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# Application of a urine and hair validated LC-MS/MS method to determine the effect of hair colour on the incorporation of 25B-NBOMe, 25C-NBOMe and 25I-NBOMe into hair in the rat.



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## Abstract

NBOMes are a group of new psychoactive substances derived from phenethylamines. Recreational abuse is thought to have begun in 2010 and they are commonly associated with the "club drug" scene. They are administered in liquid form or as blotters due to their high potency.

An LC-MS/MS method was validated using SWGTOX parameters for the detection of 25B-, 25C- and 25I-NBOMe using 25B-NBOMe-$D_3$ as internal standard for urine and hair. Calibration graphs with $R^2$ values >0.99 were observed for urine and hair for concentrations ranging from 0.1 -100 ng/mL and 0.025-2.5 ng/mg respectively. Urine LODs ranged from 5-25 pg/mL and had an LOQ of 50 pg/mL. Hair LOD and LOQs ranged from 3-5 pg/mg and 6.25-12.5 pg/mg respectively. Intra and inter-day precision was <20% and accuracy was within ± 20% for both matrices. The method was shown to be selective for both exogenous and endogenous compounds. No matrix effects were observed for either matrix. LLE recovery ranged from 90-103% for urine samples and SPE recovery ranged from 80-107% for hair samples.

Long-Evans rats (n=55) were administered 25B-, 25C- or 25I-NBOMe at doses ranging from 30-300 µg/kg over a period of 10 days. Rats were shaved prior to their first dose and re-shaved after the 10-day period. Hair was separated by colour (black: n=55 and white: n=55) and analysed using the validated LC-MS/MS method to assess the impact hair colour has on the incorporation of these drugs.

All drugs were successfully detected in black hair. 25B-NBOMe from rats receiving the highest dose and 25C-NBOMe from rats receiving the medium and high doses were quantified in white hair. 25I-NBOME was detected but fell below the limit of quantification. A dose-dependent concentration increase was observed in the black hair. All pooled urine samples tested positive for their expected NBOMes.


KEYWORDS: NBOMe, hair testing, LC-MS/MS, urinalysis

## Introduction

The analysis of hair poses significant benefits in forensic toxicology. Not only is it utilized when more conventional matrices such as blood and urine are not available, but it also provides an approximate timeline of drug use (1). This is particularly useful in cases of child custody, work place drug testing or in drug facilitated sexual assault (DFSA) cases (1–3). Each time drugs are administered to the body, they are carried through the blood and are distributed to various tissues and fluids before being eliminated. Drugs are deposited into the hair follicle via the blood and are trapped there permanently, growing out with the hair (2). This is a simplistic model of how drugs are incorporated into hair and in reality other factors such as incorporation through sweat in humans may play a part. Many factors affect the rate at which drugs will be incorporated into the hair, with hair colour having significant impact (4).

Drugs can also be present in hair samples due to contamination through smoking or handling of drugs. In order to remove this external contamination, hair samples must undergo thorough decontamination wash procedures (4–6). However, it has been shown that it may not be possible to remove all external contamination from hair. It is also important to ensure that hair is collected from acceptable sites that an adequate volume is collected and that appropriate cut-off values are set. The Society of Hair Testing (SoHT) has recommended procedures, guidelines and cut-off values to help aid with the interpretation of drug concentrations in human hair (7).

Hair analysis is not a new technique having originally been applied to forensic analysis in the 1850's when the presence of arsenic was identified in a body exhumed 11 years after burial (8). Despite this matrix having such a long history, its use is still limited due to the long sample preparation, incubation times and limited compatibility with automation. The low concentrations detected often require highly sensitive instrumentation which not all laboratories have access to such as LC-MS/MS, and interpretation can be difficult due to changes in drug concentrations with time due to cosmetic treatments (9–11).

Despite these difficulties, hair analysis in itself is relatively straight forward as no extensive metabolism or excretion occurs after the drug has been deposited. The parent drug is typically deposited at a higher concentration than the metabolite due to its more lipophilic nature. This makes it particularly applicable to New Psychoactive Substances (NPSs) where the metabolites of these substances may not always be known, or commercially available (12,13).

NBOMes are phenethylamine derivatives of the 25C-X series. The first NBOMe was mentioned in Ralf Heim's PhD thesis in 2004, with the first mention of recreational abuse appearing in 2010 (14,15). NBOMes are routinely associated with the club drug scene and are administered either in liquid form or on blotters (16). Due to the high potency of NBOMes, adverse effects and fatalities have been reported following their use (17–19). Analytical methods have been published for blood, urine, vitreous humour, brain, liver, bile and gastric contents; however no method for hair analysis has been published to date (20).

The aim of this research was to extract 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25C-NBOMe) and 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) from rat hair by using a phosphate buffer incubation and solid phase extraction (SPE) clean up method. Additional objectives included assessing any dose response relationship and determining whether the colour of hair affects drug concentration incorporated as seen with other phenethylamines (10). This method aims to assist practitioners in the detection of these compounds in work place drug testing.

## Materials and Methods

### Chemicals and reagents

4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25C-NBOMe) and 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) monohydrochloride powders (10 mg) were purchased from Cayman Chemical (MI, USA). Methanol 1 mg/mL

hydrochloride standards of 25B-NBOMe, 25C-NBOMe, 25I-NBOMe and 25B-NBOMe-$D_3$ were purchased from Sigma Aldrich (Poole,UK). Dimethylsulfoxide (DMSO) (Sigma Aldrich, MO, USA) was used as the injection vehicle in saline. Syringes and needles were purchased from Fisher Scientific (MA, USA). Needles were replaced after each injection and syringes were changed daily for each drug used. Formic acid was purchased from Sigma Aldrich (MO, USA and Poole, UK). All NBOMe reagents were purchased from Sigma Aldrich (Poole, UK). $NaH_2PO_4.H_2O$, $Na_2HPO_4$ and sodium hydroxide were purchased from BDH (Poole, UK). $NH_4CH_3CO_2$ were purchased from Sigma Aldrich (Poole, UK). All other chemicals were of analytical grade and purchased from VWR (Leicestershire, UK). UCT Cleanscreen CSDAU133 cartridges were used (PA, USA).

**Calibrators, quality control and internal standard preparation**

Stock solutions of 100,000 ng/mL for each NBOMe solution were prepared by dilution with methanol. Serial dilutions using these solutions were then carried out using methanol to produce three working solutions of 10, 100 and 1,000 ng/mL. A 1,000 ng/mL and 100 ng/mL solution of 25B-NBOMe-D3 was prepared by dilution with methanol. Calibrators and controls were prepared from different lots by different analysts.

Urine calibration standards ranging from 0.1-100 ng/mL (n=7) were extracted in duplicate. Urine quality controls (QCs) were prepared at concentrations 0.18 ng/mL (QC1), 4.20 ng/mL (QC2) and 84.0 ng/mL (QC3).

Hair calibration standards ranging from 0.025-2.5 ng/mg (n=7) were extracted in duplicate. Hair quality controls (QCs) were prepared at concentrations 0.105 ng/mg (low), 1.05 ng/mg (medium) and 2.10 ng/mg (high). All QCs were extracted in triplicate.

**Urine Sample preparation**

One mL aliquots of urine were transferred into glass culture tubes. Calibrators and QCs were then produced by adding the relevant solution volumes to each tube. For instance to produce a 0.1 ng/mL urine calibrator 10 µL of each 10 ng/mL NBOMe solution was used to spike 1 mL of blank urine. This process was repeated for all calibrators. To each tube 100 µL of 100 ng/mL 25B-NBOMe-$D_3$

internal standard (I.S.) was added, as well as 500 µL of 0.1 M NaOH solution. Extraction solvent (3 mL) was then added to each test tube (50:50 hexane: ethylacetate (EtOAc)) and samples were rotated and mixed for 10 minutes using a Denley Spiramix 10. They were then centrifuged for 5 minutes at 4000 rpm. These test tubes were then placed in the freezer (-20$^{o}$C) for 5 minutes, freezing the bottom aqueous layer. The organic top layer was then decanted into another new glass culture tube. Samples were evaporated to dryness under nitrogen at room temperature. The samples were then reconstituted with 75 µL mobile phase mixture (50% mobile phase A: 50% mobile phase B). This solution was then transferred to autosampler vials for analysis by LC-MS/MS.

**Hair Sample Preparation**

Hair calibrators, QCs and samples were washed with deionised water (3 mL) and dichloromethane (3 mL). Each wash was sonicated for 30 minutes at room temperature and any excess solvent was transferred into fresh 7 mL vials using Pasteur pipettes, for later analysis. In between each wash, any remaining solvent was evaporated to dryness using a gentle stream of nitrogen at room temperature. Hair was then cut into 1-2 mm segments and 40 mg (±1 mg) was weighed and transferred to new 7 mL vials. These were then spiked with 50µl of 25B-NBOMe-$D_3$ (1,000 ng/mL). Calibrators and QCs were made by adding the relevant solution volume. For instance to produce the 0.025 ng/mg calibrator 10 µL of 100 ng/mL of each NBOMe solution was used to spike 40 mg of hair. This process was repeated for all calibrators. To each vial, 3 mL of pH7.4 phosphate buffer were added before sonicating for 1 hour. Vials were then transferred to the oven where they were left to incubate overnight (12 hours) at 40$^{o}$C.

After incubation, the samples were centrifuged at 4000 rpm for 10 minutes. SPE cartridges were conditioned using 2 mL methanol, and 1 mL 0.1 M pH7.4 phosphate buffer. The supernatant from the centrifuged samples was transferred to the conditioned SPE cartridges. SPE cartridges were washed with 3 mL of $dH_2O$ and 1 mL of 1 M acetic acid before being dried for 10 minutes under vacuum. Methanol (3 mL) was then added to the cartridges before subsequent drying again under full vacuum for 1 minute. Elution was carried out using a 3 mL dichloromethane/isopropyl alcohol /ammonia solution, before being evaporated to dryness under a slow stream of nitrogen, at room temperature.

The samples were then reconstituted in 75 µL of mobile phase mixture (50% mobile phase A: 50% mobile phase B), vortex mixed for 30 seconds and transferred to vials for analysis by LC-MS/MS.

**Instrumentation**

Analysis of samples was carried out using an Agilent Technologies 6420 series triple quadrupole LC-MS/MS (Agilent, USA) coupled with an Agilent 1260 binary pump, autosampler, degasser and thermostated column compartment. An electrospray ionization (ESI) source was used in positive ionization mode. The column used was a Phenomenex Gemini® 5 µm C18 110 Å (15 x 2 mm) with a Phenomenex Gemini C18 guard column (4 x 2.0mm) (Cheshire, UK). The column temperature was maintained at $40^0$C. Isocratic gradient elution (50:50) was employed using a mobile phase consisting of A) 2 mM ammonium acetate in $dH_2O$ and B) 2 mM ammonium acetate in MeOH at a flow rate of 0.3 mL/min. The injection volume was 30 µl and the MS was operated in multiple reaction monitoring mode (MRM) and the total run time was 4 minutes. The optimal conditions were achieved using a gas flow of 11 L/min, a nebulizer pressure of 30 psi and a capillary voltage of 4000 V.

**Method Validation**

The method was validated according to the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines and sample analysis was completed following the guidelines produced by the Society of Hair Testing (SoHT).

Specificity and selectivity were assessed by comparing the chromatograms of urine and hair from 10 different blank sources (donors) with those of the spiked NBOMe standards. Each of the analytes and I.S. were analysed individually to observe any interferences between each analyte peak area and height. Exogenous interferences were assessed by analysing a wide range of compounds commonly encountered within the forensic and clinical setting (including paracetamol and caffeine), and comparing the resulting chromatograms with those of the target analytes and I.S.

Linearity was assessed by analysing six separate calibration curves for 25B-NBOMe, 25C-NBOMe and 25I-NBOMe. These were produced by spiking each matrix with each NBOMe at concentrations ranging from 0.025-2.5 ng/mg (hair) and 0.1-100 ng/mL (urine). All calibrators were prepared freshly

in duplicate over 6 days. Calibration curves were generated by plotting the peak area ratio of each analyte to internal standard against concentration. Blank matrix with internal standard was included with each batch but not included in the calibration curves. The correlation coefficient ($R^2$) was calculated. The $R^2$ values should be >0.99. Residual plots were also assessed to determine if any calibrators fell out with ±3 standard deviations.

The LOD and LLOQ were determined by assessing the peak area response of standards of decreasing concentration. For LOD a signal to noise ratio >3 was required and for LLOQ a signal to noise ratio >10 was required. LLOQs were also required to give reproducible data with a co-efficient of variation (CV%) <20%.

Accuracy and precision were calculated by running calibration standards alongside three replicates of each QC, low (n=18), medium (n=18) and high (n=18). Results had to have accuracy values within ±20%. The intra- and inter-day variation limit was set at <20%.

Autosampler stability was assessed by injecting the low and high QCs in triplicate over a 72-hour period. Changes in peak area ratio (PAR) were observed and were noted. Analytes were deemed stable when results were ±20% of the true value. PARs was monitored to determine if after instrument failure, analysis could be resumed without directly impacting upon the concentrations calculated. QC's were individually extracted.

Matrix effect was identified by determining the PAR of standards added to extracted blank matrix to that of the standard solutions of the same concentration. If the value obtained through this calculation was <1 then ionization suppression was observed, where as a value >1 showed ionization enhancement (21).

Recovery was calculated by dividing the peak area of extracted standard by the peak area of standard spiked after the blank matrix was extracted at the same concentration.

Carryover was identified by injecting the highest calibration standard in triplicate and analysing the following blank mobile phase injection.

Dilution integrity of urine samples was assessed by preparing 12 replicates of QC3 (84 ng/mL) and diluting these with the addition of 1 to 4 mL of blank urine, producing triplicate dilutions of 1:2. 1:3, 1:4 and 1:5. Each QC was then vortex mixed before transferring 1 mL of each diluted QC to separate culture tubes and extracting.

**Injection Vehicle**

Injection solutions were prepared by dissolving each of the individual NBOMe powders (10 mg) in DMSO (1 mL). The 30 µg/kg (low), 100 µg/kg (medium) and 300 µg/kg (high) dose solutions were then produced by serial dilution with saline solution.

**Specimens**

Male Long Evans rats were purchased from Charles River (PA, USA). Animals were housed singly in a humidity and temperature controlled laboratory with 12:12 hour light:dark cycles. Animals entered the laboratory at age 3 weeks old and weighed 28-50g at the start of the study. Animals were given 7 days to acclimatize to their surroundings before any experimental work commenced and had *ad libitum* access to food and water. All rats (n=55) were initially shaved with an electric razor. After the 1-week grace period all rats (n=55) were shaved with an electric razor along their backs, collecting both black and white hair separately in clean 7 mL vials.

Each rat was injected intraperitoneally (IP) with either the low (30 µg/kg), medium (100 µg/kg) or high dose (300 µg/kg) of a single NBOMe drug. Typical human doses range from 200-1500 µg, which is in line with the lowest dose received by the rats. After 8 days of injections the rats were re-shaved in the same place as the initial shaving, with shavings separated by dose and colour. Hair from animals receiving the same drug at the same dose was pooled. Hair samples were analysed in triplicate. All procedures were conducted during the dark cycle, under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Arcadia University.

## Results and discussion

### Method Validation Results

The method was developed and validated for 25B-NBOMe, 25C-NBOMe and 25I-NBOMe as per SWGTOX and SoHT guidelines (7,22). The MRM transitions are given in Table 1 along with the retention times of each analyte.

### Specificity and selectivity

None of the exogenous compounds tested interfered with the peak area or peak height or any of the target analytes. There was no interference observed between the internal standard and any of the analytes. Urine and hair from 10 different donors also provided no interference to the internal standard or the other analytes.

### Linearity, LOD, and LLOQ

Urine

All calibration models were linear over a wide range of concentrations 0.1-100 ng/mL with an $R^2$>0.99. The matrix LODs were determined to be 5, 10 and 25 pg/mL for 25B-NBOMe and 25C-NBOMe and 25I-NBOMe respectively. The LOQ for all analytes was 50 pg/mL.

Hair

All calibration models were linear over a wide range of concentrations 0.025-2.5 ng/mg with an $R^2$>0.99. The matrix LODs were determined to be 3 pg/mg for 25B-NBOMe and 25C-NBOMe where as 25I-NBOMe had an LOD of 5 pg/mg. The LOQ results for both 25B-NBOMe and 25C-NBOMe were 6.25 pg/mg. Again 25I-NBOMe was slightly higher with a result of 12.5 pg/mg.

### Accuracy and precision

Urine

All intra-day urine precision results were <15% as shown in Table 2. 25C-NBOMe showed the least variability averaging 2.3%, (range 1.3-3.4%). 25I-NBOMe showed the largest intra-day variation averaging 7.3% (rang 2.9-14.9%). Urine inter-day precision results showed variation <20%. 25B-

NBOMe showed the least variability averaging 7.8%, (range 4.3-12.7%). 25I-NBOMe showed the largest intra-day variation averaging 9.1% (range 4.1-15.7%). Urine accuracy results were within ±20% of the expected value. 25C-NBOMe had the highest calculated accuracy (98-114%), closely followed by 25I-NBOMe (99-118%).

Hair

All intra-day hair precision results were <15% as shown in Table 3. 25C-NBOMe showed the least variability averaging at 4.9%, (ranging 2.9-9.1%). 25B-NBOMe showed the largest intra-day variation averaging 8.2% (range 2.6-12.1%). Hair inter-day precision results showed variation <15%. All NBOMes yielded roughly the same results averaging 7.1, 8.0 and 6.2% for 25B-NBOMe, 25C-NBOMe and 25I-NBOMe respectively. Results ranged from 1.2-11.3%. Hair accuracy results were all within ±10% of the expected value. 25B-NBOMe had the highest calculated accuracy (103-109%), closely followed by 25C-NBOMe (96-109%). This is unsurprising as the I.S. used was 25B-NBOMe-$D_3$. Therefore, any differences observed in 25B-NBOMe analysis over the course of the validation should have been closely mimicked by 25B-NBOMe-$D_3$.

**Matrix effects, recovery and carryover**

No carryover was observed in any of the subsequent washes after injection of the highest calibration standard in triplicate.

Urine LLE extraction of 25B-, 25C- and 25I-NBOMe from urine was extremely efficient with % recoveries ranging from 89-102%. Urine process efficiency was also good ranging from 89.4-102.6%. Matrix effects were less than ±10 % for all urine QC's with the exception of 25C-NBOMe which saw slight enhancement although this was <±20%.

Hair extraction recovery (%) ranged from 80.5-107.5% over all compounds and QC levels. Process efficiency was excellent for both 25B and 25C-NBOMe ranging from 94-104%. It was lower for 25I-NBOMe which was only 55-68%. Matrix suppression for hair samples was minimal for all 25B and 25C-NBOMe QC's ranging from 0.93-1.12. Again 25I-NBOMe performed slightly poorer ranging from 0.77-0.82 although this was within the ±25 % limits of acceptability.

### Stability

All analytes extracted from urine showed significant degradation whilst on the autosampler after 72 hours, with all QC1 and QC3 results failing to meet the <20% acceptability cut-off. Only the 25C and 25I-NBOMe QC2 met the ±20% requirements with recoveries of 84.6% and 89.5% respectively. 25B-NBOMe was the most affected analyte over the 72-hour time period, with an average recovery of 66.0% (50.9-75.0%). 25I-NBOMe was the least affected analysed over the 72-hour time period, with an average recovery of 82.4% (77.8-89.5%) at the end of the 72-hour period. Recovery for all analytes did not decrease by more than 20% after 24 hours, with average % recovery values of 96.5, 92.3 and 92.8% respectively for all 3 QCs.

Hair autosampler stability results are shown in Table 4. Both 25C and 25I-NBOMe showed acceptable stability results over the 120-hour time period with recovery values ±20% of the original. 25I-NBOMe however, fell outside this for QC2 and QC3 with recoveries of 123.3 and 123.7%. On average 25C-NBOMe remained the truest to its original recovery with an average result of 101.7% across all 3 QCs.

It is much more likely that hair samples would not be able to be re-extracted due to limited sample volume and thus this parameter is more important for this matrix should instrument failure occur and reinjection of samples is necessary.

It is not known why there is such a marked difference between urine and hair results for autosampler stability (50.9-89.5% and 86.67-12.7% respectively for each matrix). Both used different extraction techniques and it is possible that matrix contamination is still present in urine samples extracted by LLE, which is negatively affecting the concentrations of these samples.

### Dilution Integrity

The dilution integrity for urine samples was assessed. All dilution % accuracy results fall within the selected <20% criteria with the exception of the 1:4 dilution. For this particular dilution 25I-NBOMe was out with the accepted <±20% limit, at +21.7% and 25B-NBOMe was further outside this limit at +29%. 25C-NBOMe was the only analyte to remain within ±20% for all dilutions. 25B- and 25I-

NBOMe samples should therefore only undergo a maximum of 1:3 dilutions in order to comply with the original precision and bias requirements. 25C-NBOMe is able to undergo a 1:4 dilution if necessary, whilst retaining acceptable accuracy.

**Pooled Rat hair samples**

Pooled black hair concentrations ranged from 37-92 pg/mg for rats dosed with 25B-NBOMe (n=3), 21-143 pg/mg for rats dosed with 25C-NBOMe (n= 3) and 14-92 pg/mg for rats dosed with 25I-NBOMe (n=3). Pooled white hair from rats administered 25B-NBOMe (n=3) was only positive from those receiving the highest dose (300 µg/kg), with 22 pg/mg detected. Pooled white hair from rats dosed with 25C-NBOMe (n=3) was positive from animals receiving the medium and high doses (100 µg/kg and 300 µg/kg) with 11 pg/mg and 30 pg/mg detected respectively. All white hair from rats receiving 25I-NBOMe (n=3) tested positive (above the 5 pg/mg LOD), but all were below the 12.5 pg/mg LLOQ for this method.

25C-NBOMe incorporated into black hair to the greatest extent for 300 µg/kg doses in comparison to 25B-NBOMe and 25I-NBOMe. 25C-NBOMe also incorporated into black hair of rats receiving the medium dose to the greatest degree, +10% in comparison to 25B-NBOMe and +37% in comparison to 25I-NBOMe. Of rats receiving the lowest dose of drug, hair from those administered 25B-NBOMe yielded the highest concentration of 37 pg/mg; 43% higher than the concentration detected in hair from rats dosed with 25C-NBOMe and 62% higher than the concentration detected in hair from rats dosed with 25I-NBOMe. Drugs were only detected and quantified in white hair from rats receiving the highest concentration of drug as illustrated in Table 5. Concentrations marked with an asterix (*) fell below that of the level 1 calibrator (25 pg/mg) and thus were calculated using the LOQ calibration curve which was run on the same day.

Overall, 25C-NBOMe > 25B-NBOMe > 25I-NBOMe when it came to the incorporation of each drug into black hair. This may be in part due to the electronegativity the halogen ion on each NBOMe, with chlorine > bromine > iodine (10). Chlorine has already been shown to positively affect incorporation rates, although further work would need to be carried out in order to better assess this hypothesis (10).

The basicity of a drug is an important factor affecting the incorporation of drugs into hair. As the size of atoms increase their basicity decreases, and thus it follows that 25C-NBOMe incorporates greater than 25B-NBOMe and 25I-NBOMe (23). These observations were seen regardless of hair colour with 25C-NBOMe being the only drug to be detected in white hair with a concentration above that of the level 1 calibrator.

The water and dichloromethane washes for each hair concentration were analysed as well as those from a blank rat hair sample. Both blanks (white and black) resulted in no detectable traces of NBOMes. Deionised water washes resulted in little to no NBOMe drugs detected. This was not the case with the dichloromethane washes however, which did result in identification of NBOMes, although the PARs of these results were significantly lower than that of the level 1 calibrator as shown in Figure 1. External contamination is unlikely in this case as rats do not produce sweat. This may be a result of the long sonication time which enables the solvent to extract some of the drug from the hair.

### Urine Samples

All pooled urine samples tested positive for their expected NBOMes. Due to the limited sample volume urine analysis was carried out once for each NBOMe. As seen with hair samples 25C-NBOMe provided the highest detected concentration (16 pg/mL), however this was then followed by 25I-NBOMe (12 pg/mL), and finally 25B-NBOMe (7 pg/mL). Again this may be in part due to the electronegativity the halogen ion on each NBOMe, with chlorine > bromine > iodine (10).

## Conclusion

A simple and quick quantitative LC-MS/MS method for the analysis of 3 common NBOMes was developed and validated for urine and hair. The speed of this method allows for high sample throughput, with time limiting steps being the sample preparation itself. Black hair incorporated all drugs to a higher degree than the white hair. All white hair from rats receiving 25I-NBOMe tested positive, but all were below the LOQ for this method. A dose-dependent concentration increase was observed in the black hair.

## Conflict of interest

The authors have declared no conflicts of interest.

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Table 1: LC-MS/MS precursor ions, product ions, dwell time, fragment voltage, collision energy and retention time for 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25C-NBOMe), 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) and 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine –$D_3$ (25I-NBOMe-$D_3$).

| Analyte | Precursor Ion (amu) | Product Ion (amu) | Dwell (s) | Fragment (V) | Collision Energy (V) | RT (mins) |
|---|---|---|---|---|---|---|
| **25B-NBOMe-$D_3$** | 383 | 124 | 50 | 110 | 20 | 2.2 |
| **25B-NBOMe** | 380 | 121 | 50 | 115 | 20 | 2.2 |
| | 380 | 91 | 50 | 115 | 50 | 1.9 |
| **25C-NBOMe** | 336 | 121 | 50 | 105 | 18 | 1.9 |
| | 336 | 91 | 50 | 105 | 50 | 1.9 |
| **25D-NBOMe** | 428 | 121 | 50 | 120 | 20 | 1.8 |
| | 428 | 91 | 50 | 120 | 60 | 1.8 |

Table 2: Intra-day precision, inter-day precision and accuracy of 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25C-NBOMe) and 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) in urine.

| **Urine Results** | | **25B-NBOMe** | **25C-NBOMe** | **25I-NBOMe** |
|---|---|---|---|---|
| **Intra-day Precision (%CV) (n=18)** | **QC 1 (0.18 ng/mL)** | 2.3 | 10.2 | 4.2 |
| | **QC 2 (4.2 ng/mL)** | 1.3 | 1.2 | 2.9 |
| | **QC 3 (84.0 ng/mL)** | 3.4 | 6.0 | 14.9 |
| **Inter-day Precision (%CV) (n=18)** | **QC 1 (0.18 ng/mL)** | 6.4 | 9.9 | 7.6 |
| | **QC 2 (4.2 ng/mL)** | 12.7 | 12.6 | 15.7 |
| | **QC 3 (84.0 ng/mL)** | 4.3 | 4.8 | 4.1 |
| **Accuracy (%) (n=18)** | **QC 1 (0.18 ng/mL)** | 80.3 | 105 | 118 |
| | **QC 2 (4.2 ng/mL)** | 117 | 98 | 99 |
| | **QC 3 (84.0 ng/mL)** | 104 | 114 | 113 |

Table 3: Intra-day precision, inter-day precision and accuracy of 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25C-NBOMe) and 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) in hair.

| **Hair Results** | | **25B-NBOMe** | **25C-NBOMe** | **25I-NBOMe** |
|---|---|---|---|---|
| **Intra-day Precision (%CV) (n=18)** | **QC 1 (0.105 ng/mg)** | 2.6 | 9.1 | 4.8 |
| | **QC 2 (1.05 ng/mg)** | 9.9 | 2.9 | 7.1 |
| | **QC 3 (2.10 ng/mg)** | 12.1 | 2.7 | 11.4 |
| **Inter-day Precision (%CV) (n=18)** | **QC 1 (0.105 ng/mg)** | 10.9 | 8.1 | 10.7 |
| | **QC 2 (1.05 ng/mg)** | 4.8 | 11.3 | 1.2 |
| | **QC 3 (2.10 ng/mg)** | 5.8 | 4.5 | 6.8 |
| **Accuracy (%) (n=18)** | **QC 1 (0.105 ng/mg)** | 109 | 96 | 102 |
| | **QC 2 (1.05 ng/mg)** | 104 | 105 | 110 |
| | **QC 3 (2.10 ng/mg)** | 103 | 109 | 94 |

Table 4: Autosampler results for 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25C-NBOMe) and 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) in hair.

| | | 25B-NBOMe | 25C-NBOMe | 25I-NBOMe |
|---|---|---|---|---|
| **QC1 (n=3) (Peak Area Ratio)** | **t=0 (h)** | 0.12 | 0.15 | 0.15 |
| | **t=24 (h)** | 0.17 | 0.17 | 0.14 |
| | **t=120 (h)** | 0.14 | 0.13 | 0.17 |
| | **Recovery %** | 117.0 | 86.7 | 113.0 |
| **QC2 (n=3) (Peak Area Ratio)** | **t=0 (h)** | 1.03 | 1.36 | 1.07 |
| | **t=24 (h)** | 1.46 | 1.82 | 1.00 |
| | **t=120 (h)** | 1.27 | 1.47 | 1.23 |
| | **Recovery %** | 123.0 | 108.0 | 115.0 |
| **QC3 (n=3) (Peak Area Ratio)** | **t=0 (h)** | 2.19 | 2.79 | 2.25 |
| | **t=24 (h)** | 3.15 | 3.89 | 2.05 |
| | **t=120 (h)** | 2.71 | 3.08 | 2.61 |
| | **Recovery %** | 124.0 | 110.0 | 116.0 |

Table 5: Average concentration of and standard deviation of 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25C-NBOMe) and 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) in black and white rat hair for each dose group.

| Drug | Dose (µg/kg) | Black Hair | | White Hair | |
|---|---|---|---|---|---|
| | | Average Concentration (pg/mg) | Standard Deviation (pg/mg) | Average Concentration (pg/mg) | Standard Deviation (pg/mg) |
| **25B-NBOMe (n=3)** | 30 | 37 | ±3.3 | Not Detected | |
| | 100 | 51 | ±0.7 | Not detected | |
| | 300 | 92 | ±1.5 | 22* | ±0.3 |
| **25C-NBOMe (n=3)** | 30 | 21* | ±2.8 | Not detected | |
| | 100 | 57 | ±3.3 | 11* | ±1.0 |
| | 300 | 143 | ±1.0 | 30 | ±0.4 |
| **25I-NBOMe (n=3)** | 30 | 14* | ±1.6 | Detected | |
| | 100 | 36 | ±0.8 | Detected | |
| | 300 | 92 | ±4.3 | Detected | |

## Figure

Figure 1: Peak area ratios of dichloromethane washes from rat hair samples from dosed with 300 µg/kg of 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25C-NBOMe) or 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) and the peak area ratio of a level 1 calibrator.