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Quantitative estimation of α-PVP metabolites in urine by GC-APCI -QTOFMS with nitrogen chemiluminescence detection based on parent drug calibration

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ABSTRACT

Gas chromatography (GC) hyphenated with nitrogen chemiluminescence detection (NCD) and quadrupole time-of-flight mass spectrometry (QTOFMS) was applied for the first time to the quantitative analysis of new psychoactive substances (NPS) in urine, based on the *N*-equimolar response of NCD. A method was developed and validated to estimate the concentrations of three metabolites of the common stimulant NPS α-pyrrolidinovalerophenone (α-PVP) in spiked urine samples, simulating an analysis having no authentic reference standards for the metabolites and using the parent drug instead for quantitative calibration. The metabolites studied were OH-α-PVP (M1), 2″-oxo-α-PVP (M3), and *N*,*N*-bis-dealkyl-PVP (2-amino-1-phenylpentan-1-one; M5). Sample preparation involved liquid–liquid extraction with a mixture of ethyl acetate and butyl chloride at a basic pH and subsequent silylation of the *sec*-hydroxyl and *prim*-amino groups of M1 and M5, respectively. Simultaneous compound identification was based on the accurate masses of the protonated molecules for each compound by QTOFMS following atmospheric pressure chemical ionization. The accuracy of quantification of the parent-calibrated NCD method was compared with that of the corresponding parent-calibrated QTOFMS method, as well as with a reference QTOFMS method calibrated with the authentic reference standards. The NCD method produced an equally good accuracy to the reference method for α-PVP, M3 and M5, while a higher negative bias (25%) was obtained for M1, best explainable by recovery and stability issues. The performance of the parent-calibrated QTOFMS method was inferior to the reference method with an especially high negative bias (60%) for M1. The NCD method enabled better quantitative precision than the QTOFMS methods To evaluate the novel approach in casework, twenty post- mortem urine samples previously found positive for α-PVP were analyzed by the parent calibrated NCD method and the reference QTOFMS method. The highest difference in the quantitative results between the two methods was only 33%, and the NCD method's precision as the coefficient of variation was better than 13%. The limit of quantification for the NCD method was approximately 0.25 μg/mL in urine, which generally allowed the analysis of α-PVP and the main metabolite M1. However, the sensitivity was not sufficient for the low concentrations of M3 and M5. Consequently, while having potential for instant analysis of NPS and metabolites in moderate concentrations without reference standards, the NCD method should be further developed for improved sensitivity to be more generally applicable.

1. Introduction

The continuous emergence of new psychoactive substances (NPS) pose a challenge for both toxicological analysis and case interpretation. Among the hundreds of NPS recognized during the ten previous years, the stimulant alpha-pyrrolidinovalerophenone (α-PVP) has turned out to be one of the more established drugs on the illicit market despite scheduling as a controlled substance in many countries. α-PVP belongs to the "second generation" cathinones that appeared on the illicit market in Europe in 2011 following the "first generation" cathinones 3,4-methylenedioxypyrovalerone (MDPV), 3,4-methylenedioxymethcathinone (methylone), and 4-methylmethcathinone (mephedrone). Current studies suggest that the potency of cathinones to reinforce responding in animal studies, and consequently increased

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abuse liability, is related to their capacity to inhibit dopamine uptake [1]. α-PVP has more dopaminergic than serotonergic properties compared with amphetamine analogues [2]. The Risk Assessment carried out by the European Monitoring Centre for Drugs and Drug Addiction reported 116 deaths associated with α-PVP between 2012 and 2015 in eight member states [3]. Recently, Swedish authors published a case series of consecutive patients within the STRIDA project with admitted or suspected intake of NPS presenting to hospitals for emergency treatment from January 2011 to March 2016. They detected 45 α-PVP and 17 MDPV cases in addition to 114 intoxications involving any of 11 other pyrovalerone drugs [4].

Qualitative screening for a broad range of NPS in biological samples is feasible by modern analytical techniques, notably taking advance of the specificity and flexibility of high-resolution mass spectrometry. Accurate mass-based identification of NPS in data-independent acquisition mode is particularly useful since it enables immediate suspect screening, while subsequent confirmation can be accomplished once a reference standard has been obtained [5]. However, quantification still remains a major challenge. Continuous appearance and disappearance of NPS complicates the acquisition of authentic reference standards because the standards are expensive and difficult to obtain in a sufficiently short period of time. Metabolites of NPS are rarely commercially available, and consequently expensive custom syntheses may be required to include the most important metabolites in the analysis panel.

It is well recognized that the present-day routine analytical method, liquid chromatography electrospray triple quadrupole tandem mass spectrometry (LC-ESI-MS/MS), is at best suitable only for semi-quantitative estimation of metabolite concentrations when using the parent drug for calibration, because of the large variation in responses between the compounds [6]. However, there are non-MS techniques available that provide a more universal response [7,8]. Nitrogen chemiluminescence detection (NCD) shows equimolar response to nitrogen that could be exploited in universal quantification of nitrogen containing compounds [9]. In our previous studies [10,11], we introduced a novel platform for simultaneous identification and quantification of NPS in the absence of authentic reference standards. The instrumentation consisted of gas chromatography (GC) coupled to NCD and atmospheric pressure chemical ionization quadrupole time-of-flight mass spectrometry (APCI-QTOFMS). The GC flow was divided in appropriate proportions between NCD and QTOFMS to achieve quantification and identification, respectively. We obtained promising quantitative results by analyzing blood samples that were spiked post-extraction to avoid emphasizing recovery issues [10]. In addition, we were able to show that taking advantage of the accurate mass measurement of the protonated molecule and product ions by GC-APCI-QTOFMS, the obtained MS/MS spectra were comparable to existing soft-ionization mass spectral libraries [11].

In the present study, our objective was to advance the concept further by developing a quantitative analysis method, based on the GC-NCD-APCI-QTOFMS platform, for α-PVP and its three metabolites in urine samples using the parent drug for calibration. Following liquid–liquid extraction, a derivatization step was introduced to avoid undesirable adsorption of polar metabolites in the GC inlet and column [12,13]. Thereafter, post-mortem urine samples previously found positive for α-PVP were re-analyzed by the developed method to reveal the metabolite concentrations.

2. Materials and methods

2.1. Chemicals

α-PVP and the *N*,*N*-bis-dealkyl-PVP (2-amino-1-phenylpentan-1-one; M5) were from Chiron AS (Trondheim, Norway), OH-α-PVP (M1) was from Cayman Chemical (Ann Arbor, MI, USA), and 2″-Oxo-α-PVP (M3) from Toronto Research Chemicals (Toronto, ON, Canada). The structures of α-PVP and the three metabolites used in the study are shown in Fig. 1. The derivatization agent MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) with 1% TMCS (2,2,2-trifluoro-*N*-methyl-*N*-(trimethylsilyl)-acetamide, chlorotrimethylsilane) was from Thermo Fisher Scientific (Bellefonte, PA, USA).

2.2. Sample preparation

Blank urine samples from five healthy volunteers were used for the validation experiments. The blank urines did not contain co-eluting nitrogen-containing components that would interfere with quantification at NCD. Blank urine aliquots were spiked to create external calibration curves for MS and NCD quantifications. Each reference standard was dissolved in methanol to obtain a 1 mg/mL stock solution. The stock solution was diluted to obtain a working solution containing a mixture of α-PVP and metabolites M1, M3 and M5 within a concentration range of 0.4–200 μg/mL. Blank urine (1 mL) was spiked with 25 μL of the working solution to prepare solutions ranging from 0.01 to 5 μg/mL.

Twenty post-mortem urine samples from medico-legal autopsies, previously identified as positive for α-PVP, were selected for metabolite quantification. In such samples where the analytes caused a signal saturation to the mass selective detector the urine volume was adjusted to match 0.2–1 μg/mL concentration.

The pH of the urine samples was adjusted by mixing with 400 μL of 1 M Tris buffer (pH 11) and 40 μL of 1 M NaOH in a 6-mL centrifuge tube, resulting in a pH between 10–12. The mixture was extracted with 500 μL of ethyl acetate + butyl chloride (75 + 25) in a vortex mixer for 2 min. After centrifugation, an aliquot (100 μL) of the organic phase was transferred into a conical autosampler vial. For the derivatization procedure, the organic phase was mixed with 40 μL of MSTFA + 1% TMCS silylation reagent, incubated for 15 min at 50 °C and mixed prior to GC injection.

2.3. Instrumentation

A 7890B Series GC System equipped with a 7693 Automatic Liquid Sampler and a split/splitless injector was coupled through a two-way splitter with makeup gas (He) to an Agilent 6540 UHD Accurate-Mass QTOF mass analyser and a 255 Nitrogen Chemiluminescence Detector (all Agilent Technologies, Santa Clara, CA, USA).

2.4. Nitrogen chemiluminescence detection

Pyrolysis of the analytes at NCD was carried out at 900 °C using a hydrogen flow rate of 4 mL/min and an oxygen flow rate of 9.4 mL/min. Data from NCD was collected at 50 Hz over the entire course of the analysis. OpenLab CDS Chemstation GC driver A.02.05.021 was used to control GC-NCD.

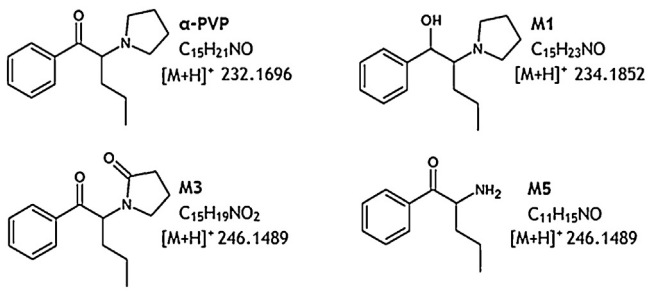

Fig. 1. Chemical structures, molecular formulas and accurate masses of the protonated molecules of α-pyrrolidinovalerophenone (α-PVP) and three of its metabolites (M1, M3, M5).

Table 1
Accuracy of quantification of parent drug-calibrated NCD method, parent drug-calibrated QTOFMS method and reference QTOFMS method calibrated with authentic reference standards.[a]

	Spiked concentration (μg/mL)	Measured concentration (μg/mL)	CV (%)	Bias (%)
GC-NCD calibrated with α-PVP				
α-PVP	0.25	0.26	8	4
	1.00	0.94	4	6
M1	0.25	0.19	13	24
	1.00	0.75	7	25
M3	0.25	0.27	8	8
	1.00	1.09	5	9
M5	0.25	0.29	8	16
	1.00	1.05	5	5
GC-APCI-QTOFMS calibrated with α-PVP				
α-PVP	0.25	0.22	18	12
	1.00	1.03	12	3
M1	0.25	0.10	12	60
	1.00	0.42	15	58
M3	0.25	0.24	13	4
	1.00	1.09	14	9
M5	0.25	0.18	12	28
	1.00	0.83	11	17
GC-APCI-QTOFMS calibrated with authentic reference standards				
α-PVP	0.25	0.22	18	12
	1.00	1.03	12	3
M1	0.25	0.25	12	0
	1.00	1.00	15	0
M3	0.25	0.22	13	12
	1.00	1.00	14	0
M5	0.25	0.21	12	16
	1.00	0.94	11	6

[a] Averaged values from five different urine samples, each measured in duplicate in six separate days.

External calibration was performed after each data acquisition sequence, using the equimolar nitrogen response to α-PVP. Three calibration points (0.25, 1 and 5 μg/mL) were measured in spiked blank urine samples after extraction and derivatization.

The amount of nitrogen was calculated by multiplying the relative amount of nitrogen in the molecule by concentration, dilution factor caused by the derivatization reagent, and injection volume.

2.5. Gas chromatography

Injector liner was a Single taper Ultra Inert liner with glass wool (Agilent 5190-2293). Analytical column was a DB-5MS (30 m × 0.25 mm id with 0.1 μm film) capillary column (Agilent Technologies). After the analytical column, the GC flow was divided between the NCD and the APCI ion source through a two-way splitter, using 0.55 m × 0.18 mm and 2 m × 0.18 mm uncoated deactivated fused-silica post-columns to obtain a 10:1 flow ratio, respectively. The splitter pressure was 15.8 psi and the flow ratio was calculated using the Effluent Splitter Calculator (with Makeup) (Agilent Technologies). In this concurrent detection, the NCD signal is detected 0.02 min earlier than QTOFMS.

GC was operated in the pulsed splitless injection mode with an equilibration time of 0.75 min and 50 mL/min purge flow to split vent at 0.75 min. A pulse pressure of 50 psi for 0.75 min was applied prior to using initial head pressure of 24.9 psi. The injector port temperature was 250 °C and the transfer line temperature 320 °C. The injection volume was 1.0 μL. The oven temperature was initially held at 100 °C for 0.75 min and then increased by 30 °C per min to 320 °C, which was held for 6 min. Helium was used as carrier gas at 1 mL/min in the constant flow mode.

2.6. Mass spectrometry

QTOFMS was operated in APCI positive ionization mode, drying gas (nitrogen) flow at 5.0 L/min and gas temperature at 365 °C. Corona discharge needle current was 1000 nA and capillary voltage 1000 V. Fragmentor voltage was 140 V and skimmer voltage 65 V.

Mass acquisition was performed in All-ions mode, and data were recorded over the m/z range of 50–400 with an acquisition rate of 5 spectra/s. Collision energy in the low energy function was 0 eV, whereas in the high energy function 10 and 20 eV were used. External mass calibration was carried out using the APCI tuning mix (Agilent Technologies). Ion m/z 257.2475 was used for internal calibration throughout the chromatographic separation. QTOFMS was operated in 2 GHz, Extended Dynamic Range mode.

All data were collected with MassHunter Data Acquisition B.04.00 software (Agilent Technologies). MassHunter Profinder B.06.00 software (Agilent Technologies) was used for initial data processing and compound identification.

2.7. Validation

Validation was performed according to the recommended guidelines [14], where appropriate. Limit of detection (LOD) was determined using the following criteria for tentative identification: peak area of >1000, retention time tolerance of 0.1 min, and mass tolerance of 1 mDa. All the quantitative data, including the limit of quantification (LOQ), bias, and precision, were gathered from parallel measurements of five calibration points ranging from 0.01 to 1 μg/mL in spiked control urine samples (Tables 1 and 2). Processed sample stability at room temperature at concentrations of 0.01 and 1.0 μg/mL was tested by measuring the difference from

Table 2
GC-NCD-APCI-QTOFMS method validation results for α-PVP, M1, M3 and M5.

Parameter	Detector	Acceptance criteria	Result
Bias	NCD	±30%	Range from −25 to 16%[a]
Carryover	QTOFMS	<10% of LOD	No carryover at 10 μg/mL[b]
LOD	QTOFMS	Identification criteria must be met	10 ng/mL
LOQ	NCD	Bias and precision criteria must be met	16 pg/N/injection[c], CV < 13%
Precision	NCD	CV ≤ 20%	Range from 4 to 13%[a]
Processed sample stability	QTOFMS	±20% compared to t_o	> 10 h[d]
Selectivity	NCD	No significant peak overlapping	9/80 (11%) of results by NCD and/or QTOFMS were disqualified
	QTOFMS	Full scan mode: Area of $[M+H]^+$ at apex must be >5-fold from all other peaks	

[a] Averaged values from five different urine samples, each measured in duplicate in six separate days.
[b] Averaged values from five different urine samples measured in triplicate.
[c] Corresponding to approximately 0.25 μg/mL of derivatized M1.
[d] Averaged values from one urine sample, measured in triplicate in one hour intervals for 10 h.

the averaged time zero (t_o) response within a 10 h interval. Selectivity was measured from actual post-mortem samples.

3. Results & discussion

3.1. Method validation with spiked urine samples

A quantitative analysis method by GC-NCD-APCI-QTOFMS was developed for α-PVP and its metabolites M1, M3 and M5 in urine samples. Liquid–liquid extraction was chosen because the technique is rather non-selective and easy to master. Among the several extraction solvents tested, a mixture of ethyl acetate and butyl chloride (75 + 25) at a basic pH was found to produce a sufficiently uniform extraction recovery for all the study compounds without excessive background noise.

The *sec*-hydroxyl- (M1) and *prim*-amino (M5) metabolites showed initially adsorption and unsymmetrical peaks in GC, and consequently a derivatization step with MSTFA to form trimethylsilyl (TMS) derivatives was included. Compound identification was based on the accurate masses of the protonated molecules for each compound by GC-APCI-QTOFMS. For M1 and M5 the addition of the TMS moiety resulted in a mass shift of +72.0396 Da to the protonated molecule. Fig. 2 shows a GC-NCD chromatogram from spiked urine samples containing α-PVP and metabolites M1, M3 and M5 at two concentration levels.

An experimental setting was designed that simulated a quantitative analysis having no authentic reference standards for the metabolites and using the parent drug instead for quantitative calibration, assuming that the extraction recovery of the parent drug equals that of the metabolites.

Table 1 shows the accuracy of quantification of the parent-calibrated NCD method, compared with that of the corresponding parent-calibrated QTOFMS method and a reference QTOFMS method calibrated with the authentic reference standards. The NCD method produced generally a better quantitative precision than the QTOFMS methods. The NCD method showed an equally good accuracy to the reference method for α-PVP, M3 and M5, but a higher negative bias (24–25%) was obtained for M1. The bias was due to a combined effect of lower extraction recovery and relative instability of the TMS derivative of M1. Cleavage of the TMS derivative was verified as an increase of free M1 with time, and consequently it is recommended to minimize the storage time of the prepared samples prior to analysis. The parent-calibrated QTOFMS method performed worse than the reference method with an especially high negative bias (58–60%) for M1. This result is obviously due to the differences in the ionization efficiency in the APCI source, in addition to the lower extraction recovery and instability issues.

Acceptance criteria for all method validation parameters determined are summarized in Table 2.

At NCD all compounds are pyrolyzed prior to the chemiluminescent detection. This ensures that the response of most nitrogen-containing compounds is equimolar regardless of the molecular structure or condition, with a notable exception of adjacent nitrogen atoms as pointed out by Yan [9]. The fact that neither of

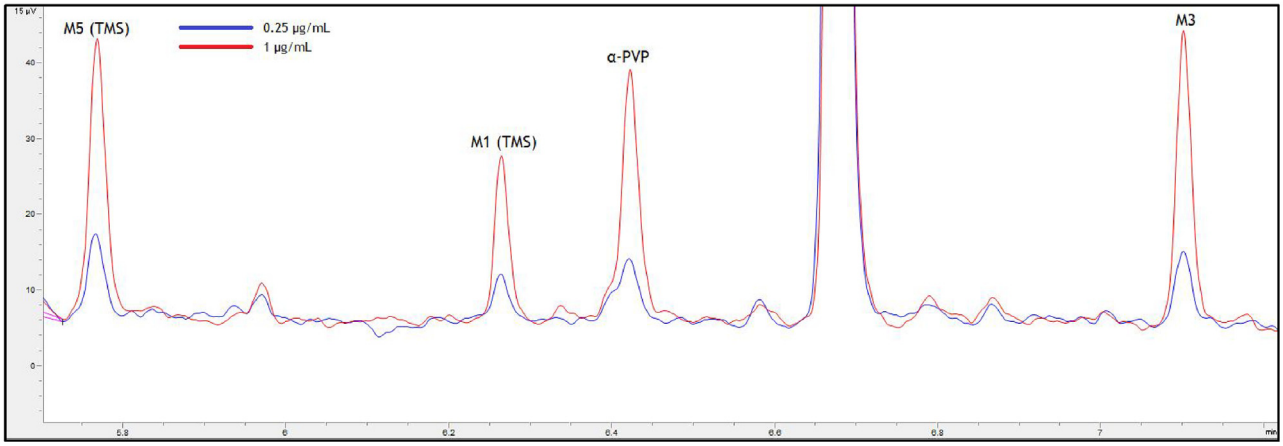

Fig. 2. GC-NCD chromatogram from spiked urine samples containing 0.25 μg/mL (blue) and 1 μg/mL (red) of α-PVP and metabolites M1, M3 and M5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Comparison of measured drug concentrations in twenty post-mortem urine samples between GC-APCI-QTOFMS calibrated with authentic reference standards and GC-NCD calibrated with α-PVP.

Sample	a-PVP (μg/mL)		M1 (μg/mL)		M3 (μg/mL)		M5 (μg/mL)	
	MS	NCD	MS	NCD	MS	NCD	MS	NCD
1[a]	8.52	8.04	6.77	5.18	0.21	[b]	0.05	[c]
2[a]	4.64	5.16	4.23	3.05	0.52	[b]	0.10	[c]
3[a]	3.03	3.01	0.97	0.71	0.35	0.38	<0.01	[c]
4[a]	2.93	3.01	3.66	2.92	0.63	0.70	0.06	[c]
5[a]	2.04	1.56	1.66	1.14	0.24	[b]	0.04	[c]
6[a]	1.25	1.51	1.53	1.06	0.27	[b]	0.03	[c]
7[a]	1.16	1.41	0.57	0.48	0.13	[c]	0.01	[c]
8	0.99	1.20	1.47	1.00	0.05	[c]	0.03	[c]
9	0.80	0.82	0.33	[b]	0.08	[c]	0.01	[c]
10	0.74	0.72	0.69	0.46	0.05	[c]	<0.01	[c]
11	0.58	0.58	0.35	0.27	0.04	[c]	<0.01	[c]
12	0.37	[b]	0.27	0.18	0.19	[b]	0.01	[c]
13	0.25	0.30	0.29	[b]	0.03	[c]	0.01	[c]
14	0.13	[c]	0.05	[c]	0.01	[c]	ND	[c]
15	0.10	[c]	0.05	[c]	<0.01	[c]	ND	[c]
16	0.09	[c]	0.14	[c]	0.01	[c]	<0.01	[c]
17	0.05	[c]	0.02	[c]	<0.01	[c]	<0.01	[c]
18	0.05	[c]	0.02	[c]	<0.01	[c]	<0.01	[c]
19	0.02	[c]	0.03	[c]	ND	[c]	ND	[c]
20	0.02	[c]	0.57	[b]	0.01	[c]	<0.01	[c]
Average bias (%)[d]		5.8		26.9		9.9		
CV (%)[e]	12	13	17	8	17	2	18	

[a] A smaller volume of urine was used to avoid signal saturation at GC-QTOFMS.
[b] Matrix interference in NCD chromatogram.
[c] Value below LOQ.
[d] Average bias (%) was calculated by comparing MS and NCD measurements.
[e] CV (%) was calculated from duplicate measurements.

the parent-calibrated methods was capable of fully compensating the extraction recovery of M1 emphasizes the importance of developing a sample preparation method as generic as possible. However, acceptable levels of accuracy were reached using the parent-calibrated NCD method contrary to the parent-calibrated QTOFMS method. Indeed, quantification of metabolites by MS in the absence of authentic reference standards is at best only semi-quantitative. A study by Hatsis et al. [6] found up to a 70-fold difference in ion response factor between metabolite and parent drug by ESI-LC-MS/MS.

3.2. Investigation of post-mortem urine samples

To evaluate the novel GC-NCD-APCI-QTOFMS platform in casework, twenty post-mortem urine samples previously found positive for α-PVP were analyzed by the parent-calibrated NCD method and the reference QTOFMS method. Table 3 shows that by using the parent-calibrated NCD method a quantitative result was obtained in 12 out of 20, 11/20, 2/20 and 0/20 cases for α-PVP, M1, M3 and M5, respectively. The highest difference in the quantitative results between the two methods was only 33%, and the NCD method's precision expressed as the coefficient of variation was better than 13%. The accuracy and precision of quantification for post-mortem samples was similar to that for the spiked urine samples by the parent-calibrated NCD method (Table 3).

M1 and M3 have been considered as the major metabolites of α-PVP in humans [15]. In our cases, the presence of these metabolites was confirmed in all but one urine sample by GC-APCI-QTOFMS. Currently, the sensitivity of NCD was not sufficient to quantify all metabolites, especially the minor metabolite M5. Moreover, interference caused by co-eluting nitrogen compounds

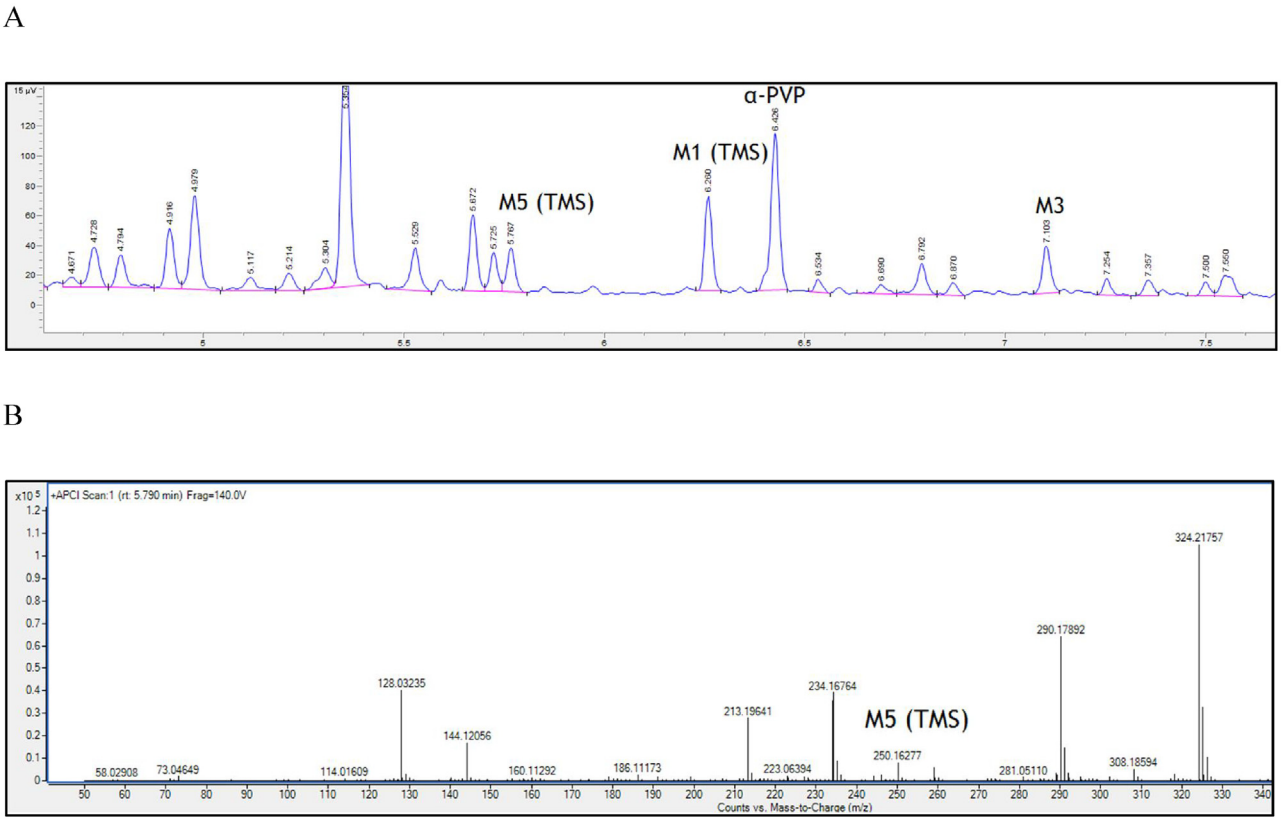

Fig. 3. Interfering compounds in post-mortem urine #7. (A) In NCD chromatogram co-eluting compounds result in overestimation of M5. (B) QTOFMS spectrum confirms that TMS derivatized peak of protonated M5 (m/z 250.1622) contributes only minimally to total NCD peak area.

prevented reliable determination in some cases with lower metabolite concentrations (Fig. 3).

According to the manufacturer, the NCD detector can detect nitrogen over the carbon background signal in the mass ratio of 10^7 to one. However, co-eluting nitrogen-containing compounds can cause overestimation in NCD quantification. Such compounds can be due to a multidrug intake or they can be common endogenous urine compounds which are prevalent at low concentrations. In Table 2 we have set criteria for assessing the quality of the quantitative data. For high-sensitivity NCD analysis, common matrix background peaks should be subtracted using a blank urine. A viable strategy to enhance chromatographic selectivity would be using two-dimensional GC [16].

4. Conclusions

This study demonstrated that the current GC-NCD-APCI-QTOFMS instrument platform has potential in the rapid quantitative bioanalysis of the main α-PVP metabolites together with the parent drug in cases where authentic reference standards are not immediately available. As conventional validated analysis necessitates information on retention time, compound specific spectral fragmentation and quantitative response obtained with an authentic reference standard, these conditions cannot be realized in case of infrequently encountered NPS metabolites. However, identifying NPS metabolites would facilitate the confirmation of the parent drugs, and metabolite quantification can aid forensic interpretation, in particular when investigating the time of drug intake utilizing metabolite to parent drug ratios. In future studies we will seek to improve the NCD method's LOQ using the large volume injection and widen the scope of substances included in the NPS screening.

Compliance with ethical standards

This article does not contain any studies with living human participants or animals performed by any of the authors. The analysis of drugs from the autopsy specimens was performed according to the request of judicial authorities.

Conflict of interest

The authors declare that they have no conflict of interest.

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