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Direct injection horse-urine analysis for the quantification and confirmation of threshold substances for doping control. IV. Determination of 3-methoxytyramine by hydrophilic interaction liquid chromatography/quadrupole time-of-flight mass spectrometry

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Levodopa and dopamine have been abused as performance-altering substances in horse racing. Urinary 3-methoxytyramine is used as an indicator of dopaminergic manipulation resulting from dopamine or levodopa administration and is prohibited with a urinary threshold of 4 μg mL^{-1} (free and conjugated). A simple liquid chromatographic (LC)/mass spectrometric (MS) (LCMS) method was developed and validated for the quantification and identification of 3-methoxytyramine in equine urine. Sample preparation involved enzymatic hydrolysis and protein precipitation. Hydrophilic interaction liquid chromatography (HILIC) was selected as a separation technique that allows effective retention of polar substances like 3-methoxytyramine and efficient separation from matrix compounds. Electrospray ionization (ESI) in positive mode with product ion scan mode was chosen for the detection of the analytes. Quantification of 3-methoxytyramine was performed with fragmentation at low collision energy, resulting in one product ion, while a second run at high collision energy was performed for confirmation (at least three abundant ions). Studies on matrix effects showed ion suppression depending on the horse urine used. To overcome the variability of the results originating from the matrix effects, isotopic labelled internal standard was used and linear regression calibration methodology was applied for the quantitative determination of the analyte. The tested linear range was 1–20 μg mL^{-1}. The relative standard deviations of intra- and inter- assay analysis of 3-methoxytyramine in horse urine were lower than 4.2% and 3.2%, respectively. Overall accuracy (relative percentage error) was less than 6.2%. The method was applied to case samples, demonstrating simplicity, accuracy and selectivity.

Keywords: 3-methoxytyramine; horse urine; doping control; direct injection analysis; hydrophilic interaction liquid chromatography; quadrupole time-of-flight mass spectrometry

Introduction

The endogenous catecholamine dopamine and its pharmacologically inert precursor, L-dopa, are both available as human pharmaceutical preparations and can be used as performance-enhancing drugs in horses. Dopamine is critically involved in the modulation of locomotor behaviour (central nervous system stimulation) and also plays a role in the regulation of cardiovascular function. Its effects include dopaminergic, α-adrenergic and β_1-adrenergic stimulation. L-dopa, which is used principally in the treatment of Parkinsonism and is converted to dopamine *in vivo*, may enhance performance through its actions on dopamine levels in the brain or on the cardiovascular system, or a combination of both.[1] Major metabolites of levodopa and dopamine, apart from catecholamines adrenaline and noradrenaline, include 3-methoxytyramine, 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid.

Previous work on the urinary excretion of 3-methoxytyramine and other dopamine metabolites in the horse and greyhound proved that the only effective method for controlling dopamine and levodopa abuse in equine and greyhound racing is a urinary threshold for 3-methoxytyramine.[2–4] Based in large part on

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the above works, a urinary allowable threshold concentration of 4 μg mL^{-1} has been established for this substance (free and conjugated) and incorporated in Article 6 of the International Agreement on Breeding and Racing.[5] For this reason, qualitative and quantitative methods need to be developed and validated by the doping-control laboratories. In 2003, several post-race equine urine samples (all from race winners) received at the Australian Racing Forensic Laboratory were found to contain threshold-exceeding 3-methoxytyramine concentrations and also irbesartan, an angiotensin II receptor antagonist. Subsequent investigations led to the conclusion that levodopa was administered to horses as a performance enhancer, but the use of irbesartan is open to speculation (probably to offset dopamine-induced hypertension or peripheral vasoconstriction).[6]

Two methods[4,6] have been reported for the doping control analysis of 3-methoxytyramine in horse urine. These methods involve gas chromatography/mass spectrometry (GCMS) as an analytical technique for the detection of 3-methoxytyramine, while liquid-liquid or solid-phase extraction and time consuming derivatization procedures are applied after urine hydrolysis.

In this paper, a direct injection LCMS method suitable for the quantitative and qualitative analysis of 3-methoxytyramine in horse-race samples is presented, using a relatively new instrumentation, a quadrupole/time-of flight mass spectrometer (QTOFMS) interfaced to a hydrophilic interaction liquid chromatographic (HILIC) system (HILIC/QTOFMS). Hydrophilic interaction liquid chromatography was selected as an alternative chromatographic technique using polar stationary phases and low aqueous/high organic mobile phases in order to achieve effective retention of such a polar analyte. Additional advantages of HILIC include increased ESI sensitivity due to high organic content in the mobile phase, higher flow rates due to lower column back-pressure and less matrix effect.[7–10] This work is a continuation of a series of studies[11,12] on the application of direct injection LCMS analysis for the quantification and identification of threshold substances in horse urine.

Experimental

Materials and reagents

3-Methoxytyramine hydrochloride and 3-methoxytyramine-1,1,2,2-d_4 hydrochloride (internal standard, IS) were obtained from Sigma-Aldrich (Steinheim, Germany) and CDN Isotopes (Quebec, Canada), respectively. Acetonitrile and water were of LCMS grade and purchased from Riedel-de Haen (Seelze, Germany). All other reagents were of analytical grade. Methanol and formic acid were purchased from Merck (Darmstadt, Germany) and ammonium formate was obtained from Acros Organics (Geel, Belgium). β-Glucuronidase from *Helix pomatia* (115 600 units mL^{-1}) was purchased from Sigma–Aldrich (Steinheim, Germany). Millex-HV 0.45 μm syringe-driven filters were obtained from Millipore (Bedford, USA).

HILIC/QTOFMS instrumentation

Liquid chromatographic conditions

An Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Waldbronn, Germany) was used for the chromatographic separation. The system consisted of a vacuum degasser, a high-pressure binary pump, a temperature-controlled autosampler set at 5 °C and a thermostated column compartment set at 35 °C. Chromatographic separation was performed on a SeQuant ZIC®-HILIC column (150 × 2.1 mm, 3.5 μm particle size, Merck, Dermstadt, Germany). A SeQuant ZIC®-HILIC guard column (20 × 2.1 mm, 5 μm particle size,) was used to prolong column lifetime. An isocratic mobile phase was used consisting of 5 mM ammonium formate and 0.05% formic acid (pH 3.5) in a mixture of acetonitrile/water 80/20 (v/v) and was pumped at a flow rate of 0.3 mL min^{-1}. The analysis run time was 10 min and injection volume was 5 μL. A divert valve was set to transmit the LC eluent directly to the mass spectrometer from 2.5 to 5.5 min; for the remaining time the LC eluent was diverted to waste.

Mass spectrometric conditions

An orthogonal accelerator quadrupole time-of-flight mass spectrometer (Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS, Santa Clara, California, USA) was used, equipped with an orthogonal electrospray ionization source (ESI), temperature-stabilized analogue-to-digital converter (ADC) operated at 2 GHz (extended dynamic range mode) and multichannel plate operated at 700 V. Ionization was performed in the positive mode and nitrogen was used as a drying and nebulizing gas. Drying gas flow and temperature were set at 10 L min^{-1} and 330 °C, respectively and nebulizer gas pressure at 40 psi. Capillary and fragmentor voltages were optimized during method development at 1500 V and 100 V, respectively. All the other MS parameters (transfer optic voltages, voltages of the ion focus and octapole lens for shaping the beam to achieve optimal parallelism and size as it enters the TOF analyzer, TOF voltages, and detector voltage) were automatically optimized by the instrument autotuning procedure, performed on a weekly basis. The QTOF-MS system was operated in targeted MS/MS mode acquiring consecutively MS data with scan rate 3 spectra s^{-1} from 110 to 1100 m/z and MS/MS product ion data with scan rate 4 spectra s^{-1} from 50 to 220 m/z.

Mass calibration was performed daily before starting the analysis set over a mass range of m/z 118.0863–2721.8950, using a reference mixture provided by the manufacturer (Product number: G1969-85000, Agilent Technologies). Reference mass correction was used during the analysis in order to achieve better mass accuracy, by introducing two reference compounds (Hexakis(1H, 1H, 3H-tetrafluoropropoxy) phosphazine and purine, Agilent Technologies) simultaneously with the samples. The reference compounds were introduced continuously to the ESI source from a second orthogonal nebulizer. All aspects of instrument control, tuning, acquisition and data analysis were controlled by Mass Hunter software (Agilent Technologies).

Stock and working standard solutions

Stock standard solutions of 3-methoxytyramine and 3-methoxytyramine-d_4 at 3 mg mL^{-1} and 1 mg mL^{-1}, respectively, were prepared by dissolving the appropriate amounts of the compounds in methanol. Working standard solutions of the analyte and the IS were prepared at 250 μg mL^{-1} and 100 μg mL^{-1}, respectively, by subsequent dilution of the above stock solutions with methanol. All solutions were stored at −20 °C.

Calibration spiked urine samples and quality control samples

In this study pooled urine was prepared from a number of horses (male, female and gelding) that presented very low concentrations

of 3-methoxytyramine from the semi-quantitative GCMS screening procedure. The pooled urine was dispensed in aliquots for each validation day (100 mL) and stored at −20 °C until use.

Calibration spiked urine standards of 3-methoxytyramine were prepared freshly every working day for the concentration levels of 1, 2.5, 5, 10, 15 and 20 μg mL^{-1} by addition of the appropriate amount of the above mentioned working standard solution at 2.5 mL pooled urine. Quality control (QC) samples were prepared in pooled urine at four concentration levels (2, 4, 12 and 18 μg mL^{-1}). All the QC samples were freshly prepared for each run. Separate stock and working standard solutions of 3-methoxytyramine were used for the preparation of calibration standards and QC samples.

Sample preparation

To 2.5 mL urine, 1 mL carbonate buffer (pH 5), 50 μL of β-glucuronidase from Helix pomatia and 50 μL of the IS working solution were added and the mixture was incubated for 2.5 h at 50 °C. The hydrolysed urine (100 μL) was transferred to an Eppendorf tube and 500 μL of acetonitrile were added. After ultra-centrifugation at 13 000 rpm (g-force 12 300) for 10 min and filtration of the supernatant with syringe-driven filters, samples were injected into the HILIC/QTOFMS system.

Matrix effects

Matrix effects were evaluated by analysing calibration curves in water samples and three horse-urine samples of different specific gravity ('high', 'medium' and 'low' specific gravity) and comparing the absolute and relative response of 3-methoxytyramine along with the slopes of the regression lines.

Validation procedure

Spiked horse-urine calibration standards at six concentration levels, ranging from 1 to 20 μg mL^{-1}, were prepared and analysed in duplicate in five different analytical runs. Calibration curves were prepared for each run on the basis of peak area ratio of 3-methoxytyramine to that of the IS versus the theoretical concentration.

Quality control samples were processed in six replicates at each concentration (2, 4, 12 and 18 μg mL^{-1}) for five different analytical runs in order to evaluate the intra- and inter-assay precision and accuracy. Precision was assessed as the percentage relative standard deviation (% RSD) for each concentration level. Maximum allowed tolerance for precision can be calculated from the Horwitz-equation RSDmax $= 2^{(1-0.5 \log C)}$ (C = concentration (μg mL^{-1}) $\times 10^{-6}$). The maximum allowed tolerances for intra- and inter-assay precision are 2/3 RSDmax and RSDmax, respectively.[13] Accuracy was defined as the relative difference between the calculated and theoretical concentration of 3-methoxytyramine. The limit of quantification (LOQ) of the method was defined as the lowest concentration where acceptable precision and accuracy could be guaranteed.

Specificity was checked by analysing horse-urine samples spiked with substances structurally related to 3-methoxytyramine and other routinely screened horse doping agents, including stimulants, narcotics, corticosteroids and diuretics. Concentrations of substances in these mixtures were 4 μg mL^{-1}. Carry over was examined each working day by analysing a blank sample after a calibration spiked standard with concentration 20 μg mL^{-1}.

Application to case samples

The proposed quantitative and qualitative methodology was applied to the analysis of three horse-urine samples that presented high concentrations of 3-methoxytyramine from the semi-quantitative GCMS screening method.

Results and Discussion

Method development

Optimization of mass spectrometric conditions

3-Methoxytyramine contains an amino group, which can be easily protonated in positive ESI mode. Fragmentor and capillary voltages were optimized by flow injection analysis using a solution containing the analyte and the IS at concentration 1 μg mL^{-1} (in mobile phase). The aim of this study was to find the best ionization conditions in order to achieve the maximum abundance of their protonated molecules $[M+H]^+$ and reduce in-source fragmentation (loss of ammonia molecule), which was observed less when capillary and fragmentor voltages were set at low values (1500 V and 100 V, respectively). For increased sensitivity and selectivity, MSMS acquisition mode of the QTOFMS instrument was preferred. 3-Methoxytyramine and the IS gave protonated molecules $[M+H]^+$ at m/z 168.1019 and 172.1270, respectively, which were chosen as precursor ions. The $[M+H]^+$ ion of 3-methoxytyramine fragmented under collision-induced decomposition to produce one dominant product ion at m/z 151.0754, formed by the loss of ammonia (17.0265 Da) at low values of collision energy voltage (CE) (2.5–10 V). Further fragmentation was observed at higher values of CE (10–25 V) resulting in product ions at m/z 151.0754, 119.0491 (loss of methanol molecule) and 91.0542 (tropylium ion). Quantification was performed at CE 7.5 V using the area of the fragments at m/z 151.0754 for 3-methoxytyramine and 155.1005 for the IS. For confirmation purposes (at least three abundant product ions)[14,15] a second acquisition was performed at CE 20 V. A full scan spectrum of a 1 μg mL^{-1} 3-methoxytyramine solution (a), along with product ion spectra of its protonated molecule under CE 7.5 V (b) and 20 V (c) are presented in Figure 1, together with the mass error results of the diagnostic ions.

Chromatography

Since reversed-phase C18 columns are highly compatible with the mass spectrometer, initial LCMS studies of 3-methoxytyramine were carried out using a C18 column (Zorbax Eclipse Plus C18, 100 × 2.1 mm i.d., 1.8 μm particle size, Agilent Technologies, Santa Clara, California, USA) with mobile phases containing mixtures of acetonitrile/water and various acidic modifiers (formic acid and/or ammonium formate, acetic acid and/or ammonium acetate). Retention of 3-methoxytyramine was poor ($k < 1.3$) even at high composition of (95%) aqueous mobile phase (water). During these experiments urine samples were diluted with water (because of the high proportion of water in mobile phase). This resulted in poor selectivity and ion suppression caused by coelution of matrix compounds, which mostly originated from the enzyme used for urine hydrolysis.

Hydrophilic interaction liquid chromatography was selected as a separation technique that allows the retention of very polar substances like 3-methoxytyramine. A zwitterionic ZIC-HILIC column with sulphobetaine groups on the surface was

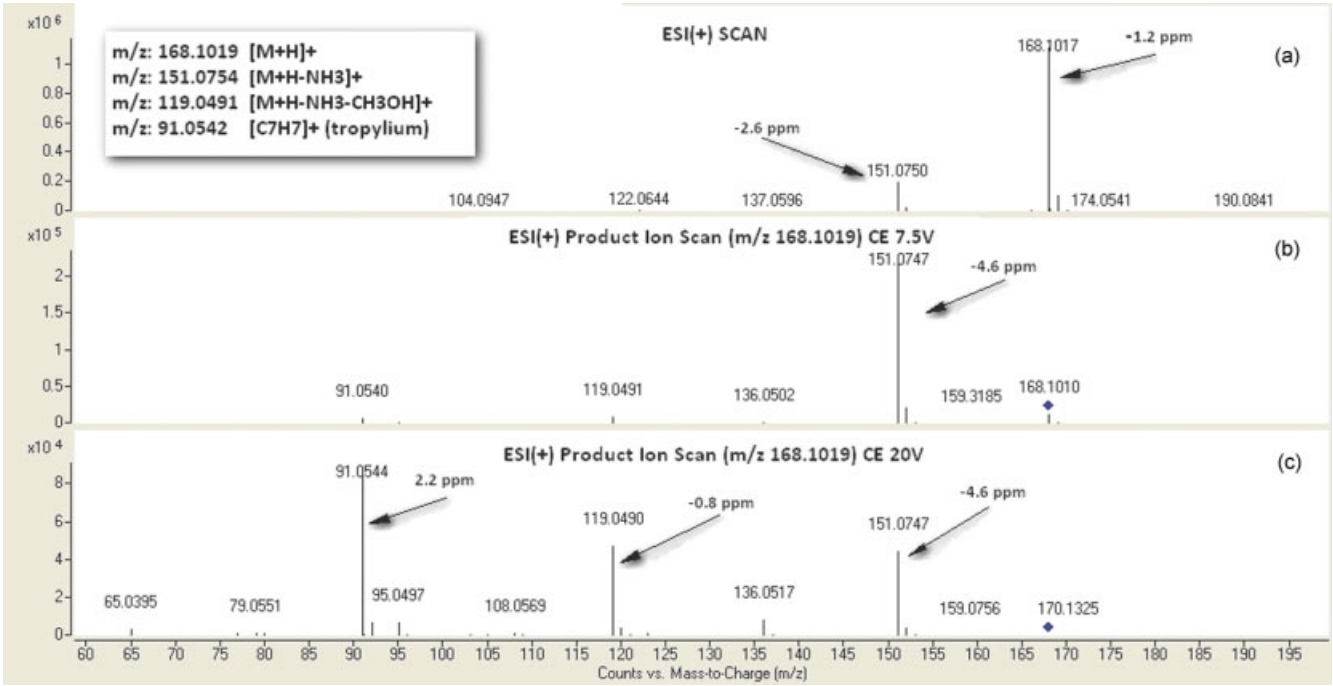

Figure 1. Full scan mass spectrum of a 1 μg ml^{-1} 3-methoxytyramine standard solution (a), along with product ion scan spectra of its protonated molecule at collision energy 7.5 V (b) and 20 V (c).

selected as stationary phase. Apart from the analyte retention, the use of a HILIC column had two advantages. In HILIC mode the weak mobile phase is organic solvent, thus it was possible to use higher amount of acetonitrile in mobile phase (80%), which resulted in more efficient desolvation of the analytes in the MS interface and higher MS intensities. In addition, for efficient HILLIC separations it is important that the injected sample is diluted with organic solvent to at least 50%. This permitted the addition of acetonitrile after urine hydrolysis and protein precipitation after ultra-centrifugation, which eliminated the matrix interferences from coeluting substances.

A ZIC column carries a sulphobetaine-type zwitterionic functionality covalently attached to porous silica to form a permanently charged, highly polar stationary phase. The ZIC-HILIC separation is mainly governed by a hydrophilic partitioning and weak electrostatic interactions allowing the use of low buffer concentration, which is normally beneficial to mass spectrometric detection. The use of buffered mobile phase was also suggested to maintain a consistent electrostatic interaction between the solute and the stationary phase for reproducible chromatographic separation of charged analytes with ZIC-HILIC.[9,16,17]

During method development, mobile phases containing a mixture of acetonitrile/water 80/20 (v/v) and various concentrations of formic acid (0–0.1%) and ammonium formate (0–20 mM) were tested in order to achieve efficient retention of the analyte, good peak shape and high MS intensity. A mixture of 5 mM ammonium formate and 0.05% formic acid was found to be the optimum mobile phase modifier. Under these conditions 3-methoxytyramine and the IS elute at 3.3 min.

Matrix effects

An important issue in method development of a quantitative analysis in complex biological matrices using liquid chromatography/electrospray ionization-mass spectrometry (LCESIMS) is the possible occurrence of matrix effects, defined as the effect of co-eluting residual matrix components on the ionization of the target analyte. Although the interferences may remain undetected because of the selectivity of the accurate mass measurement and the MS/MS detection, they do affect the reproducibility and accuracy of the developed quantitative procedure.[18,19] Horse urine is a complex matrix that contains numerous different compounds, ranging from simple inorganic salts to large proteins. In previous works with direct injection horse urine analysis[11,12] without the use of stable isotope labelled IS, great variability was observed in absolute and relative responses of the analyte and the IS originating from matrix effects, even though a fourfold sample dilution was applied, resulting in quantitative analysis using standard additions approach.

Matrix effects were evaluated by analysing calibration curves in horse urine samples of different specific gravity (as a measure of the matrix load) together with a calibration curve in water samples, and comparing the response of 3-methoxytyramine and the IS, as well as the slopes of the regression lines for the different matrices (Table 1). The use of 3-methoxytyramine-d_4 as IS resulted in precise relative responses of the analyte between different matrices at different concentration levels, although the absolute responses of the analyte and the IS presented great variability between the different urine samples. Linear regression calibration was used for quantification and pooled blank urine from different horses was used for the method's validation.

Validation and statistical analysis

Linear relationships between the ratio of the peak area signals of 3-methoxytyramine to that of the IS and the corresponding concentrations were observed in the estimated concentration range (1–20 μg mL^{-1}). The regression equations of the five calibration curves, the standard deviation values of the slopes and

Table 1. Matrix effects evaluation after analyzing calibration curves in water and different horse urine samples. Areas and area ratios for the different matrices are presented relative to the values corresponding to the water samples, normalized to 100

	Concentration (μg mL^{-1})									
	1			*5*			*20*			
Matrix	*Area 3MT*[b]	*Area IS*	*Area Ratio*	*Area 3MT*[b]	*Area IS*	*Area Ratio*	*Area 3MT*[b]	*Area IS*	*Area Ratio*	***SLOPE***
Water	100	100	100	100	100	100	100	100	100	1.093
Urine 'low' SG[a]	88	83	106	80	84	95	98	105	94	1.018
Urine 'medium' SG[a]	75	76	99	82	84	95	93	101	91	1.018
Urine 'high' SG[a]	64	58	111	48	49	98	63	69	91	1.001
% RSD	19.1	22.2	**5.4**	28.1	27.3	**1.9**	19.8	17.8	**4.3**	**4.0**

[a] Specific gravity
[b] 3-Methoxytyramine

Table 2. Analytical parameters of the calibration equations for the determination of 3-methoxytyramine

			SD[c]			
Run	*Regression Equations*[a]	r^2[b]	*Slope*	*Intercept (a)*	$S_{y/x}$[d]	a/SD_a[e]
1	$R_{3MT} = 0.5103 \times C_{3MT} + 0.056$	0.99992	0.0032	0.036	0.054	1.54
2	$R_{3MT} = 0.5351 \times C_{3MT} + 0.044$	0.9998	0.0053	0.060	0.089	0.73
3	$R_{3MT} = 0.5235 \times C_{3MT} + 0.077$	0.9998	0.0047	0.053	0.079	1.44
4	$R_{3MT} = 0.5201 \times C_{3MT} - 0.004$	0.99996	0.0024	0.027	0.040	0.16
5	$R_{3MT} = 0.5141 \times C_{3MT} + 0.012$	0.9998	0.0037	0.042	0.062	0.29

[a] Ratio of the peak area of 3-methoxytyramine (3MT) to that of the IS, R_{3MT}, versus the corresponding concentration, C_{3MT}.
[b] Correlation coefficient.
[c] Standard deviation of slope and intercept.
[d] Standard error of the estimate.
[e] Calculated t-value (theoretical value of t = 2.78 (95% level of significance, for 4 degrees of freedom).

Table 3. Accuracy and precision evaluation of QC samples for 3-methoxytyramine in horse urine (in six validation days, six replicates per day)

		Concentration (μg mL^{-1})			
Validation Run		*2*	*4*	*12*	*18*
1	Mean ± SD	1.92 ± 0.12	3.92 ± 0.12	11.79 ± 0.39	18.07 ± 0.72
	(% E_r)[a]	−3.9	−2.1	−1.8	0.4
2	Mean ± SD	1.922 ± 0.060	3.75 ± 0.12	11.64 ± 0.61	17.74 ± 0.57
	(% E_r)[a]	−3.9	−6.2	−3.0	−1.4
3	Mean ± SD	1.960 ± 0.077	3.95 ± 0.14	11.47 ± 0.49	17.82 ± 0.92
	(% E_r)[a]	−2.0	−1.4	−4.4	−1.0
4	Mean ± SD	2.035 ± 0.029	4.11 ± 0.13	11.98 ± 0.35	18.06 ± 0.33
	(% E_r)[a]	1.8	2.8	−0.2	0.4
5	Mean ± SD	2.073 ± 0.059	4.030 ± 0.097	12.00 ± 0.58	17.80 ± 0.39
	(% E_r)[a]	3.6	0.7	0.0	−1.1
Overall mean		1.983	3.95	11.78	17.90
Overall accuracy (%E_r)[a]		−0.9	−1.2	−1.9	−0.6
%$RSD_{intra-assay}$[b]		3.9	3.1	4.2	3.5
%$RSD_{inter-assay}$[b]		3.1	3.2	0.9	N.V[d]
%RSD_{max}[c]		14.8	13.7	12.0	11.4

[a] % E_r: Relative percentage error.
[b] % RSD: relative standard deviation, Intra- and inter-assay %RSD were calculated by ANOVA (degrees of freedom = 35)
[c] %RSD_{max}: maximum allowed tolerance for precision calculated from Horwitz equation $RSD_{max} = 2^{(1-0.5logC)}$ (C = concentration (μg/mL) × 10^{-6}).
[d] N.V: non variable

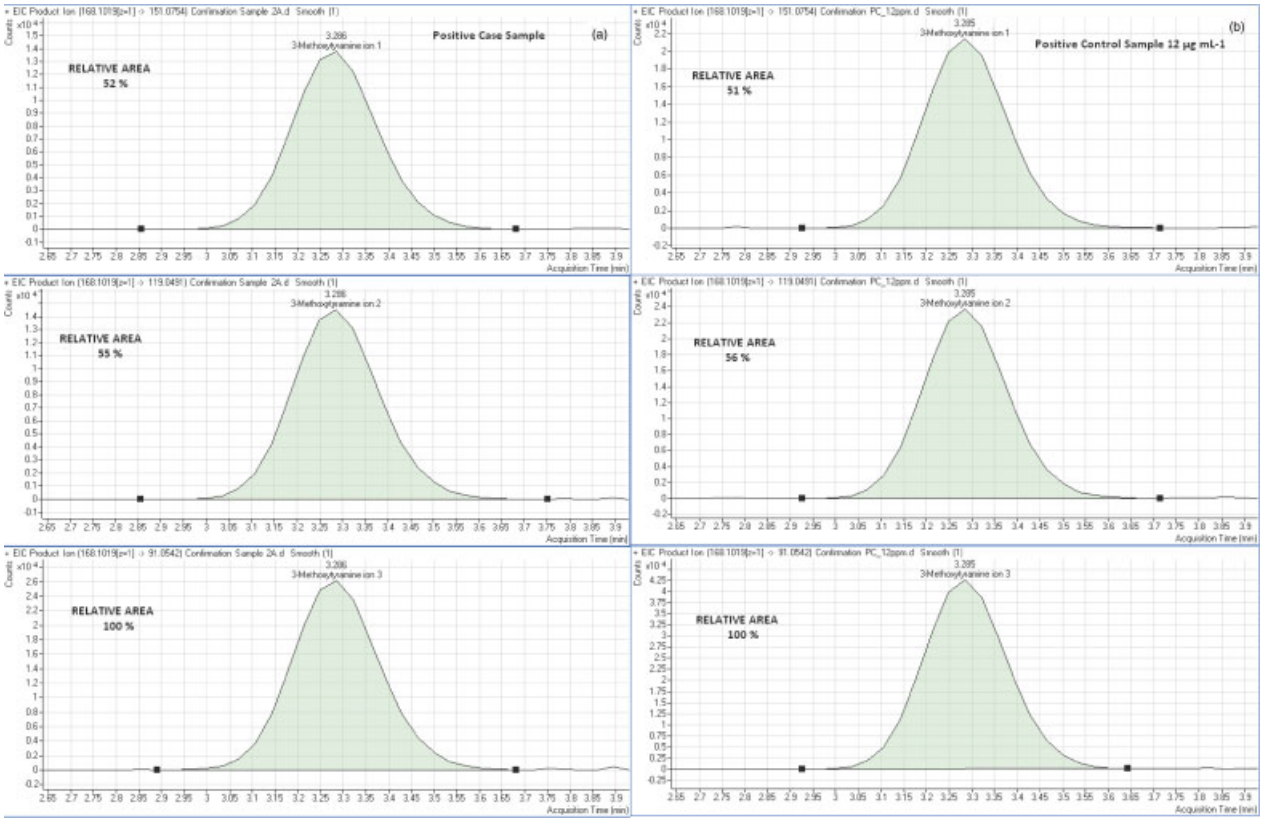

Figure 2. Representative HILIC/QTOFMS chromatograms of the diagnostic ions (50 ppm mass window) of 3-methoxytyramine obtained from a positive horse-urine sample (a) and a horse-urine sample fortified with the analyte at 12 µg mL^{-1} (b).

intercepts along with the correlation coefficients are presented in Table 2. The correlation coefficient invariably exceeded 0.9998. In all cases, back-calculated concentrations in the calibration curves were within 15% of the nominal values.

A Student's t-test was performed to determine whether the experimental intercept values of the regression equations mentioned above were significantly different from the theoretical zero value. The test is based on the calculation of the quantities $t = a/SD_a$, where a is the intercept of the regression equations and SD_a is the standard deviation of intercept. The calculated t-values are also presented in Table 2. These values did not exceed the 95% criterion of $t_p = 2.78$ for 4 degrees of freedom (n − 2, n: number of calibration points), indicating that the intercepts of all regression lines are not significantly different from zero.

One-way analysis of variance (ANOVA) was used to evaluate the intra- and inter-assay precision. Results for accuracy and precision are presented in Table 3. Intra- and inter-assay % RSD were lower than 4.2 and 3.2, respectively and did not exceed 2/3 RSDmax. Accuracy was assessed by the relative percentage error (% Er) which ranged from −6.2% to 3.6%. The LOQ for 3-methoxytyramine was determined according to the definitions of ICH Topic Q2B[20] using the equation $LOQ = 10 \times SD_a/b$, were SD_a is the standard deviation of the intercept and b is the slope of the regression line. The LOQ was found to be 0.95 µg mL^{-1}.

The selectivity towards endogenous urine compounds was tested in four different batches of horse urine that showed low levels of 3-methoxytyramine at the GCMS semi quantitative method, which is routinely used at the laboratory for the screening of basic substances in horse urine. The ion chromatograms of 3-methoxytyramine at the four batches contained no coeluting peaks greater than 8.5% of the relative response of 3-methoxytyramine at the lower calibration level (originating probably from endogenous 3-methoxytyramine), while no interfering peaks were observed at the ion chromatograms of the IS. No interfering peaks were observed after the analysis of horse-urine samples fortified with structurally related substances and other horse doping agents.

Moreover, blank horse urine samples analysed after the analysis of a sample fortified with 3-methoxytyramine at 20 µg mL^{-1} did not show any evidence of carryover signal.

Confirmation data evaluation

The confirmation method of 3-methoxytyramine was evaluated by MSMS analysis (at CE 20 V) of 24 samples prepared during the method's validation at concentrations covering the tested linear range (1–20 µg mL^{-1}) at three analytical days. Relative retention times of the analyte were found to be stable with %RSD lower than 0.3 (N = 24). The relative areas of 3-methoxytyramine diagnostic ions were calculated with regard to the area of the most abundant ion. % Relative areas (± standard deviation) of the ions at m/z 119.0491 and 151.0754 with regard to the area of ion at m/z 91.0542, were 56.3 (±1.6) and 52.2 (±2.7), respectively. The above results show that product ion peak area ratios are precise, between different days and different concentration levels of 3-methoxytyramine. Moreover, mass (± standard deviation, N= 24) of the three diagnostic ions of the analyte and the main product ion of the IS was 8.3 (±3.3) ppm (product ion MSMS scans). Mass (± standard deviation, N = 24) of the precursor ions (protonated molecules) was 6.2 (±2.3) ppm (MS scans). These results are in

agreement with instrument's specifications for accurate mass measurement in MS and MSMS acquisition mode.

Application to case samples

During method validation the intercept of all regression lines was not found to be significantly different from zero, so quantification of the case samples was performed using one-point calibration. For all samples (calibrators and unknowns) three replicates were prepared and injected in duplicate. The concentrations of 3-methoxytyramine (± standard deviation) in the three horse-urine case samples were 18.94 (±0.62) μg mL^{-1}, 11.51 (±0.35) μg mL^{-1} and 3.69 (±0.11) μg mL^{-1}. The MS/MS analysis of the suspicious urine samples at high CE (20 V) confirmed the detection of 3-methoxytyramine, with confirmation results in accordance with the criteria of the Association of Official Racing Chemists (AORC) for the identification of a prohibited substance.[14] The diagnostic ion chromatograms obtained from the confirmatory analysis of a positive sample along with a positive control sample (fortified at 12 μg mL^{-1}) are presented in Figure 2.

Conclusions

A direct injection HILIC/QTOFMS method was developed and validated for the quantification and confirmation of 3-methoxytyramine. The proposed method takes advantage of the benefits of HILIC chromatography (efficient retention of the polar analyte, MS sensitivity improvement due to high organic content of the mobile phase and less matrix effect due to protein precipitation with acetonitrile prior sample injection) and QTOF mass spectrometry (high selectivity and sensitivity due to MSMS analysis combined with accurate mass measurement). Quantification and identification of 3-methoxytyramine were performed by analysing the same aliquots at low and high CE voltage, respectively. The use of an isotopically labelled IS eliminated ion suppression and linear regression calibration was applied for the quantitative determination of the analyte. The linearity, accuracy and precision results prove the method's suitability for the doping control analysis of 3-methoxytyramine in horse urine. The area ratios of the diagnostic product ions of 3-methoxytyramine were found to be precise for confirmation purposes. The above method was applied to the quantitative and confirmatory analysis of three real samples.

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