a di-alkylated derivative. The C6 derivative had the highest sensitivity followed by the C5, C3, and C4derivatives. No derivatives were observed for C7 and C8. The norepinephrine derivatives had significantly different fragmentation patterns for each aldehyde (see Table 2 and Figure 6).

Table 1. Straight-chain aldehydes dopamine derivatization peak area count (n = 9)

Straight-chain aldehydes				
Dopamine mono-alkyl derivatives				
Aldehydes	**Mass transitions**	**Mean*e4**	**SD*e4**	**%CV**
C3	196 / 137	618.71	33.25	3.47
	196 / 91	405.93	33.56	6.49
	196 / 65	163.61	12.97	6.30
C4	210 / 137	31.89	2.62	5.38
	210 / 91	21.15	2.29	4.79
	210 / 65	13.85	0.23	0.54
Dopamine di-alkyl derivatives				
	Mass transitions	**Mean*e4**	**SD*e4**	**%CV**
C3	238 / 137	360.88	9.77	2.03
	238 / 91	321.29	10.01	2.33
	238 / 65	136.55	2.92	1.60
C4	266 / 137	33.87	4.83	10.76
	266 / 91	30.12	4.92	12.40
	266 / 65	11.46	1.95	12.63
C5	294 / 137	293.42	22.74	5.95
	294 / 91	252.19	19.17	5.76
	294 / 158	101.35	7.86	5.88
C6	322 / 137	899.14	77.46	6.39
	322 / 91	808.81	57.43	5.09
	322 / 65	346.91	30.35	6.66
C7	350 / 137	56.17	6.78	8.52
	350 / 91	57.09	6.28	9.18
	350 / 65	23.97	3.74	11.69
C8	-	-	-	-
	-	-	-	-
	-	-	-	-

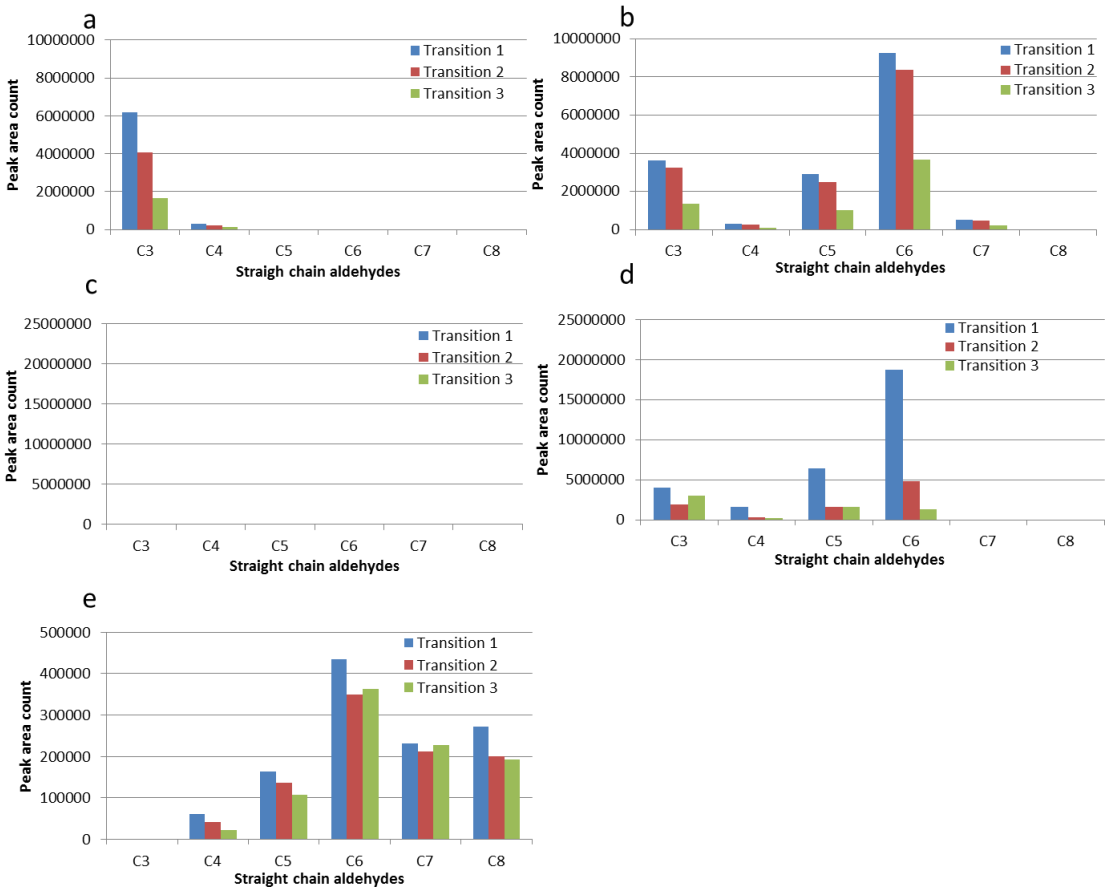

Figure 6. Evaluation of derivatized catecholamines using straight-chain aldehydes
Sensitivity measured in peak area count of derivatized dopamine (a/b), norepinephrine (c/d), and epinephrine (e) using straight-chain aldehydes was analyzed. a) Only C3 and C4 yielded dopamine mono-alkyl derivatives. b) The C6 dopamine di-alkyl derivative had the highest sensitivity. c) No mono-alkyl norepinephrine derivatives were detected. d) The C6 di-alkyl norepinephrine derivatives had the highest sensitivity. e) The C6 epinephrine derivative had the highest sensitivity.

Table 2. Straight-chain aldehydes norepinephrine derivatization peak area count (n = 9)

Straight-chain aldehydes				
Norepinephrine mono-alkyl derivatives				
	Mass transitions	**Mean*e4**	**SD*e4**	**%CV**
C3	254 / 194	402.46	9.98	2.45
	254 / 107	302.99	8.52	2.76
	254 / 77	187.91	4.80	2.55
C4	282 / 264	162.72	36.43	20.50
	282 / 137	35.04	8.07	21.15
	282 / 107	19.35	4.31	20.78
C5	310 / 292	642.58	52.50	7.87
	310 / 137	161.72	13.91	8.25
	310 / 107	165.50	7.34	5.14
C6	338 / 320	1877.26	180.62	9.78
	338 / 137	481.60	49.74	10.31
	338 / 91	133.94	13.90	10.04
C7	-	-	-	-
	-	-	-	-
	-	-	-	-
C8	-	-	-	-
	-	-	-	-
	-	-	-	-

Epinephrine

Because epinephrine is a secondary amine, the di-alkylated product was not possible. A mono-alkylated derivative was observed with all the aldehydes. The epinephrine derivatives had similar fragmentation for each aldehyde (see Table 3 and Figure 6).

Branched-chain aldehydes

Dopamine, norepinephrine, and epinephrine were derivatized using 3DB, 2MP, 2EH, HC, and 3PB. Each derivative was identified by retention time and mass

Table 3. Straight-chain aldehydes epinephrine derivatization peak area count (n = 9)

Straight-chain aldehydes				
Epinephrine mono-alkyl derivatives				
	Mass transitions	**Mean*e4**	**SD*e4**	**%CV**
C3	-	-	-	-
	-	-	-	-
	-	-	-	-
C4	240 / 166	6.04	0.34	5.62
	222 / 166	4.12	0.36	8.80
	240 / 137	2.24	0.23	10.45
C5	254 / 166	16.34	2.19	10.55
	236 / 166	16.33	2.30	11.89
	236 / 137	12.85	1.88	11.43
C6	268 / 166	42.87	1.66	3.80
	268 / 137	35.05	1.46	4.39
	250 / 137	34.97	2.16	5.95
C7	282 / 166	19.63	2.04	8.74
	282 / 137	17.39	2.62	12.43
	264 / 166	19.32	2.70	12.57
C8	296 / 166	27.19	9.71	8.04
	296 / 137	23.12	8.34	7.84
	278 / 166	21.77	8.96	9.90

fragmentation pattern; peak area counts were determined for the three transitions leading to the most abundant ions.

Dopamine

Formations of mono-alkylated and di-alkylated derivatives were observed for 3DB, 2MP, 2EH, and 3PB. HC was the only aldehyde to yield the di-alkylated dopamine derivative. Some of the dopamine derivatives had similar mass fragmentation patterns; the 3DB, 2MP, 2EH derivatives most abundant product ions were 137 and 91 while the HC and 3PB most abundant product ions were 91 and 137 (see Table 4 and Figure 7).

Norepinephrine

Formations of mono-alkylated and di-alkylated derivatives were observed for 3DB and 2MP. HC and 3PB were the only aldehydes to yield the di-alkylated norepinephrine derivative. No product was observed using 2EH. The norepinephrine derivatives had distinctively different mass fragmentation patterns for each aldehyde (see Table 5 and Figure 7).

Epinephrine

A mono-alkylated derivative was observed for all the aldehydes. HC and 3PB epinephrine derivatives had the largest peak area counts. The epinephrine derivatives had similar fragmentation patterns for each aldehyde (see Table 6 and Figure 7).

Table 4. Branched-chain aldehydes dopamine derivatization peak area count (n = 9)

Branched-chain aldehydes				
Dopamine - monoalkyl derivatives				
Aldehydes	**Mass transitions**	**Mean*e4**	**SD*e4**	**%CV**
3DB	238 / 137	3216.79	113.10	3.46
	238 / 91	2302.53	121.36	5.37
	238 / 65	975.14	45.81	4.71
2MP	238 / 137	3223.56	49.59	1.56
	238 / 91	2248.33	44.29	1.99
	238 / 119	948.39	18.61	2.00
2EH	266 / 137	236.26	19.88	8.29
	266 / 91	163.45	16.98	10.30
	266 / 65	56.34	5.01	8.87
HC	-	-	-	-
	-	-	-	-
	-	-	-	-
3PB	286 / 137	14.98	14564.49	9.44
3PB	286 / 91	14.44	13503.25	9.06
3PB	286 / 65	5.49	4064.24	6.87
Dopamine - di-alkyl derivatives				
	Mass transitions	**Mean*e4**	**SD*e4**	**%CV**
3DB	322 / 137	1025.33	57.96	5.90
	322 / 91	965.79	61.26	6.37
	322 / 65	397.99	22.96	6.15
2MP	322 / 137	190.65	11.54	5.91
	322 / 91	134.51	8.51	6.35
	322 / 65	52.17	4.57	8.89
2EH	378 / 137	49.01	6.83	15.14
	378 / 91	46.55	6.53	14.65
	378 / 119	14.59	2.08	15.34
HC	390 / 91	299.55	225.88	4.79
	390 / 137	205.87	166.46	7.55
	390 / 254	136.09	114.38	5.26
3PB	418 / 91	74.43	5.40	7.43
	418 / 137	64.92	3.94	6.33
	418 / 282	36.79	1.97	5.81

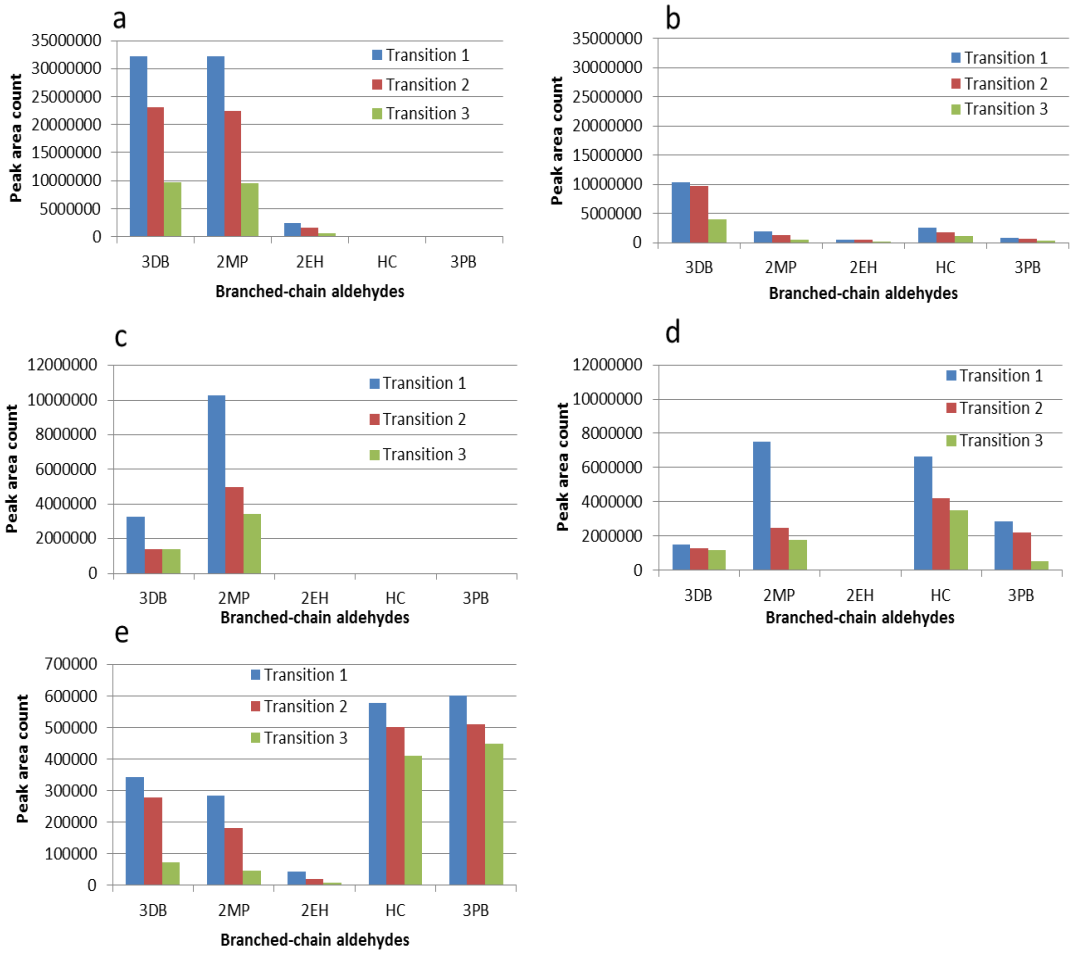

Figure 7. Evaluation of derivatized catecholamines using branched-chain aldehydes
Sensitivity measured in peak area count of dopamine (a/b), norepinephrine (c/d), and epinephrine (e) using branched-chain aldehydes was analyzed. a) 3DB, 2MP, 2EH, and 3PB were the only aldehydes that yielded dopamine mono-alkyl derivatives. b) HC was the only aldehyde to yield the dopamine di-alkyl derivative. However, the 3DB dopamine di-alkyl derivative had the highest sensitivity. c) 3DB and 2MP were the only aldehydes that yielded norepinephrine mono-alkyl derivatives. d) HC and 3PB were the only aldehydes to yield the complete norepinephrine di-alkyl derivatives. 2MP and HC norepinephrine derivatives had the highest sensitivity. e) HC and 3PB epinephrine mono-alkyl derivatives had the highest sensitivity.

Table 5. Branched-chain aldehydes norepinephrine derivatization peak area count (n = 9)

Branched-chain aldehydes				
Norepinephrine mono-alkyl derivatives				
Aldehydes	**Mass transitions**	**Mean*e4**	**SD*e4**	**%CV**
3DB	254-107	327.57	10.49	3.19
	254-57	138.92	7.46	5.38
	254-77	141.57	6.27	4.39
2MP	254-152	1028.35	26.78	2.60
	254-107	499.66	13.20	2.70
	254-135	341.18	10.52	3.07
2EH	-	-	-	-
	-	-	-	-
	-	-	-	-
HC	-	-	-	-
	-	-	-	-
	-	-	-	-
3PB	302-91	5245.87	695.39	14.86
	306-65	1332.41	181.59	15.06
	302-77	833.17	91.10	11.21
Norepinephrine di-alkyl derivatives				
	Mass transitions	**Mean*e4**	**SD*e4**	**%CV**
3DB	338-57	152.33	6.21	4.08
	338-106	129.96	6.93	5.37
	338-77	118.80	3.89	3.28
2MP	254-152	765.05	23.83	3.11
	254-107	260.18	12.65	4.91
	254-135	182.62	10.79	5.96
2EH	-	-	-	-
	-	-	-	-
	-	-	-	-
HC	406-388	649.64	36.40	5.56
	406-91	416.45	14.90	3.54
	406-137	334.71	18.91	5.68
3PB	302-91	311.24	25.03	8.19
	306-65	247.17	25.26	10.27
	302-77	57.58	4.43	7.84

Table 6. Branched-chain aldehydes epinephrine derivatization peak area count (n = 9)

Branched-chain aldehydes				
Epinephrine mono-alkyl derivatives				
Aldehydes	**Mass transitions**	**Mean*e4**	**SD*e4**	**%CV**
3DB	250 / 166	38.48	2.69	6.41
	268 / 166	30.27	2.46	7.72
	250 / 107	8.24	0.57	6.44
2MP	268 / 166	28.41	1.96	6.69
	250 / 166	18.03	1.29	6.60
	268 / 107	4.60	0.27	5.86
2EH	296 / 166	4.30	0.38	8.99
	278 / 166	1.98	0.17	8.35
	296 / 107	0.73	0.13	14.47
HC	302 / 137	57.58	4.61	7.90
	284 / 137	50.22	3.83	7.18
	302 / 180	41.10	3.37	7.97
3PB	316 / 137	60.06	7.01	10.45
	316 / 180	51.10	5.61	10.35
	298 / 137	44.89	3.05	6.45

Optimization of a hydrocinnamaldehyde derivatization protocol

$NaBH_3CN$ concentration

HC was used to derivatize the catecholamines in one reaction at different $NaBH_3CN$ concentrations including 25 mmol/L, 50 mmol/L, 100 mmol/L, 250 mmol/L, and 400 mmol/L. The ion corresponding to the mass transition with the largest peak area count of each derivative formed at each concentration was analyzed (see Figure 8).

Hydrocinnamaldehyde concentration (in methanol)

HC was used to derivative the catecholamines in one reaction at different concentrations including 250 mmol/L, 500 mmol/L, 1000 mmol/L, and 2000 mmol/L. The ion corresponding to the mass transition with the largest peak area count of each derivative formed at each concentration was analyzed (see Figure 8).

Time and temperature

Catecholamines were derivatized using HC in one reaction at 37˚C for 15, 20, and 30 minutes and at 25˚C for 10, 15, and 20 minutes. The ion corresponding to the mass transition with the largest peak area count of each derivative formed at each time and temperature was analyzed (see Figure 8).

Comparison of aldehydes - hexanal versus hydrocinnamaldehyde

Catecholamines were derivatized with hexanal (C6) and hydrocinnamaldehyde (HC) in one reaction using their optimal sample preparation protocols. Each catecholamine aldehyde derivative was identified by retention time and mass

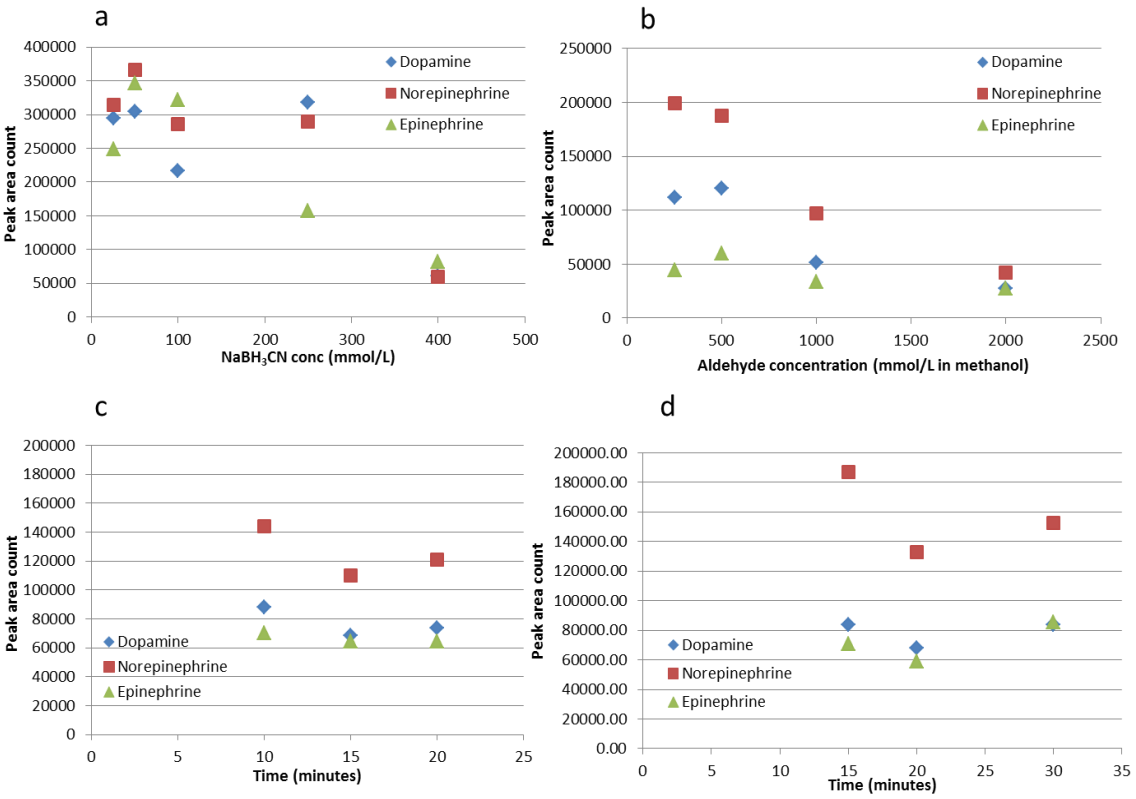

Figure 8. Hydrocinnamaldehyde derivatization optimization (n = 2)
$NaBH_3CN$ concentration, aldehyde in methanol concentration, and incubation time and temperature were evaluated for optimal hydrocinnamaldehyde derivatization conditions. The experiments were performed on the catecholamines in one reaction. a) The HCderivatives sensitivity initially increases with higher $NaBH_3CN$ concentration and peaks at 50 mmol/L before decreasing. b) The HC derivatives sensitivity generally decreases with higher aldehyde (in methanol) concentration. c) At 25˚C, the HC derivatives sensitivity decreases from 10 to 15 minutes and slightly increases at incubation time of 20 minutes. d) At 37˚C, the HC derivatives sensitivity decreases from 15 to 20 minutes and slightly increases at an incubation time of 30 minutes.

fragmentation pattern (see Figures 9 and 10); peak area counts were analyzed for the most abundant ions (see Table 7 and Figure 11).

Method application using hydrocinnamaldehyde

HC was used to derivatize one patient pool and five individual repeat patients' samples. Derivatized catecholamines were identified by retention time and mass fragmentation pattern; peak area count was determined for the most abundant mass transition.

Five calibrators including 0 pmol/L, 50/200 pmol/L, 200/800 pmol/L, 500/2000 pmol/L, and 5000/20,000 pmol/L were used (first calibrator concentration for dopamine /epinephrine and second concentration for norepinephrine). The measured calibrator concentrations were compared to the expected values for accuracy (see Table 8) and their calibration curves created (see Figure 12). Quality controls (low and high) and a patient pool (analyzed triplicate) were used to evaluate precision (see Tables 9 and 10). The five repeat concentrations were compared to the initial concentration for accuracy (see Table 11).

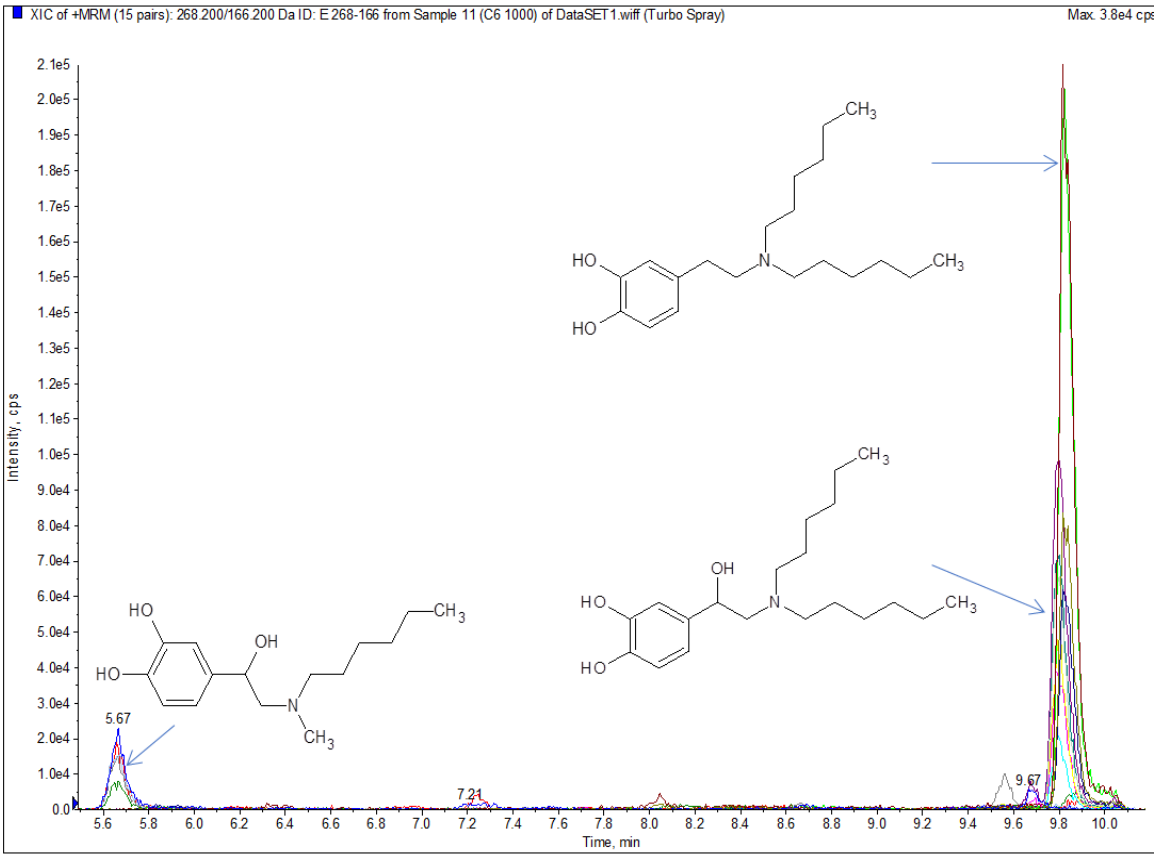

Figure 9. Hexanal (C6) catecholamines derivatives chromatogram

C6 was used to derivative catecholamines in one reaction. The catecholamines retention times (RT) were as follows: epinephrine (5.65 minutes), norepinephrine (9.80 minutes), and dopamine (9.83 minutes).

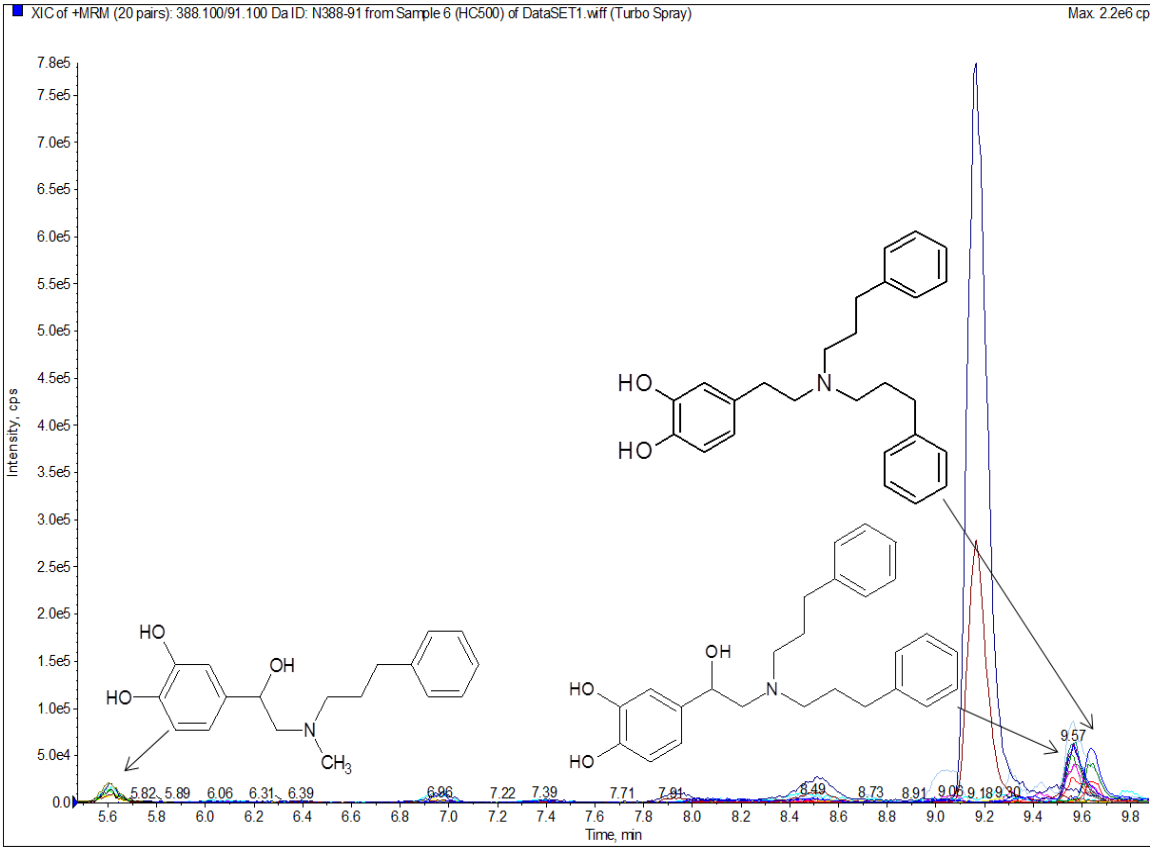

Figure 10. Hydrocinnamaldehyde (HC) catecholamines derivatives chromatogram
HC was used to derivative catecholamines in one reaction. The catecholamines retention times (RT) were as follows: epinephrine (5.61 minutes), norepinephrine (9.57 minutes), and dopamine (9.64 minutes).

Table 7. Hexanal (C6) versus hydrocinnamaldehyde (HC) catecholamines derivatization peak area count (n = 3)

C6 (hexanal)					
Catecholamines	**Mass transitions**	**Mean peak area count**	**Mean RT**	**SD**	**%CV**
Dopamine	322 / 91	942713.00	9.83	43345.70	4.60
Norepinephrine	338 / 137	485490.00	9.80	25758.75	5.31
Epinephrine	268 / 166	91138.67	5.65	13018.78	14.28
HC (hydrocinnamaldehyde)					
Catecholamines	**Mass transitions**	**Mean peak area count**	**Mean RT**	**SD**	**%CV**
Dopamine	390 / 91	321800.00	9.64	43186.48	13.42
Norepinephrine	406 / 91	559937.33	9.57	24845.50	4.44
Epinephrine	284 / 137	152473.67	5.61	11966.49	7.85

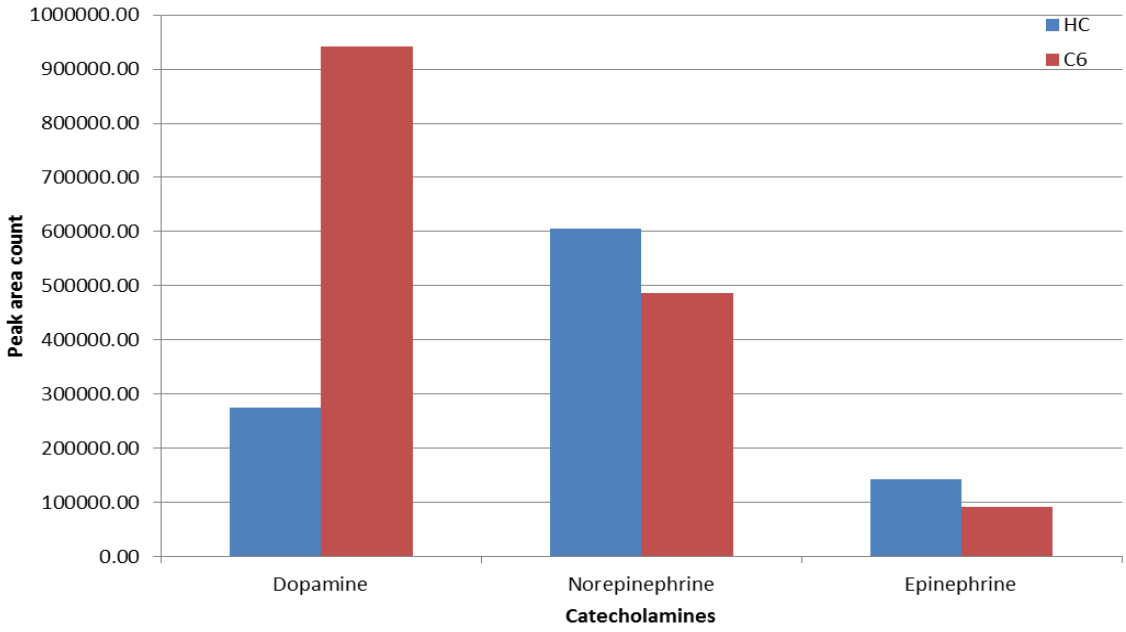

Figure 11. Catecholamines derivatization comparison using hexanal (C6) and hydrocinnamaldehyde (HC) (n = 3)
Catecholamines were derivatized in one reaction using HC and C6 optimized derivatization protocols. The C6 dopamine drivative had ~ x3.5 times the sensitivity of the HC derivative. The HC norepinephrine and epinephrine derivatives had higher sensitivity compared to their C6 derivatives.

Table 8. HC method application – expected and observed calibrator concentrations (n = 1) (ND; not detected)

Dopamine		**Norepinephrine**		**Epinephrine**	
Expected (pmol/L)	Observed (pmol/L)	Expected (pmol/L)	Observed (pmol/L)	Expected (pmol/L)	Observed (pmol/L)
0	0	0	0	0	0
50	ND	200	136	50	32
200	ND	800	583	200	154
500	694	2000	2626	500	585
5000	4980	20000	19947	5000	4994

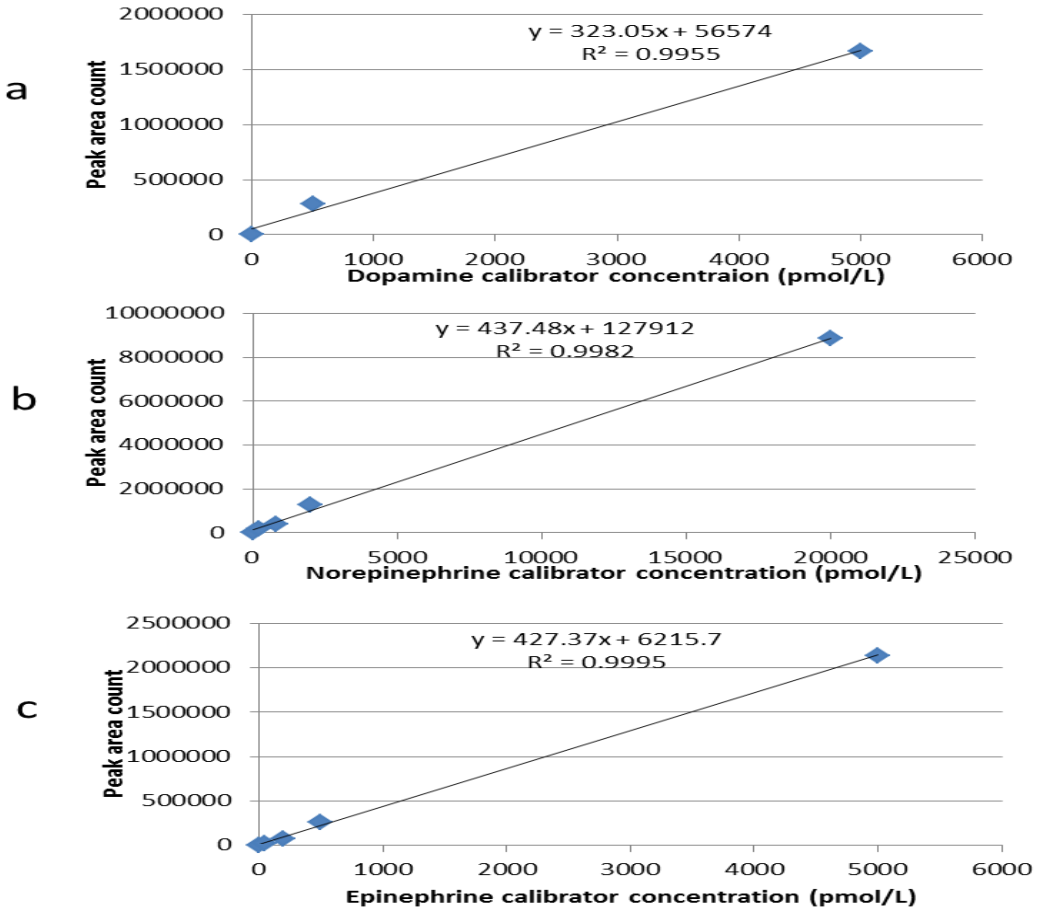

Figure 12. Method application using hydrocinnamaldehyde (HC) – catecholamines standards calibration curves

Calibrators were analyzed in the method application using HC for patient sample concentration quantitation. A) The dopamine calibrators (50 and 200) pmol/L were not detected. However, the calibration curve was linear at 0 pmol/L, 500 pmol/L, and 5000 pmol/L (R^2 = 0.9955). b) The norepinephrine calibration curve was linear (R^2 = 0.9982). c) The epinephrine calibration curve was linear ((R^2 = 0.9995).

Table 9. HC method application – controls concentrations and retention times (RT) (n = 1)

Hydrocinnamaldehyde derivatized catecholamines controls				
	Low (pmol/L)	RT	High (pmol/L)	RT
Dopamine	344	9.87	189	9.86
Norepinephrine	1320	9.80	293	9.84
Epinephrine	95	5.80	184	5.79

Table 10. HC method application – patient pool concentrations (n = 1)

Patient pool	Dopamine (pmol/L)	RT	Norepinephrine (pmol/L)	RT	Epinephrine (pmol/L)	RT
1	123.41	9.91	3644.87	9.82	387.62	9.82
2	292.44	9.94	3372.24	9.81	371.44	9.81
3	27.10	9.90	3657.51	9.83	373.02	9.83
Mean	147.65	9.92	3558.20	9.82	377.36	9.82
SD	134.32	0.02	161.18	0.01	8.92	0.01
CV	90.97	0.21	4.53	0.10	2.36	0.10

Table 11. HC method application – method comparison using five plasma patient samples (n = 1)

Method comparison		
	Initial concentration pmol/L (HPLC-EC)	Repeat concentration pmol/L (LC-MS/MS) HC derivatization
Dopamine	144	48.5
	<130	ND
	<130	ND
	<130	62.5
	300	766.1
Norepinephrine	2246	2789
	2282	897
	2955	3626
	4303	4017
	3192	3585
Epinephrine	87	97.1
	371	123.7
	131	106.7
	<55	64.8
	278	293.8

DISCUSSION

It has been reported that reductive amination is a simple, fast, cheap, and mild derivatization technique that can significantly improve sensitivity for measurement biogenic amines, including catecholamines in LC-MS/MS (23). Derivatizing catecholamines using aldehydes increases sensitivity as follows: they transform catecholamines into larger molecules whose protonated forms are more stable and easily reach the detector once ionized, they increase the hydrophobicity (nonpolar surface area) of the catecholamines which allows them to migrate to the surface of the liquid droplets in the ionization chamber, therefore increasing the rate of ionization, and they transform the catecholamines into tertiary amines which are easily protonated due to the increased electronegative induction on the nitrogen by the added alkyl chains (17).

In this study, reductive amination of catecholamines was evaluated using a series of six straight-chain and five branched-chain aldehydes to determine which was the most effective in increasing sensitivity in LC-MS/MS analysis. The straight-chain aldehydes included: propionaldehyde (C3), butyraldehyde (C4), valeraldehyde (C5), hexanal (C6), heptaladehyde (C7), and octanal (C8). The branched-chain aldehydes were 3,3-dimethylbutyraldehyde (3DB), 2-methylpentanal (2MP), 2-ethylhexanal (2EH), hydrocinnamaldehyde (HC), and 3-phenylbutyraldehyde (3PB). The aldehydes were used to derivatize, dopamine, norepinephrine, and epinephrine individually in triplicate on three different days. Thereafter, the most effective straight-chain and branched-chain

aldehydes were used to derivatize all three catecholamines in one reaction.

Straight-chain aldehydes

In dopamine derivatization, a mix of the mono-alkyl (one alkyl chain added) and di-alkyl (two alkyl chains added) derivatives was observed indicating incomplete derivatization. Dopamine is a primary amine (RNH_2) with two hydrogen atoms that can be replaced in a derivatization reaction. C3 and C4 were deemed ineffective for dopamine derivatization due to their tendency to form mixed derivatives (mono and di-alkyls). The longer straight-chain aldehydes including C5, C6, and C7 only yielded di-alkyl derivatives. The dopamine C6 derivative had the highest sensitivity (peak area count), indicating that C6 was the most effective aldehyde for dopamine derivatization. No derivatized products were observed for C8, possibly due to its low solubility in aqueous solutions.

Unlike the dopamine derivatization, all the straight-chain aldehydes successfully transformed norepinephrine into the complete di-alkyl derivative. Norepinephrine is also a primary amine (RNH_2) with two hydrogen atoms that can be replaced in a derivatization reaction. A possible explanation for this occurrence could be the structural difference between dopamine and norepinephrine. Compared to dopamine, norepinephrine has a hydroxyl group on the alpha carbon of the ethylamine chain that possibly provides steric hindrance to self-reaction between the electron rich catechol group and the imine formed in reductive amination. This limits formation of side products and increases reaction efficiency (complete derivatization). The norepinephrine C6 derivative had the highest sensitivity followed by C5, C3, and C4. The C6 aldehyde

appeared to be the most effective aldehydes for norepinephrine derivatization. No derivatives were detected for C7 and C8 that could possibly be due to their low solubility in aqueous solutions.

Unlike the other two catecholamines, epinephrine is a secondary amine (R_2NH) with one hydrogen atom that can be replaced in a derivatization reaction. The aldehydes successfully transformed epinephrine into the final mono-alky derivative. Sensitivity increased with increase in chain length from C3 to C6 and decreased for C7 and C8, which could be due to their low solubility in aqueous solutions. The C6 derivative had the highest sensitivity.

Hexanal (C6) performed exceptionally and consistently well with all three catecholamines and appeared to be the most effective straight-chain aldehyde for catecholamine derivatization. This conclusion was based on the following observations: C6 completely converted dopamine and norepinephrine into the final product (di-alkyl derivative); C6 converted epinephrine into the final product (mono-alkyl derivative); there was low background noise in the C6 chromatograms; the C6 derivatives had reproducible peak shapes as seen in the low derivative peak area counts coefficient of variation (% CV); and the C6 derivatives had the highest sensitivity for all three catecholamines.

We evaluated LC-MS/MS fragmentation of the straight-chain aldehyde derivatives and the aldehyde physical and chemical properties to understand C6's efficiency in catecholamine derivatization. The dopamine derivatives shared the same most abundant product ion (m/z = 137). The norepinephrine derivatives, however, had different abundant product ions. The epinephrine derivatives shared the same most

abundant product ion (m/z = 166). There was no difference in the C6 derivatives fragmentation compared to the other straight-chain aldehydes. This suggests that fragmentation did not play a significant role on the sensitivity.

This observation also suggests that C6's physical and chemical properties are more favorable for catecholamines derivatization compared to the other straight-chain aldehydes. It has been reported that increasing the chain length of a derivatizing agent correlates with higher sensitivity in the LC-MS/MS (15). The derivatized catecholamines sensitivity was higher with longer aldehyde chain length as expected from C3 to C5 and peaked with C6. However, the sensitivity dropped for the C7 and C8 derivatives, which conflicts with previous reports. A reasonable explanation could be the low solubility of C7 and C8 in aqueous solutions that would affect their availability in the reaction. Therefore, one may suggest that C6 possesses the optimum chain length and solubility for the derivatization of catecholamines. C6's chain length may provide steric hindrance during derivatization, thus limiting formation of side products.

In reductive deamination derivatization reaction, the carbonyl in the aldehyde is converted to an imine (nitrogen double bonded to a carbon) after addition of an alkyl chain. Through transfer of electrons, the primary amine in the catecholamine (in the case of dopamine and norepinephrine) is converted to a secondary amine bonded to a partially positively charged carbon atom. In one reaction scenario, the electron rich catechol group of the catecholamines can easily attack the partially positive carbon and form a cyclized bi-product. However, an aldehyde with a longer alkyl chain in constant motion during the reaction can provide steric hindrance to the formation of cyclized bi-products (see Figure 13). Bi-products including cyclized derivatives decrease the amount of aldehyde

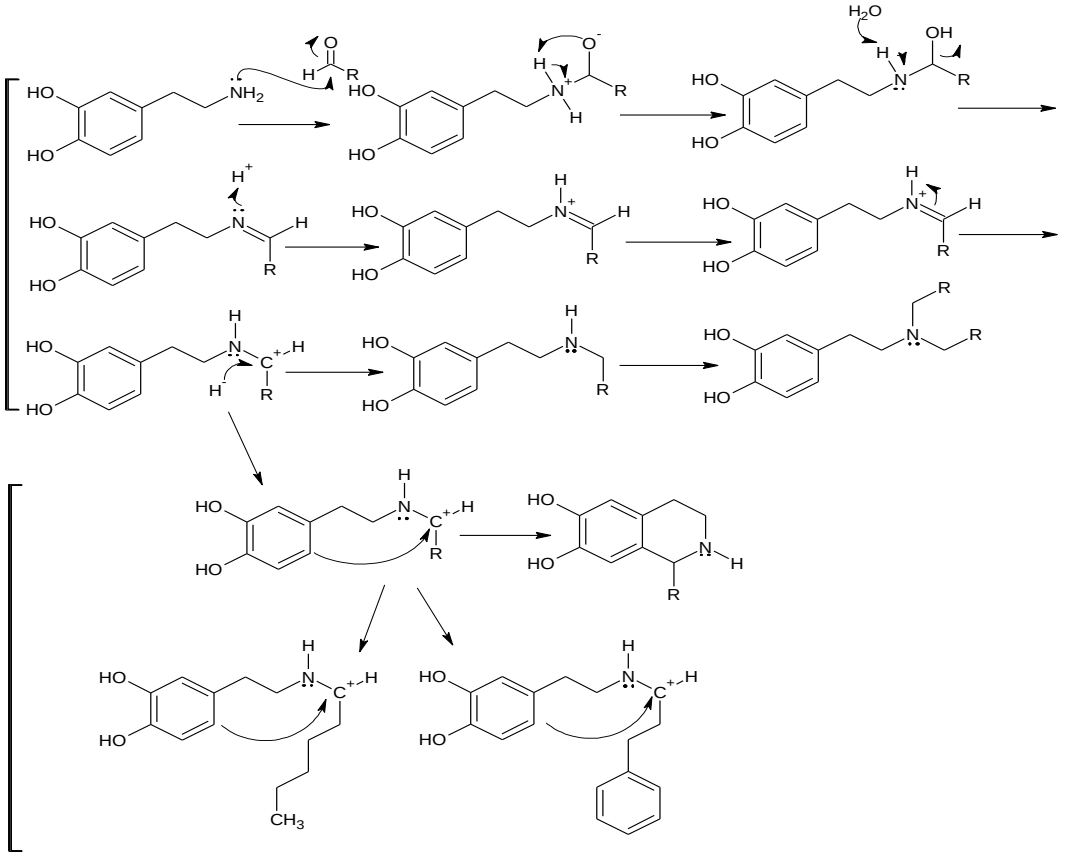

Figure 13. Reductive amination using hydrocinnamaldehyde (HC) and hexanal (C6)
Reductive amination involves the conversion of a carbonyl into an imine which is later reduced by the hydride ions from the reducing agent completing the addition of an aldehyde alkyl chain. This figure illustrates the reductive amination mechanism of the dopamine (norepinephrine and epinephrine follow the same mechanism). a) The reductive amination proceeds as expected with the action of the reducing agent to complete the addition of an aldehyde alkyl chain. b) Formation of cyclized byproducts is also possible depending on the structural properties of the aldehyde as well as concentration of the reducing agent. Cyclized byproducts tend to form less with C6 and HC due to the steric hindrances of their long/wavy and bulky alkyl chains, respectively.

and catecholamines present for reaction, leading to incomplete derivatization and lower sensitivity. This theory may explain why we saw an increase in sensitivity with increase in chain length from C3 to C6 for most of the catecholamines (the longer the chain, the greater the steric hindrance). Furthermore, the theory may explain why we saw a mix of the mono-alkylated and di-alkylated derivatives with the shorter chains (C3 and C4) which do not provide sufficient steric hindrance.

Branched-chain aldehydes

In dopamine derivatization, a mix of the mono-alkyl and di-alkyl derivatives was observed, indicating incomplete derivatization. The branched-chain aldehydes 3DB, 2MP, 2EH, and 3PB were deemed ineffective due to their tendency to form mixed derivatives (mono and di-alkyls). This may be due to the steric hindrance (access to the aldehyde carbonyl) caused by the following: the three methyl groups on the beta carbon in 3DB, the methyl group on the alpha carbon in 2MP, the ethyl chain on the alpha carbon in 2EH, and the phenyl and methyl groups on the beta carbon in 3PB which limit access to their carbonyls leading to incomplete derivatization. However, the largest steric hindrance was possibly observed in 2EH due to its longer ethyl chain on the alpha carbon resulting in the lowest derivative sensitivity. Only HC yielded the complete di-alky derivative. Compared to the other branched-chain aldehydes, HC only has a phenyl ring on the beta carbon which is farthest from the carbonyl and consequently has less steric hindrance.

In norepinephrine derivatization, a mixture of mono-alkyl and di-alkyl derivatives was observed, indicating incomplete derivatization. 3DB and 2MP were deemed

ineffective due to their tendencies to form mixed derivatives (mono and di-alkyls). No derivatives were detected using 2EH. The large steric hindrance in 3DB, 2MP, and 2EH could be a reason for these results. HC and 3PB yielded only the di-alky derivatives due to their lower steric hindrance.

Despite HC's formation of the complete dopamine and norepinephrine derivatives (lower steric hindrance to the aldehyde carbonyl), the 3DB and 2MP derivatives had higher sensitivity in general. This may be explained by HC's larger molecular volume which may cause crowding during the derivatization of dopamine and norepinephrine (require addition of two alkyl chains), leading to lower reaction rate (low formation of derivatives). The same trend was observed with 3PB, which has a comparable molecular volume to HC.

In epinephrine derivatization, the HC and 3PB derivatives had the largest sensitivity followed by 3DB, 2MP, and 2EH. 3DB and 2MP were less effective due to their steric hindrance (2 methyl groups on beta carbon) and smaller molecular volume. 2EH had the lowest effect on sensitivity due to the greater steric hindrance (ethyl group on the alpha carbon). HC and 3PB derivatives have larger molecular volumes, which significantly increases their IE. In addition, epinephrine is a secondary amine with one replaceable hydrogen; therefore, there were no overcrowding of added alkyl chains compared to the other catecholamines

Among the branched-chain aldehydes, hydrocinnamaldehyde (HC) was selected as the most promising aldehyde for catecholamines derivatization. This conclusion was based on the following observations: HC was the only branched-chain aldehyde to effectively transform dopamine and norepinephrine into the final product (di-alkyl

derivative), HC converted epinephrine into the final product (mono-alkyl), the HC epinephrine derivative had the second largest sensitivity, and the HC derivatives had reproducible peak shapes as observed in the low derivative peak area counts % CVs. Although the HC dopamine and norepinephrine derivatives did not have the largest sensitivity, it was still preferred for its reaction efficiency (no mixed derivatives).

We evaluated LC-MS/MS fragmentation for the branched-chain aldehydes derivatives and the aldehyde physical and chemical properties to understand HC's efficiency in catecholamine derivatization. HC and 3PB catecholamines derivatives had different abundant product ions compared to the other branched-chain aldehydes. The larger molecular volume and lesser steric hindrance (to the aldehyde carbonyl) of HC play a role its sensitivity effectiveness and reaction efficiency (complete derivatives). Similar to C6, HC's bulky size and larger molecular volume provides steric hindrance to possible formation of cyclized bi-products in reductive amination, leading to higher sensitivity and efficiency. There were no data available on its solubility.

Hydrocinnamaldehyde optimization

The hydrocinnamaldehyde derivatization protocol using all three catecholamines in one reaction was optimized for higher sensitivity. Experiments were performed to optimize $NaBH_3CN$ (reducing agent), HC concentration (in methanol), and incubation time and temperature.

$NaBH_3CN$ acts as a reducing agent by reducing the imine in reductive amination. At low $NaBH_3CN$ concentrations, there is a higher chance of imine reduction by the electron rich catechol group in the catecholamines, leading to formation of cyclized bi-

products. At high $NaBH_3CN$ concentrations, there is possibility of side reactions with the other reagents due to its reducing potential, which can lead to suppression of the derivatized catecholamines in the mass spectrometer and consequently low sensitivity. $NaBH_3CN$ (50 mmol/L) was selected as the optimal concentration because of the low variation in individual catecholamines sensitivity combined with high sensitivity. In addition, there was high precision (low % CV) in derivative formation at this concentration.

HC acts as the derivatizing agent by adding its alkyl chain/s to the catecholamines amine group. At very low HC concentrations, the catecholamines react with the aldehyde present at different rates, leading to significant variation in sensitivity. At high concentrations, the sensitivity decreases due to the excess aldehyde, which can engage in side reactions, leading to less efficiency (incomplete reactions/mixed derivatives). The optimal aldehyde concentration in methanol was 500 mmol/L because of the low variation in individual catecholamine sensitivity combined with overall high sensitivity. In addition, the highest precision (low % CV) in derivative formation was observed at this concentration.

Incubation time and temperature were optimized for the highest sensitivity. Incubation at 25˚C was carried out for 10, 15, and 20 minutes. There was variation observed in individual catecholamine sensitivity at each time and a minor difference in the overall sensitivity for the three times. Incubation at 37 ˚C was carried out at 15, 20, and 30 minutes. There was greater variation in individual catecholamines sensitivity at each time compared to 25 ˚C. The higher temperature possibly increases the rate of reaction, which can lead to variation in catecholamines reactions with the aldehyde and

consequently sensitivity. There was also a slight difference in the overall sensitivity between the three times. Despite the greater variation, incubation at 37 ˚C and 30 minutes was selected as the optimal temperature because of the following reasons: the assay measures catecholamines in plasma which is a body fluid, there was a smaller variation in individual catecholamine sensitivity coupled with overall high sensitivity, and the highest precision (low % CVs) in derivative formation was observed at this time and temperature.

Hydrocinnamaldehyde versus hexanal

HC and C6 were used to derivatize all three catecholamines in one reaction using their optimized derivatization protocols. The C6 dopamine derivative had approximately x3.5 more sensitivity compared to the HC dopamine derivative. The C6 and HC derivatives fragmentation did not have any significant differences. Their transitions led to the same abundant product ion (m/z 91) (see Figure 14). Therefore, other factors including reaction conditions, reagent concentrations, incubation time and temperature, and solubility may favor C6's affinity for dopamine.

The HC norepinephrine and epinephrine derivatives had higher sensitivity compared to their C6 derivatives. The HC MS/MS fragmentations were significantly different compared to the C6 derivatives. The HC norepinephrine and epinephrine derivatives fragment to one main abundant ion (m/z = 91) while the C6 derivatives produce multiple ions of similar intensity (see Figures 15 and 16). HC's larger molecular volume and hydrophobicity may also explain the increased effect on sensitivity. Interestingly, this trend was not observed for the dopamine derivative.

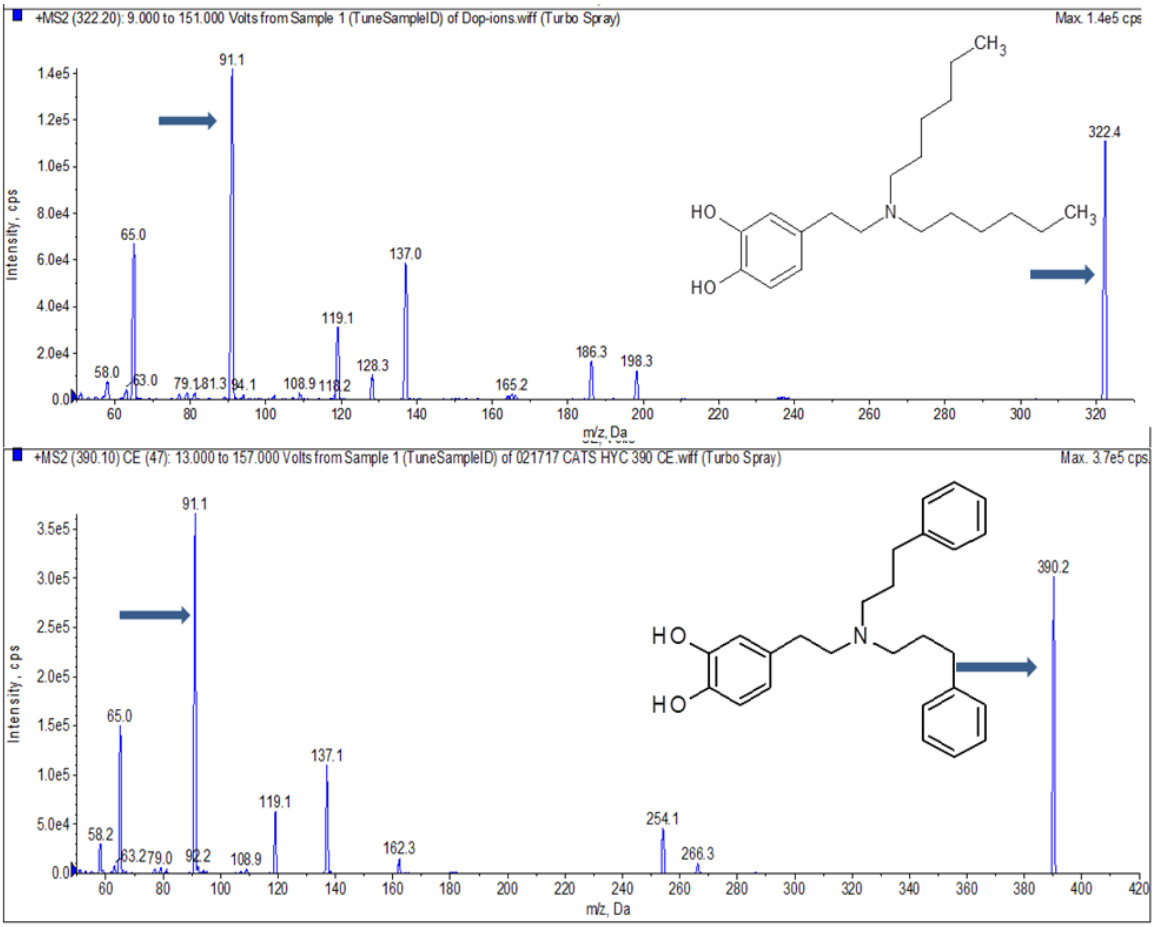

Figure 14. C6 and HC dopamine derivatives fragmentation

The C6 and HC derivatives fragment to one main abundant product ion. There is no significant difference in their fragmentation.

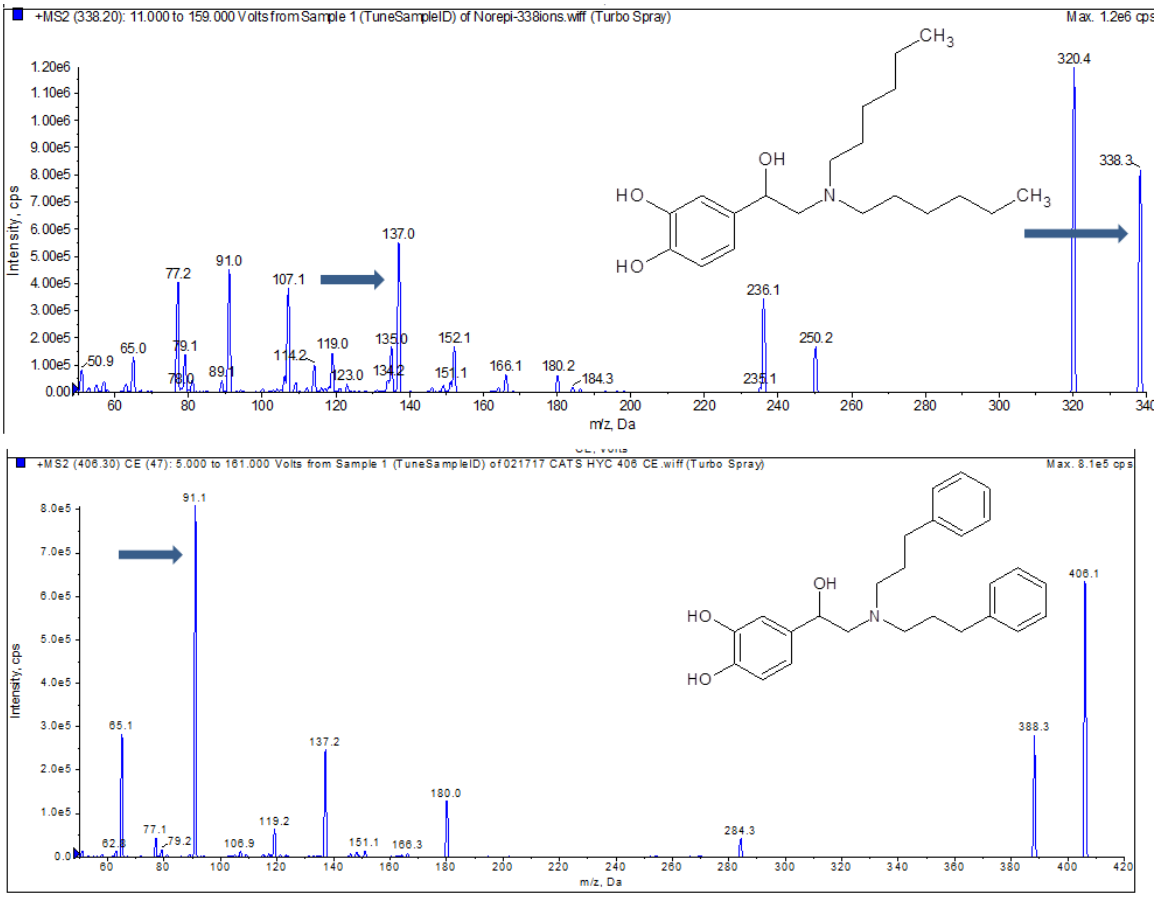

Figure 15. C6 and HC norepinephrine derivatives fragmentation

The C6 derivative fragments to multiple ions of similar intensity while the HC derivative fragments to one main abundant product ion (m/z = 91). Their fragmentations are significantly different.

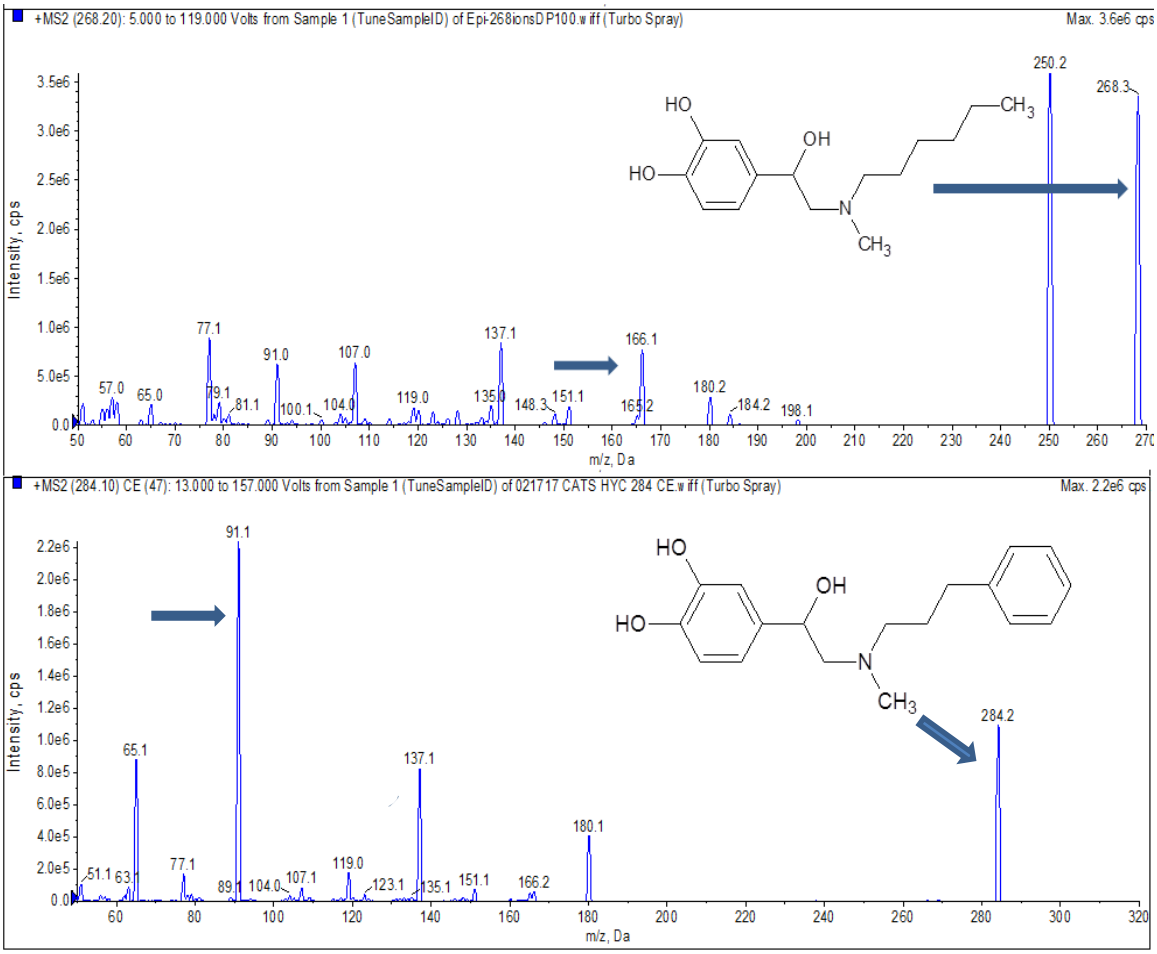

Figure 16. C6 and HC epinephrine derivatives fragmentation
The C6 derivative fragments to multiple ions of similar intensity while the HC derivative fragments to one main abundant product ion (m/z = 91). Their fragmentations are significantly different.

Hydrocinnamaldehyde method application

An HC method application experiment on five repeat patients' samples and one patient pool was performed to evaluate its sensitivity and use in a clinical setting. The experiment was performed in singlicate. A five point calibration curve (0, 50/200, 200/800, 500/2000, 5000/20000) pmol/L was used to analyze the method's accuracy, linearity, and to quantitate patient samples. Controls (low and high) and one patient pool which was analyzed in triplicate were used to analyze the method's precision.

The dopamine calibrator derivatives were not detected at lower concentrations (50 and 200 pmol/L), indicating inadequate lower limit of quantitation (LLOQ). The norepinephrine/epinephrine calibrator derivatives were detected at all the concentrations. Although the catecholamines calibration curves appeared to be linear ($R^2 \geq 0.99$), multiple calibration curves will need to be studied to confirm the methods accuracy.

The dopamine controls (low = 344 pmol/L, high = 189 pmol/L) were not within the assay's established ranges (636 – 954 pmol/L) for the low control and (1723 – 2763 pmol/L) for the high control. The norepinephrine low control (1320 pmol/L) was within range (1094 – 1637 pmol/L) but the high control (293 pmol/L) was not (5752 – 9293 pmol/L). Both the epinephrine controls (low = 94.6 pmol/L, high = 184.4 pmol/L) were not within the established ranges (325 – 487 pmol/L) for the low and (4342 - 6718 pmol/L) for the high. These results may have been possibly compromised due to the lack of isotopically labelled internal standards. In addition, the lack of LLOQ studies may explain why dopamine was not detected at the low concentrations.

The initial patient sample concentrations (analyzed using HPLC-EC) were compared to the repeat concentrations obtained using the HC LC-MS/MS method. Three of the initial dopamine concentrations were below the current method's analytical measurement range (AMR) and therefore were not evaluated, while the mean dopamine concentration bias was 44.5% for the other two samples. The mean norepinephrine concentration bias was -1.6%. One initial epinephrine concentration was below the AMR and therefore was not evaluated; the mean epinephrine concentration bias for the remaining four samples was -17.1%. The dopamine and epinephrine mean concentration biases were not within the current assay's acceptance criteria (+/- 15%).

One plasma patient pool was analyzed in triplicate and the dopamine, norepinephrine, and epinephrine concentration s were evaluated for precision. The %CV's were as follows: dopamine (91.0%), norepinephrine (4.5%), and epinephrine (2.4%). The HC method showed good precision for norepinephrine and epinephrine derivatization.

The HC method application portion of our study had a few limitations. To accurately confirm the method's accuracy, linearity, and precision multiple experiments need to be performed (only one experiment was performed). In addition, collecting patient samples for this study was challenging since most patients provide minimal sample volume for the current assay's analysis. As a result, the experiment was performed using patient samples that had gone through multiple freeze thaw cycles, which may have compromised our results. Lastly, the lack of isotopically labelled internal standards (IS) in the optimization of the HC derivatization protocol and HC method application experiments may have compromised our results. IS were not used in this study due to their high cost.

A few more optimization experiments and studies would be required to fully optimize the HC method for use in a clinical setting. For example, optimization experiments on reaction conditions including buffers, chromatographic conditions, and incubation temperature (> 30 ºC) could be conducted.

Despite these limitations, our derivatization experiments using 11 aldehydes produced precise results as seen in the low % CVs. The experiments were performed on each catecholamine in triplicate on three different days for accuracy and precision. We

also confirmed that HC could effectively increase the sensitivity of catecholamines in plasma samples as seen in the method application experiment.

CONCLUSION

It has been reported that measurement of catecholamines in plasma using LC-MS/MS is preferred for its high specificity and throughput. However, development of catecholamines assays using LC-MS/MS has been challenging due to their low concentrations in plasma. Scientists have reported that pretreatment of catecholamines is required before analysis in LC-MS/MS. The reductive amination strategy using aldehydes was reported as a simple, mild, low cost, and specific technique for derivatizing biogenic amines including catecholamines. In this study, a series of straight-chain and branched-chain aldehydes were evaluated to determine the most effective one in terms of increase in sensitivity. C6 and HC were the most effective straight-chain and branched-chain aldehydes for catecholamines derivatization. An HC method application using patient samples confirmed its performance in a clinical setting. However, preliminary results indicated that extra optimization experiments are required for higher sensitivity.

We believe that our study confirmed and added to the available information on the reductive amination derivatization strategy for increasing LC-MS/MS sensitivity in small polar amines. We are confident that our study will contribute to the development of analytical measurement methods of biogenic amines including catecholamines using LC-MS/MS.

APPENDIX

Table 12. Straight-chain aldehydes catecholamines derivatives mass spectrometry methods

Straight-chain aldehydes mass spectrometry method parameters					
Dopamine derivatives					
Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
C3 (mono-alkyl)					
196.0	196.0 / 194.3	120	10	14.65	11.64
	196.0 / 137.1	120	10	20.32	15.12
	196.0 / 119.2	120	10	30.11	14.88
	196.0 / 91.1	120	10	38.11	10.23
	196.0 / 65.0	120	10	57.57	7.09
	196.0 / 57.0	120	10	23.85	7.54
C3 (di-alkyl)					
238.5	238.5 / 209.0	100	10	17.0	10.08
	238.5 / 137.0	100	10	25.43	15.06
	238.5 / 119.1	100	10	34.88	12.95
	238.5 / 91.1	100	10	46.71	10.98
	238.5 / 73.0	100	10	27.33	16.78
	238.5 / 65.1	100	10	71.35	7.63
C4 (mono-alkyl)					
210.1	210.4 / 137.0	80	10	20.75	8.04
	210.4 / 119.0	80	10	30.92	12.99
	210.4 / 91.1	80	10	38.72	9.19
	210.4 / 65.0	80	10	64.05	7.15
C4 (di-alkyl)					
266.0	266.0 / 137.1	115	10	30.04	14.93
	266.0 / 130.1	115	10	25.02	13.54
	266.0 / 119.0	115	10	38.02	12.83
	266.0 / 91.1	115	10	53.98	10.79
	266.0 / 65.2	115	10	81.17	6.45
C5 (mono-alkyl)					
224.2	224.4 / 154.2	260	10	26.31	8.96
	224.2 / 126.1	260	10	28.45	13.22
	224.2 / 98.0	260	10	29.04	10.88
	224.2 / 82.1	260	10	44.21	9.83
	224.2 / 67.2	260	10	36.17	8.90

Table 12 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
	C5 (di-alkyl)				
294.1	294.1 / 158.2	180	10	27.69	9.19
	294.1 / 137.0	180	10	30.23	8.05
	294.1 / 119.1	180	10	38.61	13.39
	294.1 / 91.1	180	10	54.68	10.57
	294.1 / 65.0	180	10	84.03	13.04
	C6 (mono-alkyl)				
238.1	238.1 / 236.3	160	10	15.64	13.32
	238.1 /137.0	160	10	23.04	8.08
	238.1 / 119.2	160	10	33.78	7.68
	238.1 / 91.0	160	10	47.10	9.98
	238.1 / 82.1	160	10	29.92	10.12
	238.1 / 65.0	160	10	65.93	7.72
	C6 (di-alkyl)				
322.0	322.0 / 198.3	185	10	29.61	19.30
	322.0 / 186.2	185	10	30.17	18.43
	322.0 / 137.1	185	10	33.85	15.77
	322.0 / 119.0	185	10	42.18	6.95
	322.0 / 91.1	185	10	60.98	10.01
	322.0 / 65.0	185	10	90.01	7.09
	C7 (mono-alkyl)				
252.2	252.2 / 223.3	85	10	14.01	12.87
	252.2 / 167.3	85	10	20.40	18.31
	252.2 / 137.2	85	10	24.69	13.16
	252.2 / 91.2	85	10	51.12	5.25
	252.2 / 65.1	85	10	74.27	7.16
	252.2 / 57.1	85	10	35.08	6.93
	C7 (di-alkyl)				
350.0	350.0 / 321.1	145	10	13.94	10.62
	350.0 / 137.2	145	10	35.81	7.94
	350.0 / 119.1	145	10	45.76	13.16
	350.0 / 91.1	145	10	73.84	10.87
	350.0 / 65.2	145	10	95.73	7.82
	350.0 / 57.2	145	10	60.95	9.72
	C8 (mono-alkyl)				
266.4	266.4 / 237.3	75	10	15.47	21.37
	266.4 / 137.0	75	10	24.84	14.68
	266.4 / 118.9	75	10	37.15	14.10
	266.4 / 91.1	75	10	15.22	10.23
	266.4 / 64.9	75	10	74.88	28.07
	C8 (di-alkyl)				
378.3	378.3 / 242.3	150	10	35.90	14.43
	378.3 / 137.1	150	10	38.60	15.27

Table 12 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
	378.3 / 119.0	150	10	45.88	6.85
	378.3 / 91.1	150	10	66.08	5.19
	378.3 / 65.2	150	10	107.97	8.23
Norepinephrine derivatives					
C3 (mono-alkyl)					
212.0	212.0 / 194.0	73	10	15.04	11.58
	212.0 / 144.2	73	10	18.90	14.21
	212.0 / 106.9	73	10	36.59	11.97
	212.0 / 85.9	73	10	10.08	5.19
	212.0 / 77.0	73	10	59.70	8.06
	212.0 / 72.0	73	10	23.29	6.46
C3 (di-alkyl)					
254.1	254.1 / 236.1	120	10	20.90	12.98
	254.4 / 194.2	120	10	30.04	18.06
	254.1 / 107.0	120	10	42.76	6.11
	254.1 / 91.1	120	10	36.14	10.47
	254.1 / 77.1	120	10	69.73	9.85
	254.1 / 65.0	120	10	72.90	12.02
C4 (mono-alkyl)					
226.8	226.8 /159.0	180	10	10.66	16.07
	226.8 /129.2	180	10	29.92	13.24
	226.8 / 91.0	180	10	16.95	11.28
	226.8 / 73.0	180	10	36.47	8.65
	226.8 / 55.0	180	10	51.24	6.09
C4 (di-alkyl)					
282.1	282.1 / 264.2	130	10	22.28	8.22
	282.1 / 137.1	130	10	34.83	8.02
	282.1 / 128.2	130	10	23.17	13.99
	282.1 / 107.1	130	10	46.80	12.94
	282.1 / 91.1	130	10	47.30	11.00
C5 (mono-alkyl)					
240.4	240.4 / 222.2	80	10	15.56	7.06
	240.4 / 172.9	80	10	10.32	10.15
	240.4 / 104.9	80	10	17.06	11.91
	240.4 / 91.0	80	10	17.95	12.18
	240.4 / 77.1	80	10	61.34	8.17
C5 (di-alkyl)					
310.0	310.0 / 292.4	150	10	22.82	9.25
	310.0 / 137.1	150	10	35.70	15.11
	310.0 / 107.0	150	10	49.17	6.08
	310.0 / 91.1	150	10	53.89	10.43
	310.0 / 77.1	150	10	81.17	8.80
	310.0 / 77.1	150	10	81.17	

Table 12 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
		C6 (mono-alkyl)			
254.4	254.4 /236.0	90	10	15.23	14.05
	254.4 / 209.2	90	10	19.27	20.21
	254.4 / 187.0	90	10	10.26	20.97
	254.4 / 105.0	90	10	18.04	13.06
	254.4 / 91.1	90	10	33.55	5.24
	254.4 / 77.2	90	10	66.56	8.22
		C6 (di-alkyl)			
338.2	338.2 / 320.4	190	10	24.94	10.17
	338.2 / 224.0	190	10	31.78	13.46
	338.2 / 137.1	190	10	39.14	13.72
	338.2 / 91.1	190	10	51.14	10.60
	338.2 / 77.1	190	10	98.16	8.70
	C7 (mono-alkyl)				
268.1	268.1 / 250.3	91	10	16.08	8.00
	268.1 / 107.0	91	10	37.92	11.98
	268.1 / 91.1	91	10	37.12	5.21
	268.1 / 77.1	91	10	71.74	8.82
	268.1 / 57.1	91	10	40.92	6.87
	C7 (di-alkyl)				
366.1	366.1 / 348.4	176	10	26.91	10.98
	366.1 / 137.1	176	10	42.34	16.15
	366.1 / 107.1	176	10	57.01	12.94
	366.1 / 91.1	176	10	62.25	11.54
	366.1 / 77.1	176	10	95.95	9.08
	C8 (mono-alkyl)				
282.5	282.5 / 226.0	80	10	33.34	12.56
	282.5 / 105.0	80	10	30.70	13.94
	282.5 / 91.1	80	10	33.96	9.85
	282.5 / 77.2	80	10	89.16	9.49
	282.5 / 64.9	80	10	83.92	18.66
	C8 (di-alkyl)				
394.4	394.4 / 376.3	93	10	29.09	12.12
	394.4 / 221.0	93	10	13.51	12.88
	394.4 / 137.1	93	10	45.23	9.73
	394.4 / 104.8	93	10	33.62	6.26
	394.4 / 91.1	93	10	40.54	12.27
	394.4 / 72.0	93	10	47.06	9.43
		Epinephrine derivatives			
	C3 (mono-alkyl)				
226.6	226.6 / 166.1	87	10	26.95	18.0
	226.6 / 137.1	87	10	28.73	15.56
	226.6 / 107.1	87	10	37.90	11.9

Table 12 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
	226.6 / 91.0	87	10	15.99	8.61
208.1	208.1 / 166.2	155	10	25.08	9.49
	208.1 / 137.1	155	10	27.12	7.93
	208.1 / 107.1	155	10	34.56	13.54
	208.1 / 91.1	155	10	40.64	10.10
	208.1 / 77.0	155	10	62.54	8.65
	C4 (mono-alkyl)				
240.2	240.2 / 222.1	90	10	18.09	21.6
	240.2 / 166.1	90	10	28.59	17.42
	240.2 / 137.0	90	10	30.02	14.90
	240.2 / 107.0	90	10	40.0	12.11
	240.2 / 105.0	90	10	17.64	13.0
	240.2 / 91.0	90	10	44.76	11.07
	240.2 / 77.1	90	10	67.33	9.70
222.2	222.2 / 166.1	180	10	26.7	18.0
	222.2 / 137.0	180	10	27.76	15.5
	222.2 / 107.0	180	10	37.58	12.09
	222.2 / 91.0	180	10	41.25	10.79
	222.2 / 77.1	180	10	66.33	8.66
	C5 (mono-alkyl)				
254.2	254.2 / 108.1	110	10	25.32	19.14
	254.2 / 166.1	110	10	29.48	19.0
	254.2 / 137.1	110	10	29.58	16.79
	254.2 / 107.1	110	10	40.9	11.79
	254.2 / 91.1	110	10	49.8	9.82
	254.2 / 77.1	110	10	73.12	9.59
236.0	236.0 / 180.1	185	10	22.58	22.18
	236.0 / 166.1	185	10	27.18	19.80
	236.0 / 137.1	185	10	27.02	13.81
	236.0 / 107.0	185	10	38.22	12.08
	236.0 / 91.1	185	10	41.04	11.59
	236.0 / 77.1	185	10	66.3	9.20
	C6 (mono-alkyl)				
268.2	268.2 / 166.2	100	10	30.50	17.00
	268.2 / 137.1	100	10	30.50	15.50
	268.2 / 107.1	100	10	42.50	13.00
250.2	250.2 / 166.2	190	10	28.00	17.00
	250.2 / 137.1	190	10	29.50	15.00
	C7 (mono-alkyl)				
282.1	282.1 / 180.1	120	10	28.49	17.23
	282.1 / 166.1	120	10	31.01	16.0
	282.1 / 151.0	120	10	39.57	17.95
	282.1 / 107.1	120	10	43.46	14.0

Table 12 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
	282.1 / 151.0	120	10	39.57	17.95
	282.1 / 107.1	120	10	43.46	14.0
263.9	263.1 / 180.1	171	10	26.42	18
	263.1 / 166.1	171	10	28.85	18.40
	263.1 / 151.0	171	10	37.41	15.52
	263.1 / 137.1	171	10	29.55	13.17
	263.1 / 135.2	171	10	33.85	16.0
	263.1 / 107.1	171	10	41.60	11.73
	C8 (mono-alky)				
296.2	296.2 / 180.0	101	10	29.93	18.43
	296.2 / 166.0	101	10	33.04	17.62
	296.2 / 137.1	101	10	34.31	16.16
	296.2 / 107.1	101	10	45.77	11.59
	296.2 / 99.1	101	10	51.13	10.76
	296.2 / 77.1	101	10	86.13	8.87
278.1	278.1 / 180.1	207	10	27.91	10.04
	278.1 / 166.1	207	10	31.02	18.01
	278.1 / 137.1	207	10	31.02	14.02
	278.1 / 107.7	207	10	42.27	12.45
	278.1 / 91.1	207	10	52.12	10.42
	278.1 / 77.1	207	10	76.80	8.50

Table 13. Branched-chain aldehydes catecholamines derivatives mass spectrometry methods

Branched-chain aldehydes mass spectrometry method parameters					
Dopamine derivatives					
Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
3DB (mono-alkyl)					
238.1	238.1 / 137.1	100	10	25.77	16.13
	238.1 / 119.1	100	10	35.29	14.16
	238.1 / 102.2	100	10	21.24	6.02
	238.1 / 91.0	100	10	46.18	10.32
	238.1 / 65.0	100	10	68.06	7.25
3DB (di-alkyl)					
322.4	322.4 / 238.1	190	10	34.84	26.72
	322.4 / 137.0	190	10	36.43	7.96
	322.4 / 119.0	190	10	44.75	13.04
	322.4 / 91.1	190	10	64.80	10.70
	322.4 / 65.0	190	10	90.90	7.17
2MP (mono-alkyl)					
238.1	238.1 /137.1	70	10	23.75	14.44
	238.1 /119.1	70	10	37.54	14.97
	238.1 /102.1	70	10	20.15	6.05
	238.1 / 91.0	70	10	48.74	10.17
	238.1 / 65.1	70	10	71.64	7.98
2 MP (di-alkyl)					
322.1	322.4 / 137.1	140	10	32.60	15.95
	322.4 / 119.1	140	10	43.79	7.04
	322.4 / 102.1	140	10	30.99	12.97
	322.4 / 91.0	140	10	55.51	8.90
	322.4 / 65.0	140	10	89.82	8.03
2 EH (mono-alky)					
266.0	266.0 / 137.2	100	10	28.34	12.94
	266.0 / 130.0	100	10	20.54	15.02
	266.0 / 119.3	100	10	36.53	12.89
	266.0 / 91.1	100	10	53.39	5.30
	266.0 / 65.0	100	10	71.97	8.90
	266.0 / 57.2	100	10	34.09	6.79
2 EH (di-alkyl)					
378.2	378.2 / 266.2	110	10	30.63	15.85
	378.2 / 137.1	110	10	38.55	27.93
	378.2 / 130.1	110	10	23.26	3.94
	378.2 / 119.1	110	10	55.25	10.88
	378.2 / 91.2	110	10	65.96	9.93
	378.2 / 65.0	110	10	116.25	9.13
HC (mono-alkyl)					
288.5	288.5 / 270.1	72	10	15.82	24.91

Table 13 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
	288.5 / 137.1	72	10	30.78	136.0
	288.5 / 91.0	72	10	25.71	10.45
	288.5 / 65.0	72	10	90.79	7.09
			HC (di-alkyl)		
406.1	406.1 / 388.2	137	10	24.33	12.14
	406.1 / 180.2	137	10	34.44	16.89
	406.1 / 137.1	137	10	42.09	15.00
	406.1 / 119.1	137	10	49.08	14.82
	406.1 / 91.1	137	10	80.99	10.79
	406.1 / 65.0	137	10	123.15	7.02
			3PB (mono-alkyl)		
286.2	286.2 / 198.3	108	10	33.36	23.50
	286.2 / 137.2	108	10	27.43	13.95
	286.2 / 91.0	108	10	55.13	11.19
	286.2 / 65.2	108	10	82.36	7.05
	286.2 / 57.3	108	10	59.96	6.99
			3PB (di-alkyl)		
418.2	418.2 / 282.3	166	10	35.93	31.90
	418.2 / 137.1	166	10	38.45	15.84
	418.2 / 119.0	166	10	48.73	14.39
	418.2 / 91.1	166	10	68.97	10.99
	418.2 / 65.1	166	10	129.12	7.13
			Norepinephrine derivatives		
			3 DB (mono-alkyl)		
254.2	254.2 / 107.0	90	10	41.61	11.99
	254.2 / 91.2	90	10	34.58	10.99
	254.2 / 79.1	90	10	54.14	10.00
	254.2 / 77.0	90	10	67.31	8.88
	254.2 / 57.2	90	10	47.30	6.90
			3 DB (di-alkyl)		
338.2	338.2 / 106.9	170	10	54.40	11.23
	338.2 / 91.0	170	10	68.56	10.83
	338.2 / 79.2	170	10	78.13	8.89
	338.2 / 77.1	170	10	92.42	9.77
	338.2 / 65.0	170	10	98.56	7.26
	338.2 / 57.0	170	10	67.15	6.31
			2 MP (mono-alkyl)		
254.3	254.3 / 152.1	110	10	24.82	16.26
	254.3 / 135.2	110	10	30.65	7.77
	254.3 / 91.0	110	10	35.34	10.16
	254.3 / 77.1	110	10	66.05	8.44
	254.3 / 65.1	110	10	78.09	7.36
			2 MP (di-alkyl)		

Table 13 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
338.0	338.0 / 320.3	165	10	24.86	10.10
	338.0 / 236.0	165	10	32.25	13.86
	338.0 / 152.0	165	10	37.08	16.03
	338.0 / 107.1	165	10	53.07	6.19
	338.0 / 91.1	165	10	64.77	10.54
	338.0 / 77.1	165	10	89.91	8.26
		2 EH (mono-alkyl)			
282.2	282.2 / 105.0	82	10	34.96	11.05
	282.2 / 91.0	82	10	35.93	5.16
	282.2 / 77.0	82	10	90.05	8.54
	282.2 / 65.2	82	10	87.21	7.11
	282.2 / 50.9	82	10	132.88	24.17
		2 EH (di-alkyl)			
394.2	394.2 / 105.1	130	10	46.72	11.82
	394.2 / 91.2	130	10	48.00	10.77
	394.2 / 77.3	130	10	109.2	9.36
	394.2 / 65.1	130	10	125.62	7.16
	394.2 / 57.2	130	10	68.67	7.13
		HC (mono-alkyl)			
288.5	288.5 / 270.1	72	10	15.82	24.91
	288.5 / 226.8	72	10	8.81	23.13
	288.5 / 137.1	72	10	30.78	13.60
	288.5 / 91.0	72	10	25.71	10.45
	288.5 / 65.0	72	10	90.79	7.09
		HC (di-alkyl)			
406.1	406.1 / 388.2	137	10	24.33	12.14
	406.1 / 180.2	137	10	34.44	16.89
	406.1 / 137.1	137	10	42.09	15.00
	406.1 / 119.1	137	10	49.08	14.82
	406.1 / 91.1	137	10	80.99	10.79
	406.1 / 65.0	137	10	123.15	7.02
		3 PB (mono-alkyl)			
302.0	302.0 / 166.1	120	10	23.21	17.85
	302.0 / 137.0	120	10	33.68	8.08
	302.0 / 77.1	120	10	84.06	8.72
	302.0 / 65.0	120	10	87.36	7.09
		3 PB (di-alky)			
434.1	434.1 / 180.2	175	10	35.95	10.23
	434.1 / 137.1	175	10	43.26	7.97
	434.1 / 105.1	175	10	73.26	12.07
	434.1 / 77.1	175	10	127.05	8.94
	434.1 / 65.0	175	10	137.93	7.03
Epinephrine derivatives					
		3 DB			

Table 13 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
	268.0 / 166.3	140	10	30.74	17.53
	268.0 / 128.1	140	10	30.90	16.83
	268.0 / 105.1	140	10	36.07	11.43
	268.0 / 91.0	140	10	36.58	10.33
250.0	250.0 / 166.1	190	10	29.00	9.06
	250.0 / 107.1	190	10	42.91	11.58
	250.0 / 91.2	190	10	53.09	7.43
	250.0 / 79.2	190	10	53.10	8.33
	250.0 / 77.1	190	10	72.07	8.20
			2 MP		
268.0	268.0 / 180.0	108	10	25.79	10.24
	268.0 /166.0	108	10	27.98	9.42
	268.0 / 137.1	108	10	31.62	8.00
	268.0 / 107.1	108	10	43.80	12.07
	268.0 / 91.1	108	10	50.27	10.87
250.0	250.0 / 180.0	180	10	23.31	19.09
	250.0 / 166.1	180	10	26.42	9.68
	250.0 / 137.1	180	10	28.77	7.85
	250.0 / 107.0	180	10	39.97	6.15
	250.0 / 91.1	180	10	49.70	10.55
			2 EH		
296.0	296.0 / 180.0	100	10	26.85	19.21
	296.0 /166.0	100	10	30.30	16.84
	296.0 /151.0	100	10	40.71	15.13
	296.0 /137.1	100	10	34.73	14.06
	296.0 /123.1	100	10	64.35	13.00
	296.0 /107.0	100	10	45.32	12
278.0	278.0 / 180.0	177	10	24.85	10.32
	278.0 / 166.0	177	10	27.55	17.64
	278.0 / 151.1	177	10	37.49	15.23
	278.0 / 131.1	177	10	45.44	14.63
	278.0 / 123.0	177	10	60.70	13.39
	278.0 / 107.1	177	10	42.77	12.60
			HC		
302.0	302.0 / 180.0	110	10	27.51	18.59
	302.0 / 166.1	110	10	34.50	9.48
	302.0 / 151.1	110	10	30.53	8.85
	302.0 / 137.1	110	10	33.92	15.97
	302.0 / 119.1	110	10	44.31	6.97
	302.0 / 107.1	110	10	46.97	6.08
284.0	284.0 / 180.0	182	10	25.43	19.10
	284.0 / 166.1	182	10	31.07	9.26
	284.0 / 151.1	182	10	29.77	16.88
	284.0 / 137.1	182	10	30.39	14.58

Table 13 continued

Precursor ion (m/z)	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
	284.0 / 107.1	182	10	44.63	6.05
			3 PB		
316.0	316.0 / 180.0	133	10	25.44	18.82
	316.0 / 151.2	133	10	30.56	16.83
	316.0 / 137.1	133	10	33.17	14.14
	316.0 / 119.1	133	10	45.89	6.81
	316.0 / 105.1	133	10	44.45	6.10
	316.0 / 103.1	133	10	69.63	5.84
298.0	298.0 / 180.2	190	10	23.33	17.99
	298.0 / 151.2	190	10	28.42	14.56
	298.0 / 137.1	190	10	30.92	7.85
	298.0 / 119.1	190	10	41.75	14.17
	298.0 / 105.1	190	10	39.94	13.09
	298.0 / 103.1	190	10	70.88	12.50

Table 14. Hydrocinnmaldehyde catecholamines derivatives mass spectrometry methods

Hydrocinnamaldehyde mass spectrometry method parameters					
Dopamine derivative					
Precursor ion					
	Mass transitions	DP (v)	EP (v)	CE (v)	CEX (v)
390.1	390.1 / 91.1	173	10	70.20	10.89
	238.1 / 65.0	173	10	104.98	7.00
	390.1 / 137.1	173	10	37.74	16.82
	390.1 / 119.1	173	10	43.95	12.46
Norepinephrine derivative					
388.1	388.1 / 91.1	226	10	81.22	10.87
	388.1 / 65.1	226	10	125.79	7.76
	388.1 / 137.1	226	10	40.32	15.88
	388.1 / 180.2	226	10	33.93	18.95
406.3	406.3 / 91.1	170	10	83.89	10.66
	406.3 / 65.1	170	10	122.34	7.04
	406.3 / 137.2	170	10	42.76	7.67
	406.3 / 180.0	170	10	34.90	10.31
Epinephrine derivative					
302.2	302.2 / 91.1	140	10	61.95	11.15
	302. 2 / 65.1	140	10	94.39	7.17
	302.2 / 137.2	140	10	34.01	17.00
	302.2 / 180.0	140	10	27.0	10.27
284.1	284.1 / 91.1	190	10	53.94	10.90
	284.1 / 65.1	190	10	83.95	7.38
	284.1 / 137.2	190	10	30.28	8.08
	284.1 / 180.0	190	10	23.88	21.92

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