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Development of a nano-electrospray MS^n method for the analysis of serotonin and related compounds in urine using a LTQ-orbitrap mass spectrometer

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ABSTRACT

Serotonin, its key metabolite hydroxyindole acetic acid (5-HIAA) and dopamine have been shown to be potential biomarkers whose levels in serum and urine can be correlated with certain psychiatric and physiological disorders and illness, including depression, schizophrenia, anxiety and dementia. Recently we have published elsewhere that 5-HIAA has been identified as a potential biomarker for Attention Deficit Hyperactivity/Hyperkinetic Disorder (AD-HKD).

This study describes a versatile and validated method for the analysis of these three compounds in urine using a nanoelectrospray-MS^n method interfaced with an LTQ Orbitrap mass spectrometer. No chromatographic separation is required prior to nanoelectrospray infusion.

Good linear calibrations were obtained for analytes in urine (with serotonin and dopamine giving $R^2 = 0.9999$ and 5-HIAA having a lower R^2 value of 0.9955). Acceptable intraday repeatability was achieved for all analytes with RSD values ($n = 5$) ranging from 4.4% to 6.2% (57, 65 and 52 nmol/L for serotonin, dopamine and 5-HIAA respectively) to 2.1–8.1% (2837, 3268, 2618 nmol/L for serotonin, dopamine and 5-HIAA respectively). Excellent limits of detection (LOD) and limits of quantitation (LOQ) were achieved with spiked samples for all compounds; with LODs of 9–12.9 nmol/L and LOQs of 27.2–57.7 nmol/L for analytes in urine. An appropriate sample clean-up procedure for urine was developed to ensure highest recovery and reproducibility on analysis.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) a tryptophan-derived biogenic amine, acts as a neurotransmitter in the central and peripheral nervous systems in the body. Serotonin can be found widely in nature, it is present in animal and plant tissues; venoms and stings. It controls many brain functions, regulates blood pressure and smooth muscle contraction; it has been strongly implicated in several pathological conditions such as essential hypertension, migrane, depression, schizophrenia, anxiety, anorexia nervosa, dementia, and carcinoid syndrome as well as being associated in the regulation of sleep, aggression, temperature, sexual behaviour, and pain sensation [1–7]. Serotonin has been linked to Alzheimer's disease and vascular dementia along with other neurotransmitters such as dopamine [8]. Also of interest is a metabolite of serotonin, hydroxyindole acetic acid (5-HIAA). Serotonin undergoes oxidative deamination to form an intermediate aldehyde, 5-hydroxyindole-3-acetaldehyde which is then subsequently oxidized to form 5-hydroxyindole acetic acid (5-HIAA), which is the predominant serotonin metabolite in urine [9].

Dopamine is also a neurotransmitter which consists of its own system of neurons. It has been shown that serotonin can influence the dopaminergic system. [10] A dramatic loss of dopamine (DA) in the substantia nigra is constantly observed in the post mortem brains of patients with Parkinson's disease [11]. The dopaminergic system is thought to affect a wide range of behaviours and functions, including cognition, motor function, brain-stimulation reward mechanisms, eating and drinking behaviours, sexual behaviour, neuroendocrine regulation, and selective attention [12].

Lam et al. [12] suggested there may be a dynamic balance between serotonergic and dopaminergic systems, and that this varies between patients, as well as between drugs. The authors also commented that as it is widely accepted that autism is a neurobiological disorder, although specific biomarkers have yet to be found, serotonin may play an important role in the development of autism. Before assuming its role as a neurotransmitter in a mature brain, serotonin regulates both the development of serotonergic neurons as well as the development of target tissues, such as the hippocampus and the cerebral cortex. High levels of serotonin during early development may cause a loss of serotonin terminals and subsequent neuronal development.

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Disruption of normal monaminergic neurotransmission has been seen as the crucial mechanism to be linked with ADHD pathphysiology [13]. Although many studies have shown that dopamine plays an important role [14] other monoamines like noradrenaline and serotonin have also been linked to this condition [15]. Spivak et al. reported that a chronic deficit of serotonin may contribute to the clinical symptoms of the disorder [16,17]. We have recently published a report that identifies 5-HIAA as a possible biomarker in urine for the diagnosis of ADHD in children [18]. This study compared levels of serotonin, dopamine and 5-HIAA in urine samples received from a test group of children diagnosed with severe ADHD with a matched control group. The results showed a marked difference in 5-HIAA levels between the two groups.

The majority of analytical methods for the determination of serotonin and related indole compounds are liquid chromatography (LC) methods connected to different detection systems like UV [19], fluorescence [20–22], electrochemical detectors [23,24] or mass spectrometry [25–28]. The most common non-LC method is by immunoassay.

The method described herein is a nano ESI-MS method which has been developed for the analysis of serotonin, along with dopamine, and its metabolite hydoxyindole acetic acid (5-HIAA) in urine. The method uses nanoelectrospray technology for ionization of analytes and an LTQ Orbitrap for analysis. This method is robust and accurate as well as sensitive and quick. Accurate analysis of each sample by standard addition is accomplished in 12 mins (6×2 min; 5 spiked additions); triplicate analysis ($n = 3$) of each standard is acquired within 36 min. Standard addition compensates for any matrix effects such as ion suppression or enhancement that may be encountered with clinical samples. This is a significant reduction in analysis time in comparison with the previously developed LC/ESI–MS/MS method by this group [18], which required 20 min per injection (1 h for triplicate injections), and a similar standard addition experiment by LC/ESI–MS/MS with triplicate analysis of each standard taking 300 mins.

Nanoelectrospray ionization (nano-ESI), coupled to a mass spectrometer, is an important and widely used approach employed for many applications particularly large-scale protein identification. The Advion NanoMate system is a robotic system that provides an automated nano-electrospray ion source for mass spectrometers. It has potential advantages over HPLC-ESI, including higher-throughput which increases the sensitivity of the method, automated analysis with no carry-over [29], flexible MS acquisition time, and a constant spray of sample into the mass spectrometer [30]. Nano-electrospray facilitates the analysis of trace compounds in low sample volumes. It also has the ability to reduce or completely remove ion suppression effects, Schmidt et al. [31] found that ion suppression effects decreased significantly as flow rates were reduced to very low levels and completely disappeared at levels of a few nL/min. A fast and simple method was developed for the analysis of beta-casomorphins in dairy products using the Advion NanoMate system coupled to a ion trap mass spectrometer [32]. In this manuscript, the Nanomate allows for the infusion of upto 10 μL of sample to be sprayed continuously for up to 15 min into the mass spectrometer to facilitate the gathering of high resolution data. The use of the NanoMate system in place of the conventional HPLC system allows for reduced sample volumes, faster analysis times and the elimination of mobile phase solvents. The relatively simple infusion system allows for straightforward optimisation and facile troubleshooting compared with the more complex liquid chromatography system.

The description of the first Orbitrap MS was provided by Markarov et al. [33] the instrument is now used for several mainstream analytical applications [34–38]. The Orbitrap provides excellent high resolution and mass accuracy data and when coupled to external accumulation devices such as the linear trap, facilitates multiple levels of fragmentation (MS^n) to enable structure elucidation of target analytes [39,40].

2. Materials and methods

2.1. Materials

Chemicals used in this study included formic acid purchased from Merck (Darmstadt, Germany), ammonium acetate and acetic acid were purchased from Sigma–Aldrich (Dublin, Ireland). Hydrochloric acid and all HPLC grade solvents (methanol, water, acetonitrile) were purchased from Labscan (Dublin, Ireland). Nitrogen gas was purchased from Irish Oxygen (Cork, Ireland). All standards were purchased from Sigma Aldrich (Dublin, Ireland) and deuterated compounds from CDN Isotopes (Quebec, Canada). Isolute C18 cartridges were purchased from Biotage (Sweden). Supelco HybridSPE-precipitation cartridges were obtained from Sigma–Aldrich (Dublin).

2.2. Chip based nanoelectrospray emitters

Nanoelectrospray infusion analysis was achieved using a NanoMate system (Advion BioSciences, Ithaca, New York) an automated chip based nanoelectrospray device using a disposable ESI-Chip with a fully integrated array of 400 nozzles etched from the surface of a silicon wafer. This system holds a 96-well plate, a rack of 96 disposable conductive pipette tips and the nanoelectrospray mentioned above. During infusion analysis, the system sequentially picks up a pipette tip, aspirates 1–5 μL of sample from one position of the 96-well plate, and then delivers the sample to the inlet side of the ESI chip. In this study, 5 μL of sample solution was sprayed through a nozzle on the nano ESI chip. The electrospray process was initiated by applying 1.8 kV and approximately 0.7 psi nitrogen head pressure to the sample in the pipette tip to ensure constant sample flow to the chip. These settings gave an approximate sample flow rate of 150–200 nL/min for samples prepared in methanol. The Nanoelectrospray chip was positioned directly in front of the aperture in the curtain plate. Samples were infused for 2 mins but instrument acquisition was carried out for 2.2 mins. This allowed for the creation of the flat top peak needed for the integration of the target compounds, ensuring that exactly 2 mins of data was recorded for each analyte. The extra 0.2 mins also gives time for the Nano-mate and the LTQ Orbitrap MS to align up for the next samples infusion and contact closure activation event during samples infusion sequences. The 96-well sample tray was maintained at 4 °C.

2.3. LTQ orbitrap mass spectrometer

A tandem MS method was developed for the quantification of serotonin, dopamine and 5-HIAA in urine on a LTQ Orbitrap XL (Thermo, USA). Quantitation was carried out using the MS^2 fragment mode which typically results in increased selectivity by reducing background ions from the sample matrix. A resolution of 30,000 was applied for quantitation. For precise identification of analytes, high mass accuracy was achieved with analysis in full scan mode at 100,000 resolution. Also, a data dependant scan was performed by deploying the higher energy collision induced dissociation (HCD) facility on the MS instrument for each sample. This gives a spectrum showing low mass fragments similar to spectrums obtained from triple stage quadrupoles. This allows more fragment ions to be observed than regular CID activation for MS^2 and so at least 3 m/z fragments for each compound can be seen for confirmation of identity. Xcalibur software (version 2.4) was used for instrument control, data acquisition and data analysis. The

Table 1
MS parameters for NanoESI-MS method along with precursor and product ions for each analyte and Relative Collision Energy (RCE) for MS^2 scan for CID and HCD activation.

MS parameters	
Capillary temp	250 °C
Capillary volt	49 V
Tube lens	125 V
Ion optics	
Multipole 00 offset	−4.75 V
Lens 0 voltage	−3.5 V
Multipole 0 offset	−5.5 V
Lens 1 voltage	−16 V
Gate lens voltage	−58 V
Multipole 1 offset	−6 V
Multipole RF Amplitude	400 (V p-p)

	$[M+H]^+$	MS^2 product	RCE (MS^2) CID (%)
Serotonin	177	160	20
Serotonin-d_4	181	164	20
Dopamine	154	137	23
5-HIAA	192	146	35
5-HIAA-d_2	194	148	35

	$[M+H]^+$	MS^2 product	RCE (MS^2) HCD (%)
Data Dependant Scan	177	160, 132, 115	75
	154	137, 119, 91	70
	192	146, 118, 91	90

mass spectrometer was operated in positive mode. Multiple tandem MS spectra, produced by collision-induced dissociation (CID), were obtained using the $[M+H]^+$ ion. The mass spectrometer conditions are given in Table 1 along with MS^2 and data dependant scan collision energies using CID and HCD respectively. This optimisation was carried out by infusion of a 1 μg/mL standard of serotonin. For internal calibration to provide data of high mass accuracy, a lock mass was used, the internal standard for serotonin, serotonin-d_4 (m/z 181.127895) was used for this lock mass to automatically correct for any mass deviation from the instrument.

2.4. Standard and sample preparation

Standards (serotonin, dopamine, and 5-HIAA) in solvent (methanol) were prepared from a stock mixture of all compounds (10 μg/mL) by serial dilution. The deuterated internal standards were subsequently added with final internal standard concentrations for serotonin-d_4 and hydroxyindole acetic acid-d_2 being 1.42 μmol/L and 2.62 μmol/L, respectively.

On collection of urine samples (10–40 mL), HCl (1 M, 0.5 mL) was added as a preservative to prevent oxidation of the analytes and samples were stored at −24 °C. Samples were centrifuged ($3000 \times g$) and the internal standards (serotonin-d_4 and 5-HIAA-d_2, 200 μL) were applied to the urine samples (200 μL). Water acidified to pH 3.5 with acetic acid (800 μL) was applied and the samples were then vortexed. The SPE procedure comprised the following steps: the Isolute C18 cartridges were conditioned with methanol (3 mL), followed by water (pH 3.5, 3 mL), the sample containing water (1.2 mL) was then loaded onto the cartridge, washed with a methanol solution (5%, 1 mL) and eluted with methanol with 0.1 M ammonium acetate (5 mL). Samples were then dried under nitrogen and reconstituted in methanol (200 μL). Each sample was passed though a Millex-HN, 0.4 μm, 13 mm syringe filter (Millipore, MA, USA) before analysis.

For analysis by standard addition, samples were diluted by a factor of 10. 60 μL of each sample was placed into six 2 mL amber LC vials. Aliquots (10 μL) of a standard stock solution, containing the three compounds of interest, were added to each vial ranging from zero to five 10 μL aliquots of stock solution. Each 10 μL aliquot contained 37.5, 55.8 and 209.7 nmol/L of dopamine, serotonin, and 5-HIAA respectively. A flow diagram of the sample preparation procedure can be seen in Fig. 1. Methanol was then added to each vial to make up to a final volume of 120 μL. Concentration of stock solution was calculated from standard calibration curve.

3. Results and discussion

The objective of this study was to develop and validate a robust and rapid screening method for the detection of key biomarker compounds in clinical urine samples. In clinical applications the turnaround of sample analysis is necessary to ensure correct diagnosis and that appropriate treatments can be implemented in a timely fashion. For this reason mass spectrometry is now becoming a common tool for the rapid and trace identification of biomarker compounds in clinical applications [41–45]. Recently we have published elsewhere that 5-HIAA has been identified as a potential biomarker for Attention Deficit Hyperactivity/Hyperkinetic Disorder (AD-HKD)[18]. That method involved analysing urine samples by LC–tandem mass spectrometry (LC–QqQMS). However, this manuscript presents a much faster alternative using nano-electrospray-MS to screen urine samples thereby removing the requirement for liquid chromatography. It achieves this while maintaining excellent sensitivity, accuracy and precision. Additionally it has been shown that the nano-spray interface is more tolerant toward salt contamination of the analyte source (a common problem with urine samples) [31].

3.1. MS spectrum of compounds

The ESI mass spectra of serotonin, dopamine and 5-HIAA are relatively simple and can be observed in Fig. 2. The molecular-ion species formed in positive mode are protonated molecules. High background signals and potential interferences from the urine samples mean that it is prudent to perform MS^n scans on target analytes to enable accurate identification. In addition high resolution data in full scan experiments can enhance identification of analytes. The

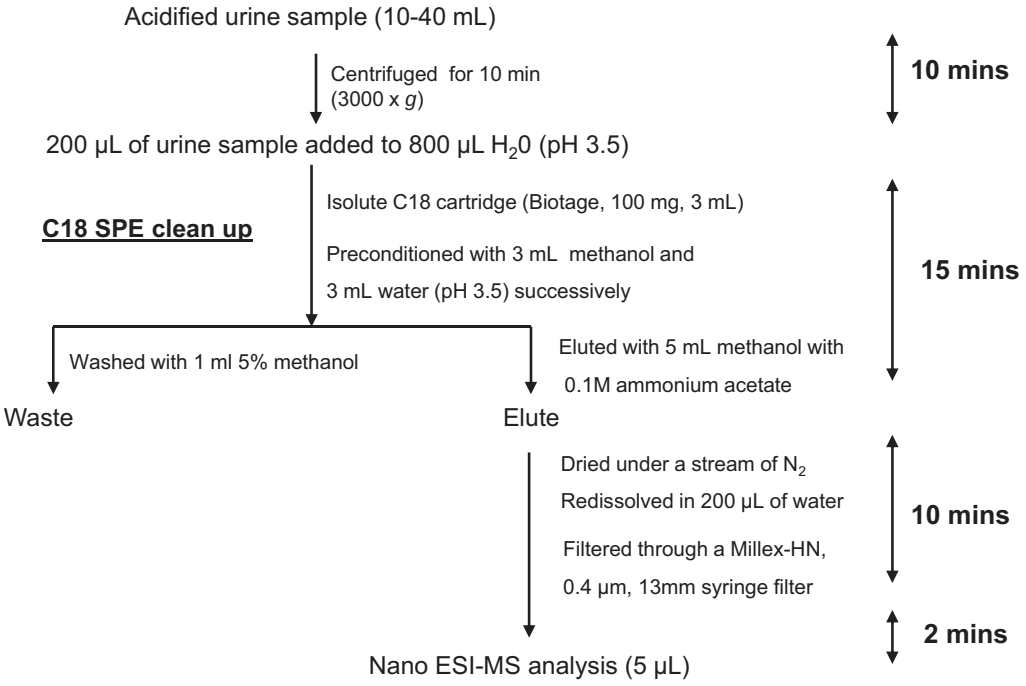

Fig. 1. Flow diagram of sample preparation procedure for extraction of a urine sample, showing volumes and time required for the method.

LTQ Orbitrap gives high mass accuracy data to four decimal places. Ions with a mass of 500 Da or less require an accuracy of 0.0025 Da to allow for definitive assignment of elemental composition [46]. When dealing with small mass molecules such as serotonin in complex matrices the ability of obtaining a mass to at least four decimal places is a prerequisite.

3.2. Fragmentation studies

In this study, multiple tandem MS (MS^n) was used for the repeated trapping and fragmenting of ions. Once the precursor ion was selected, the effect of varying the energy of the helium collision processes was determined by plotting the relative abundances of the product ions versus % RCE (Fig. 3) for each analyte. Fig. 3a, c and e shows the product ions plotted for MS^2 CID experiments for serotonin, dopamine and 5-HIAA. The most abundant product ion for the compounds of interest are observed in the MS^2 spectra. MS^3 experiments were also carried out but sensitivity was much lower (90% reduction in signal) than for MS^2 experiments. Quantitation was carried out on the most intense ion transition for each compound; these were 177/160 at 20%, 154/137 at 23% and 192/146 at 35% for serotonin, dopamine and 5-HIAA respectively. Fig. 3 b, d and f shows the HCD collision energy diagram for each of the analytes. This mode of activation uses higher energy CID to produce more extensive fragmentation and hence smaller fragments than regular MS^2 CID. Using a HCD scan event on these analytes allow for more identification points for the analytes, as even more product ions are produced. Ideally 3 fragment ions are required for confidence in identification of analytes, as recommended in the EC council directive 2002/657/EC (implementing the 96/23) [47] and as applied by Allis et al. [48] to the area of the trace identification of microcystins toxins in potable water. Fig. 3b shows the HCD collision diagram for serotonin, along with the HCD collision energy required for optimum product ion intensity. For serotonin the diagram shows that the optimum collision energy is 75% giving ions at m/z 160, 132 and 115 as the most abundant product ions. For dopamine and 5-HIAA observed fragments include m/z 137, 119 and 91 for dopamine at 70% and m/z 146, 173, and 118 for 5-HIAA at 90%.

Fig. 4a–c illustrates the fragmentation pathways of serotonin, dopamine and 5-HIAA along with the HCD spectra for each analyte. The fragmentation pathways show the molecular formulas for each of the fragments along with the PPM errors between the theoretical and measured values for each of the masses. Internal calibration, to ensure mass accuracy, was performed by using the mass of the internal standard serotonin-d_4 to correct any deviation in the mass calibration of the instrument. PPM error is seen to increase with smaller fragment ions.

3.3. Detection method study

A study was performed to assess which detection and activation method and type of scan gave the best sensitivity for this infusion method. This was achieved by comparing calibration curves ($n = 5$) of MS^2 and MS^3 CID scans using both the linear ion trap (ITMS) and Orbitrap (FTMS) as well as a HCD scan detected by the Orbitrap MS for each of the compounds, these results can be seen in Fig. 5a–c. This graph shows increased sensitivity when using detection by FTMS (fourier transform MS) over ITMS (ion trap MS) using CID. It also shows a slight increase in response when using MS^2 compared with MS^3. HCD activation gives a lower response than that observed for CID activation. All calibrations are standardised by using an internal standard. Use of an internal standard enables standard correction for any instrumentation variation that may occur from standard to standard. This is particularly important in methods where the standard/sample is infused into the MS and where there is a possibility for inconsistencies in the sample spray. The internal standard compensates for the reduction in signal intensity typically observed in the MS^3 mode. Therefore, it can be seen that the ideal method that gives the highest response using internal calibration is FTMS detection with MS^2 using CID activation. These parameters were chosen for the analytical method.

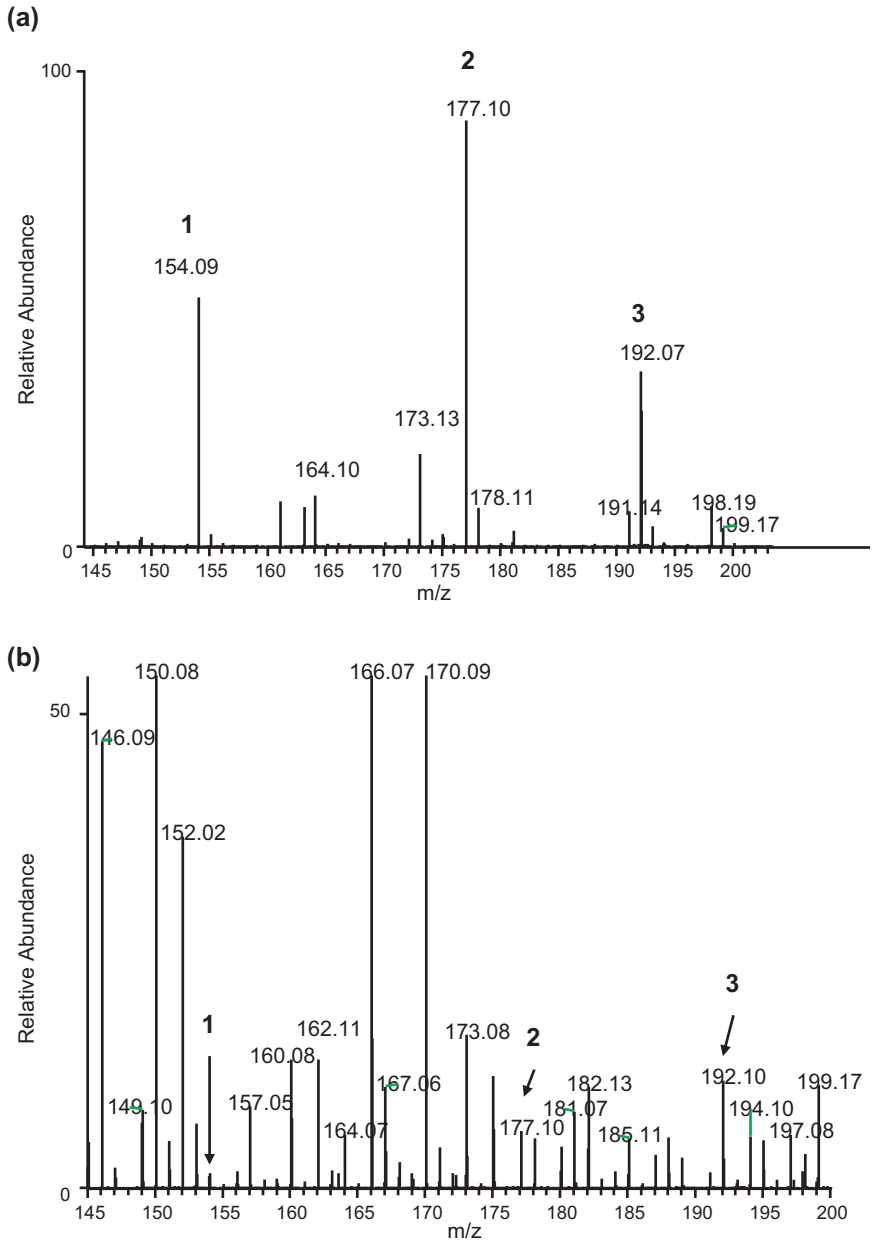

Fig. 2. (a) Spectrum of dopamine (1306 nmol/L) (1), serotonin (1135 nmol/L) (2) and 5-HIAA (1046 nmol/L) (3) in standard. (b) Spectrum of the same compounds in a urine sample (358 nmol/L, 268 nmol/L and 15,796 nmol/L) for serotonin, dopamine and 5-HIAA respectively. Analysis was carried out in the linear ion trap mass analyser and shows the number of matrix ions are in close proximity to the analytes. The Orbitrap detector allows for improved resolution between these analytes.

3.4. Resolution study

The resolution study was carried out on the Orbitrap MS by preparing calibration curves of serotonin analysed at four different resolution settings 15,000, 30,000, 60,000, and 100,000. High sensitivity is required as detection with FTMS has higher limits of detection and limits of quantitation than ITMS. Therefore, when using FTMS for quantitation a compromise is required between high resolution, high sensitivity and optimum number of scans that is required for a method.

Fig. 5d–f shows the results of the resolution study using serotonin, this demonstrates that resolutions of 15,000 and 30,000 have a higher sensitivity than 60,000 or 100,000. Table 2 shows the number of scans carried out in 2 min, scans per scan event

Table 2
Comparison of scan numbers, times and cycles using different resolutions.

Resolution	No of scans (2 min)	No of scans/scan event ($n = 5$)	Scans/s	Scan cycle (s)
100,000	60	12	0.5	10
60,000	105	21	1.14	4.39
30,000	170	34	1.42	3.52
15,000	205	41	1.71	2.93

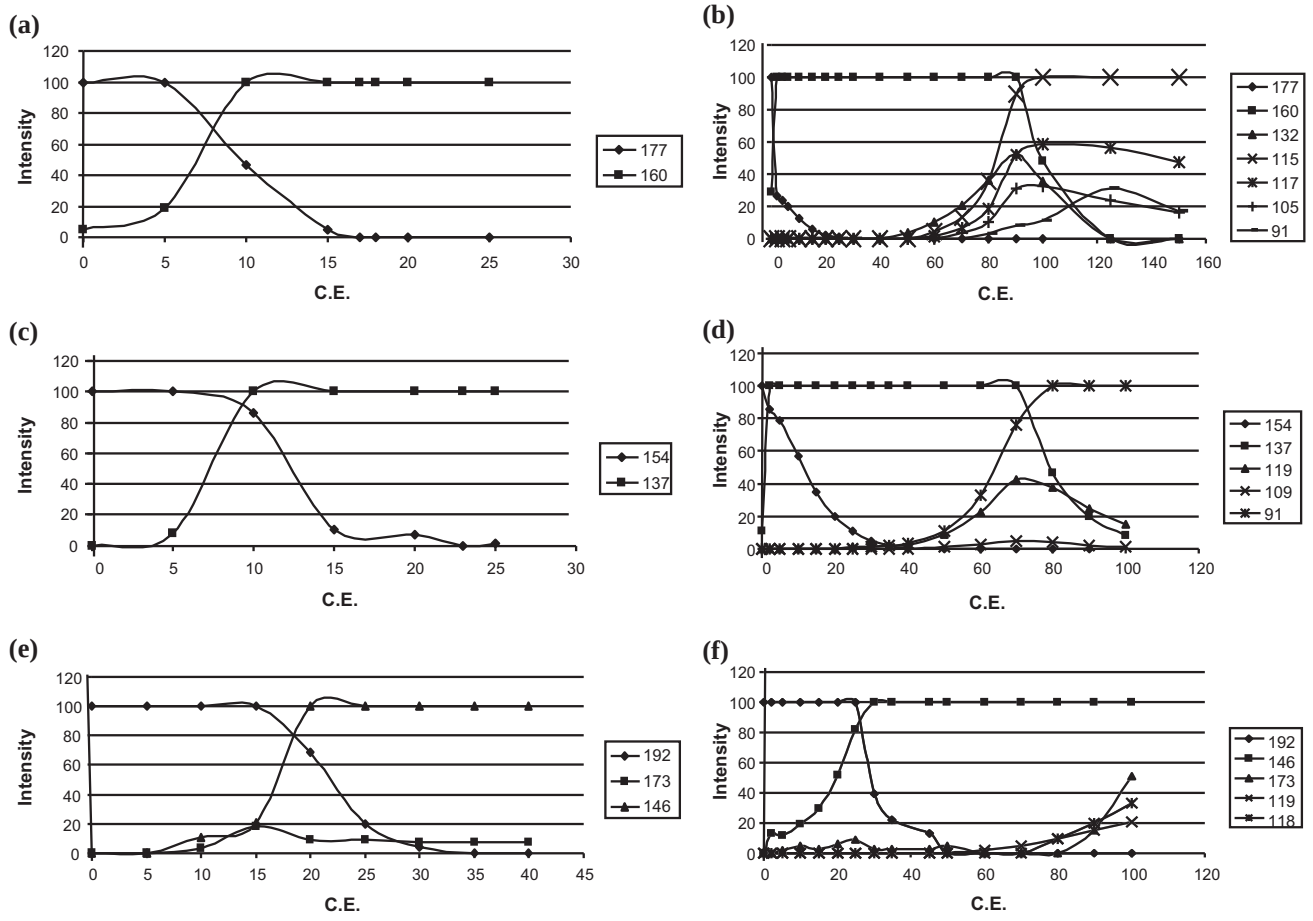

Fig. 3. Collision energy diagrams illustrating the relative abundances of ions against the collision energy applied. (a), (c) and (e) are MS^2 CID experiments on serotonin, dopamine and 5-HIAA respectively. (b), (d) and (f) are HCD experiment serotonin, dopamine and 5-HIAA respectively. For the HCD experiment it can be seen that this high energy activation method provides many characteristic fragment ions which allows for more confirmation of the identity of the analytes compared to the CID method.

(the number of scan events in this method equals 5, representing the target compounds), scans per second and the scan cycle for all 5 scan events. This indicates that a resolution of 100,000 provides just 12 scans over the 2 min period, and this could lead to considerable variation in signal response between samples even with the use of an internal standard. There is a greater reduction in scan number between 30,000 and 60,000 resolution (difference of 65 scans, 13 scans per analyte) compared to the difference between 15,000 and 30,000 (35 scans, 7 scans per analyte) and also between 60,000 and 100,000 (45 scans, 9 scans per analyte). An increase in scan numbers allow for more data points to be collected which in turn leads to better quantification. Also the more data points collect during analysis, the more reproducible the results and the better the sensitivity. Precision results showed a decline with an increase in resolution. Average %RSD results over a concentration range of 28–2837 nmol/L were $4.89 \pm 2.47\%$ for 15,000, $3.3 \pm 2.6\%$ for 30,000, $4.59 \pm 8.04\%$ for 60,000, and $7.75 \pm 4.16\%$S for 100,000 resolution. Considering all factors a resolution of 30,000 was chosen as a reasonable compromise. At 30,000 resolution, serotonin with a m/z of 177.1028 can effectively be resolved from another compound with a m/z difference of $\geq$5.9 mmu in comparison with 15,000 resolution which can separate to a m/z difference of $\geq$11.8 mmu.

3.5. High mass accuracy evaluation

A study was carried out to establish the capability of the Orbitrap MS for achieving consistent high mass accuracy measurements over an extended time period. In other words to determine the robustness and stability of the instrument. The analysis was carried out at 100,000 resolution, in full scan mode over a calibration range of 28–2837 nmol/L. Each infusion (5 μL) lasted 2 mins with 17 scans (counted scans) over this time period using 7 standards, each analysed in triplicate. This gave a total scan number of 360 over an analysis time of 46.2 min. Fig. 6 shows the results of this evaluation. Fig. 6a shows the mass accuracy for repetitive measurements of the $[M+H]^+$ of serotonin measured in PPM error. It can be seen that the majority of the measurements lie within the range of ±0.2 PPM. Fig. 6b illustrates the effect on PPM error over the linear range of 28–2837 nmol/L. The average measurement of PPM error which can be seen to be ±0.1 PPM over most of the calibration range is stable, but it can be seen that the variation in measurements increases as concentration decreases with the most significant effect seen at the 28 nmol/L concentration with a PPM error of ±0.3 PPM. In conclusion the Orbitrap MS provides acceptable accuracy, stability and reproducibility.

3.6. Validation

3.6.1. Recovery

Recovery experiments were carried out using known concentrations of standards spiked into urine matrices. This allowed evaluation of the sample preparation which used Isolute C18 solid phase extraction cartridges. Results for the SPE recovery

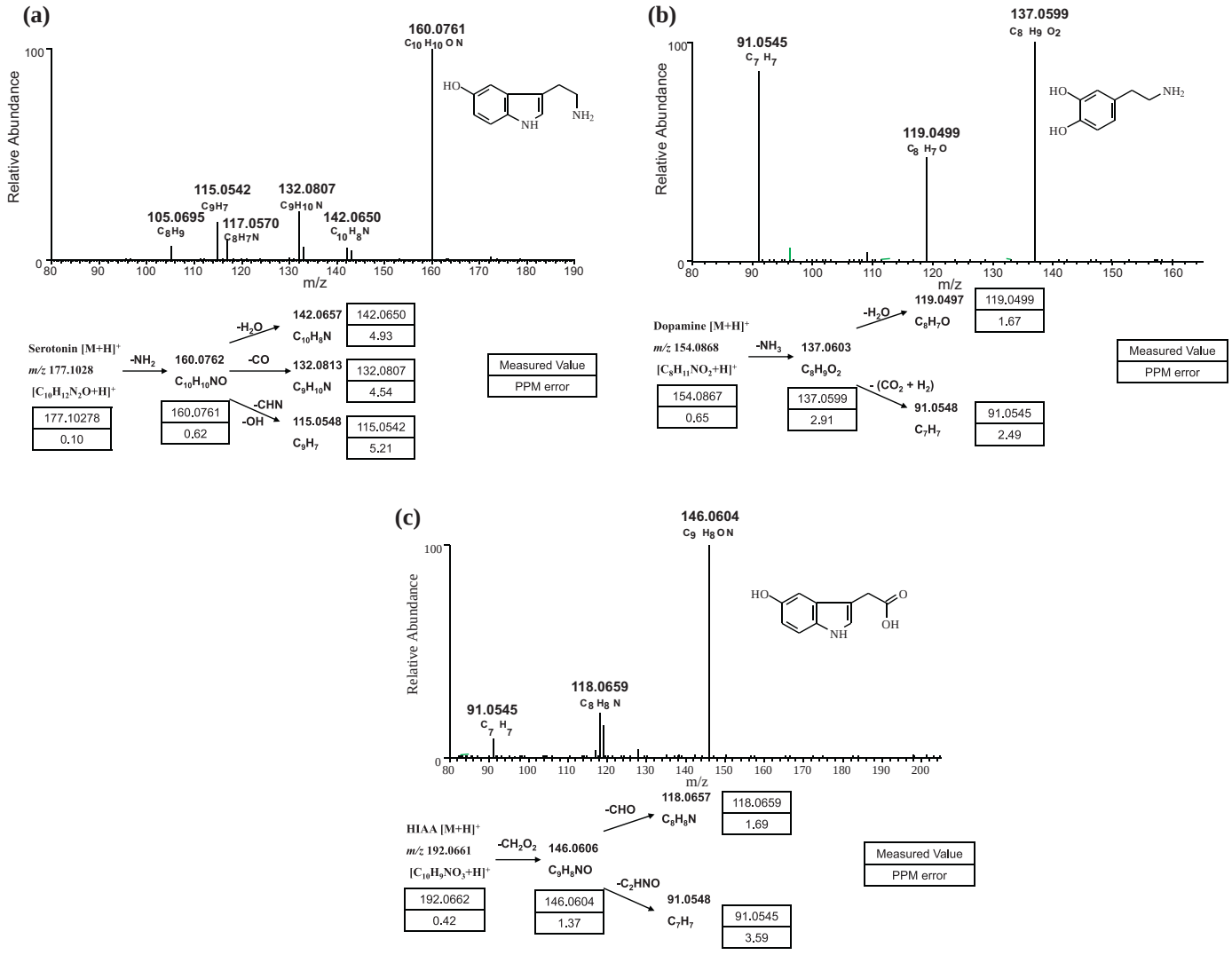

Fig. 4. (a) Fragmentation pathway for serotonin along with the HCD spectra. (b) Fragmentation pathway for dopamine along with the HCD spectra. (c) Fragmentation pathway for 5-HIAA along with the HCD spectra.

experiment are shown in Table 3. The result of the recoveries for serotonin, dopamine and 5-HIAA are quite good: 104%, 92% and 97% for dopamine (1306 nmol/L), 5-HIAA (1046 nmol/L) and serotonin (1135 nmol/L) respectively. Results for serotonin and 5-HIAA were above 100% for the 2270 and 2092 nmol/L spikes with 109 ± 6% and 115 ± 3%, respectively, possibly due to less signal suppression at higher concentrations. Results for the 284 nmol/L spike for serotonin gave a reduced recovery (86 ± 9%) than higher concentrated spiked samples but may be more prone to suppression effects at the lower end of the concentration range (Table 3).

3.6.2. Specificity

Any analytical method must be able to distinguish between the analyte under investigation and other substances that may be present in the sample. This method incorporates a few techniques to create a highly selective method. Firstly, high mass accuracy allows for extremely accurate identification of analyte peaks. In

Table 3
Recovery results for Serotonin, 5-HIAA and Dopamine.

	nmol/L added	nmol/L	±nmol/L	Recovery %	± (%)
Serotonin	0	116.9	25.5		
	284	354.2	24.4	85.67	8.67
	1135	1213.7	99.9	96.65	8.79
	2270	2593.5	141.9	109.11	6.26
5-HIAA	0	734.1	47.1		
	1046	1692.6	35.0	91.63	3.33
	2092	3132.5	60.7	114.64	2.90
Dopamine	0	75.6	9.8		
	653	696.9	23.5	95.18	3.65
	1306	1437.4	73.1	104.31	5.61

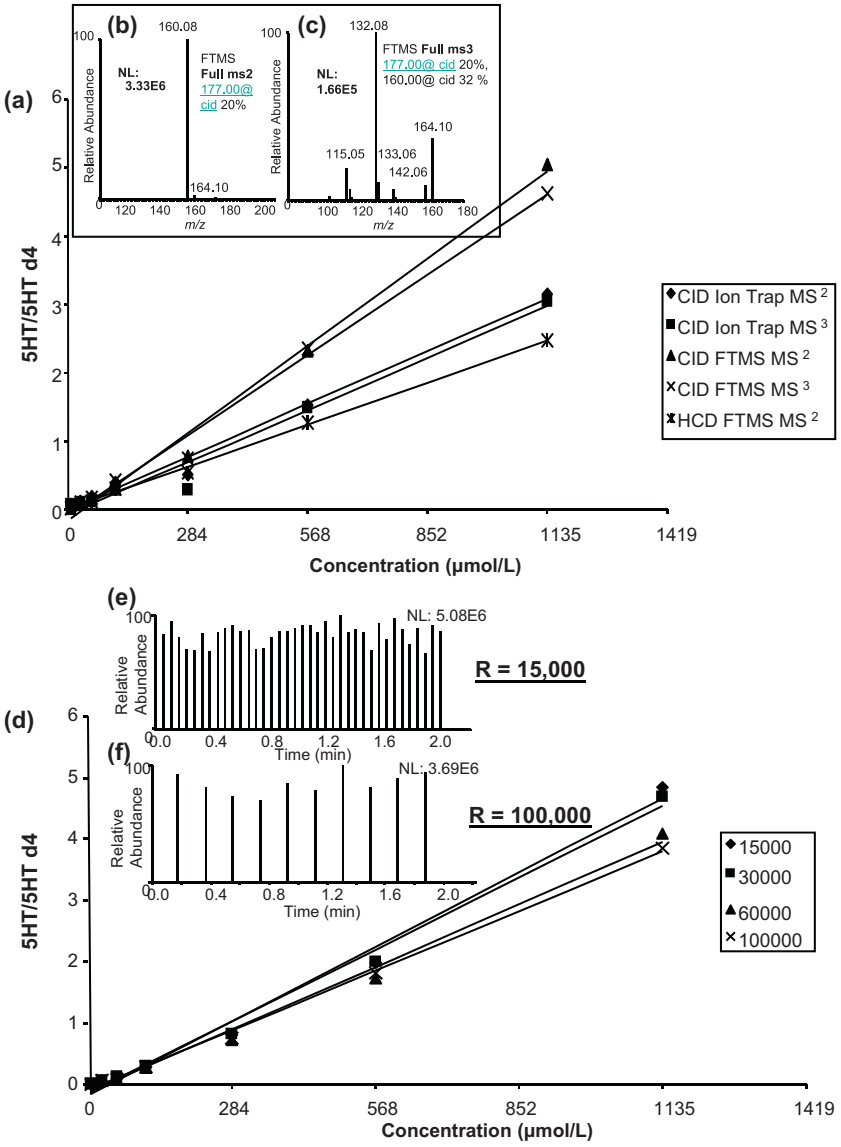

Fig. 5. (a) Serotonin detection method study comparing different activation methods (CID and HCD) and different scans (MS^2 and MS^3) and different detectors (ion trap and FTMS). (b) and (c) are MS^2 and MS^3 scans respectively analyses by FTMS. (d) Resolution study for serotonin comparing the sensitivity of the method at different resolution settings (15,000, 30,000, 60,000, 100,000). (e) and (f) compare the number of scans over the 2 min acquisition time for resolution of 15,000 and 100,000 respectively.

this method a full scan is carried out at 100,000 resolution which can separate individual m/z peaks to a difference of 0.002 amu for serotonin with a m/z of 177.1028. There were very few compounds present in the urine samples tested that were within the mass region of the analytes. All samples analysed were seen to have a PPM error of less then 3%. This translates to a difference in mass readings of ~0.0006 amu for serotonin.

The MS^2 scans allowed for increased specificity as it selects for fragmentation only the $[M+H]^+$ precursor ion to give a single intense fragment ion (e.g. m/z 177 → 160 for serotonin). A resolution of 30,000 was used for the MS^2 CID scans, which provided reliable data for each target compound. While MS^3 fragmentation would increase the specificity of the method, the signal intensities of the product ions were too low to give good accuracy and precision at low concentration levels.

Finally, a data dependant scan using HCD activation was used on each analyte signal to obtain smaller fragment ions similar to that observed in a triple stage quadrupole scan. These fragment ions were used as additional identification points for the analytes (Fig. 4).

3.6.3. Linearity

The linear range of this method was investigated for serotonin, 5-HIAA and dopamine in urine. The concentration range in this study was from 2.837 to 0.057 nmol/L for serotonin, 3.268–0.065 nmol/L for dopamine and 2.618–0.052 nmol/L for 5-HIAA in urine. Table 4 shows the linear range, equation of the line and correlation coefficient for each compound in urine. This method showed good linearity for urine matrix with correlation coefficients of 0.9999 for serotonin and dopamine and 0.9955 for 5-HIAA ($n = 5$).

3.6.4. Limits of detection and quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were evaluated from the signal to noise ratio (S/N). The LOD and LOQ were calculated for each compound in both urine. The S/N was established from the standard deviation of the Y-intercept in 6 low range calibrations which is then divided by the slope of the average calibration of the 6 calibrations. This value was then multiplied by 3.3 for the LOD and 10 for the LOQ [49]. Table 4 shows LOD and LOQ values for serotonin, 5-HIAA and dopamine in urine

Table 4
Linearity, LOD, LOQ, accuracy and precision results for serotonin, 5-HIAA and dopamine.

Urine	Linear range/calibration curve					Accuracy				Intra + interday precision		
	Equation of the line	Linear range μmol/L	R^2	LOD nmol/L	LOQ nmol/L	Theoretical concentration	Measured concentration nmol/L	RE%	RSD % $n=5$	Concentration nmol/L	Intraday RSD% $n=5$	Interday RSD% $n=9$
Serotonin	769,253x − 1,740,789	2.837–0.057	0.9999	12.9	39.0	2837	2782.8	1.9	5.9	2837	3.8	9.5
						1135	1123.7	1.0	7.2	1135	4.3	6.5
						284	262.6	7.5	3.8	568	4.6	9.0
										284	4.9	12.6
										113	7.6	13.4
										57	5.9	9.2
5-HIAA	267,751x − 3,877,841	2.618–0.052	0.9955	19.0	57.7	2618	2541.9	2.8	3.1	2618	8.1	11.1
						1046	998.1	4.6	2.9	1046	4.7	14.5
						262	237.3	9.3	0.6	523	1.7	10.2
										262	5.4	10.3
										105	1.3	11.5
										52	6.2	10.3
Dopamine	37,663x+ 284,341	3.268–0.065	0.9999	9.0	27.2	3268	3256.0	0.3	0.5	3268	2.1	10.6
						1306	1306.7	0.1	6.6	1306	3.7	9.7
						326	311.5	4.6	1.4	653	2.1	8.0
										32650	1.2	9.5
										131	2.6	7.3
										65	4.4	16.0

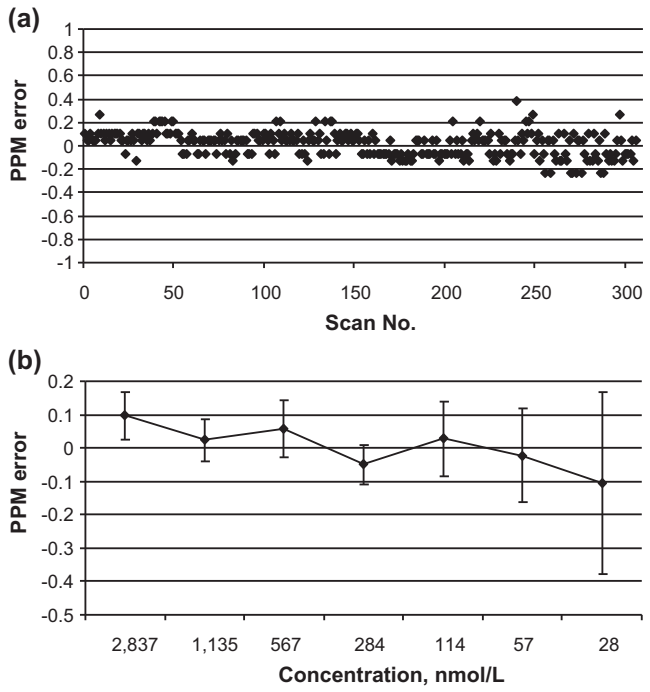

Fig. 6. (a) Graph showing the stability of the mass accuracy (PPM) for serotonin over 360 scans. (b) Graph showing the PPM error variation over the linear range (28–2837 nmol/L).

matrices. The LODs and LOQs for each compound are well below the normal levels usually found in urine samples which are reported as 25–66 μmol/mol, 0–3.9 mmol/mol and 28.8–177.8 μmol/mol of creatinine for serotonin, 5-HIAA and dopamine, respectively [50]. However, in this method, for the analysis of all analytes in urine the sample was diluted by a factor of 20 to allow it to be infused effectively through the very small nozzles on the NanoMate system. While this brought the concentration of the first standard in the standard addition experiment to a concentration level near the limit of quantitation for both serotonin (39 nmol/L) and dopamine (27.2 nmol/L), reproducible linearity was achieved for all the target analytes throughout this study (Table 4).

3.6.5. Accuracy and precision

The accuracy of this method was expressed as the percentage relative error (%RE) of three different concentrations spiked into blank urine. These concentrations were 2837, 1135 and 284 nmol/L for serotonin, 2618, 1046 and 262 nmol/L for 5-HIAA and 3268, 1306 and 326 nmol/L for dopamine. The accuracy compares the true value and the value obtained by analysis. The %RE were very good in the urine matrix giving serotonin values ≤2% for 2837 and 1135 nmol/L spikes and a higher value of 7.5% for 284 nmol/L. Dopamine showed excellent accuracy for all concentrations with very low values for the 3268 and 1306 nmol/L concentrations giving 0.3% and 0.1%, respectively. 5-HIAA showed slightly reduced accuracy with %RE values <5% for 2618 and 1046 nmol/L and 9.3% for the 262 nmol/L (Table 4).

The precision of the method was evaluated using both intra-day and interday studies. A sample number of five and nine were used for intra-day and interday reproducibility study, respectively. Precision was studied using 6 concentrations over the linear ranges. Table 4 shows a list of the percentage relative standard deviations (%RSD) for the concentrations analysed. For the intra-day assay the %RSD values are very low for all compounds in the urine matrices even at low concentrations with the majority of values being under

Table 5
Serotonin, 5-HIAA and Dopamine concentrations (nmol/L) in urine by standard addition using NanoESI-MS along with measured mass of analytes and mass accuracy represented as PPM error.

	Sample			
	1	2	3	4
Serotonin (nmol/L)	1090.7	357.5	679.6	425.6
Measured Mass	177.1023	177.1023	177.1024	177.1023
PPM error	2.82	2.82	2.26	2.82
5-HIAA (nmol/L)	N/A	15,796	13,714	13,243
Measured mass		192.0656	192.0655	192.0657
PPM error		2.08	2.60	1.56
Dopamine (nmol/L)	144.9	267.7	1286	574.5
Measured mass	154.0865	154.0864	154.0863	154.0864
PPM error	1.95	2.60	3.24	2.60

5%. The interday assay showed increased %RSD values showing an average variability of *ca.* 10% for each compound.

3.7. Analysis of compounds in urine using standard addition

Analysis of urine samples ($n > 30$) was carried out by standard addition. As previously stated, each analysis consisted of a MS^2 scan for each analyte and deuterated internal standard at 30,000 resolution, a full scan at 100,000 resolution for high mass accuracy data and a data dependant HCD scan for analyte identification. The results for four samples can be seen in Table 5.

4. Conclusion

This method provides a precise, accurate and rapid new approach for the analysis of serotonin, dopamine and 5-HIAA using an infusion method that requires small amounts of sample and has a 2.2 min run time. It has good selectivity ensuring accurate identification of the target analytes. The method also demonstrates the high mass accuracy capability of the LTQ Orbitrap. Standard addition calibration was used for quantitation of the target analytes in urine samples to ensure accurate results. Standard addition also compensates for any matrix effects that may be caused from components in the sample matrix. The method has been comprehensively validated and an instrument qualification evaluation study to assess consistent mass accuracy and resolution was carried out. The method described provides a useful protocol or template by which an Orbitrap MS instrument and its scan selection may be evaluated. Most importantly the method presented illustrates the power of nanoelectrospray technology when combined with MS for the determination of key neurotransmitters in urine. It also provides equal or better sensitivity, accuracy and precision in comparison with traditional LC/MS/MS methods, with faster analysis times and the added benefit of high mass accuracy when used with the LTQ Orbitrap.

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