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RESEARCH PAPER

Enhancing the sensitivity of the LC-MS/MS detection of propofol in urine and blood by azo-coupling derivatization

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Abstract
Propofol is a low-polarity, volatile molecule that is difficult for an electrospray ion source (ESI) to ionize in either negative ion mode (NIM) or positive ion mode (PIM), which hampers its detection via liquid chromatography–mass spectrometry. The aim of the present study was to use a new derivatization agent to improve ionization efficiency and to develop an efficient liquid chromatography–multiple mass spectrometry (LC-MS/MS) determination of propofol in urine and blood, taking advantage of an electrophilic aromatic substitution. An azo-coupling reaction with a diazonium salt from aniline was performed to introduce a protonation site into the molecule. The diazonium salt was generated by aniline in water solution by HCl and sodium nitrite; derivatization was achieved by stirring a mixture of the diazonium salt and propofol in sodium hydroxide solution for 30 min below 5 °C. A liquid-liquid extraction with dichloromethane and ethyl acetate was performed to obtain the azo derivative (molecular composition: $C_{18}H_{22}ON_2$; molecular weight: 282 Da) in high yield. The compound provided very high ionization yields in both PIM and NIM ESI, and the protonated or deprotonated molecule gave intense signals. The transitions *m/z* 283→77, 241 and *m/z* 281→176, 161 were chosen for the PIM and NIM, respectively, in order to develop quantitative methods of detecting propofol in urine and blood via triple-quadrupole LC-MS/MS. These methods proved to be highly sensitive, with limits of quantification of 0.4 pg/mL and 0.1 ng/mL obtained in the NIM when analyzing 1 mL of urine and 100 μL of blood, respectively.

Keywords Propofol · Azo coupling · Derivatization · Urine · Blood · LC-MS/MS

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Introduction

Propofol is a short-acting hypnotic agent that is commonly used to induce and maintain anesthesia. It was introduced in 1989 under the trade name Diprivan®, and has since surpassed other injectable anesthetics in terms of frequency of use due to its favorable side-effect profile (lower incidences of nausea and vomiting as well as postoperative drowsiness) [1]. However, propofol is a potent central nervous system and respiratory depressant, and overdosing on this agent can result in respiratory failure and cardiac complications, with potentially lethal consequences [2–4]. Propofol dependence was not recognized until 1992, but it has received considerable attention in recent years. Generally, this drug generates a short ecstatic and euphoric feeling due to its fast onset, and has fewer side effects than other such substances [5].

Taking into account these characteristics of propofol, it is clearly important to develop highly sensitive analytical methods of determining this agent, especially for forensic purposes. Various analytical methods such as liquid chromatography–tandem mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS) [6–8] for the detection of propofol and its metabolites in biological matrices [8–11] have been reported in the literature. GC-MS is generally used for its high separation capacity and detection sensitivity towards volatile compounds, and it requires a sample pretreatment step such as extraction and derivatization [12]. In recent years, several LC-MS/MS analytical methods have

been utilized to detect phase II metabolites of propofol [11–13], such as propofol glucoronide. However, propofol and its phase I metabolites are difficult to ionize, making it harder to detect them by LC-MS/MS. This low ionization efficiency (IE) is due to their nonpolar nature and an absence of groups that are easy to ionize. Thus, many different chemical derivatizations have been applied in attempts to enhance the IE [14, 15], and the extent to which they achieve this is strictly related to the introduction of charged (i.e., quaternary ammonium, phosphonium) or chargeable (i.e., amino) groups [16–20]. Moreover, derivatization reagents are designed to generate appropriate structures for MS/MS detection, as they are efficiently fragmented by collision-induced dissociation (CID), providing specific product ions. All of the chemical derivatizations described in the literature for propofol have focused on the hydroxyl group (–OH); there have been no derivatizations of other functional groups, such as the aromatic ring. The latter is activated by the –OH, and can act as the substrate for electrophilic aromatic substitution (EAS).

In this paper, a new derivatization method for propofol that takes advantage of an EAS reaction with a diazonium salt is described for the first time. This reaction is called azo coupling (AC), and is the most widely used industrial reaction for the production of dyes and pigments, but it has never been applied previously for analytical purposes. AC leads to the formation of a compound with two nitrogen atoms bound together (–N=N–, called the azo group) in the *para* position. This chemical modification leads to an increase in the IE of the derivatization compound, together with new fragmentation pathways in both ionization modes. From a chemical point of view, the electrophilic species is the diazonium cation, as generated by the treatment of aromatic amines (aniline in this study) with sodium nitrite under strongly acidic conditions (diazotization reaction, Fig. 1a). AC is well suited to use as a derivatization reaction, as it possesses many advantages: low reagent cost, substantial IE and fragmentation pattern enhancement, versatility, and mild operative conditions. The aim of the study described here was to develop two new LC-MS/MS analytical methods of detecting propofol in urine and blood that utilize the AC reaction to derivatize propofol. These methods have much lower limits of quantification than recent methods reported in the literature.

Materials and methods

Chemicals

Dichloromethane (DCM), diethyl ether (Et_2O), and methanol (MeOH) were purchased from Panreac Quimica S.L.U. (Castellar del Vallès, Spain). Ethyl acetate (AcOEt), sodium acetate, glacial acetic acid, hydrochloric acid (HCl), sodium nitrite ($NaNO_2$), formic acid, and *n*-hexane (Hex) were obtained from J.T. Baker (Deventen, Netherlands). Sodium hydroxide (NaOH) was supplied by Carlo Erba Reagenti (Milano, Italy). LC-MS CHROMASOLV® methanol was purchased from Sigma–Aldrich (St. Louis, MO, USA). Water was obtained from B. Braun (Milano, Italy). Aniline was acquired from Riedel-de Haën (Seelze, Germany). Propofol, propofol-d_{17} (used as an internal standard, IS), and methanolic standards were purchased from Chemical Research 2000 s.r.l. (Rome, Italy) and diluted to the appropriate concentrations with MeOH. Blank urine and whole-blood samples were provided by laboratory personnel and volunteers.

Preparation of reagents

A working solution of aniline chloride ($ArNH_3Cl$) was prepared by adding 370 μL of HCl and 100 μL of aniline to 500 μL of water. Diazonium salt (ArN_2^+) was freshly obtained by mixing 1 mL of water, 10 μL of 1.1 M $NaNO_2$ (≈10 μmol), and 10 μl of the working solution created previously (corresponding to≈10 μmol of aniline chloride) under magnetic stirring for 10 min in an ice-salt bath. The temperature should not exceed 5 °C to avoid the hydrolysis of the salt to phenol and nitrogen.

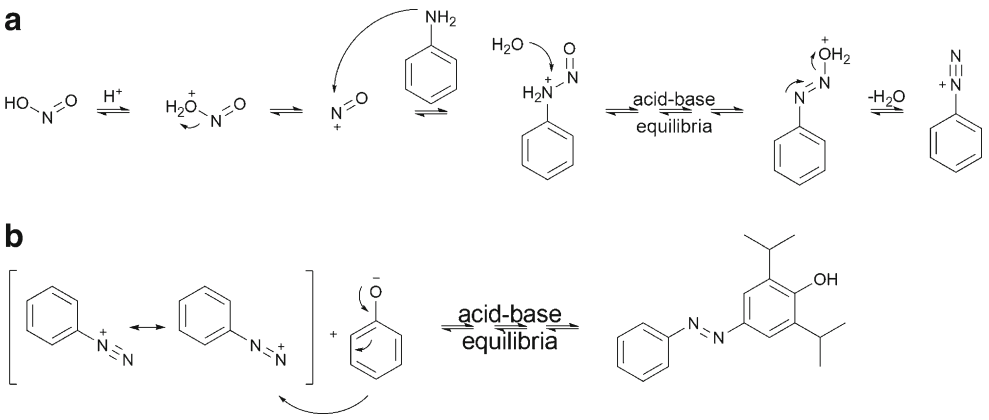

Fig. 1 Mechanisms for the diazotization (**a**) and azo-coupling (**b**) reactions

Sample pretreatment

Urine specimen

One milliliter of urine was added to 300 μl of 6 M HCl and 10 μl of a 50 ng/μL solution of IS and then incubated at 100 °C for 60 min. After cooling at room temperature, the mixture was neutralized with 6 M NaOH and the pH was adjusted by adding 200 μl of phosphate buffer (pH 8). A liquid–liquid extraction was performed by adding 3.0 mL of a DCM/AcOEt 8:2 (v:v) mixture. After centrifugation at 4000 rpm for 5 min, the lower organic layer was transferred into a tube and dried under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 100 μL of MeOH, added to 1 mL of 2 N NaOH, and stored at 0 °C.

Blood specimen

Protein precipitation was achieved by adding 100 μL of a whole-blood sample to 790 μL of MeOH and 10 μL of a 10 ng/μL solution of IS. After centrifugation at 4000 rpm for 5 min, 450 μL of the supernatant were added to 1 mL of 2 N NaOH and the resulting solution was then stored at 0 °C.

Derivatization

Ten milliliters of salt solution (≈100 nmol) were added dropwise to a cooled propofol solution. A color change to light yellow suggested that a reaction was occurring. The reaction was maintained under magnetic stirring and in an ice-salt bath for 30 min. After warming at room temperature, the derivatization compound was obtained by liquid-liquid extraction (LLE) with 5 mL of a DCM/EtOAc 8:2 (v:v) mixture. After centrifugation at 4000 rpm for 5 min, the lower organic layer was transferred to a tube and dried under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 100 μL of LC-MS CHROMASOLV® methanol. A 5-μL aliquot was injected into the LC/ESI-MS/MS system.

LC-MS/MS

Analysis was conducted using an HPLC Agilent 1290 Infinity system (Agilent Technologies, Palo Alto, CA, USA) interfaced with an Agilent 6460 Triple Quad LC/MS (Agilent Technologies), coupled at an electrospray ion source (ESI). The column used was a Zorbax SB-C18 Rapid Resolution HT (2.1 × 50 mm, 1.8 μm, Agilent Technologies), heated at 30 °C. The mobile phase initially consisted of 5 mM aqueous acid formic (A) and acetonitrile (B) 90:10. Gradient elution was experimentally carried out by increasing the %ACN to 90 % within 3 min, then it was run isocratically for 1 min; the post-time was fixed at 3 min. The flow rate was 0.4 mL/min and the injection volume was 5 μL. Upon applying this elution profile to underivatizated propofol and to azo-propofol, a retention time shift of about +0.500 min was observed, since they eluted on average at 3.018 and 3.535 min, respectively (Fig. 2). The ESI configuration was: gas temperature 325 °C; gas flow rate 10 l/min; nebulizer 20 psi; capillary 4000 V. Ionization was performed in both negative ion mode (NIM) and positive ion mode (PIM). The first mass measurements and peak identifications were carried out in selected-ion monitoring (SIM) mode for the $[M+H]^+$ (*m/z* 283) and $[M-H]^-$ (*m/z* 281) ions. The identification of target peaks above interferences was quite easy to achieve by comparing chromatograms of blank and spiked water solutions. This approach allowed us to discard impurity-related peaks, which were considered to be those found in the spectra from both solutions. No significant production of particular byproducts was observed in the matrix samples. Fragmentation patterns, necessary for multiple reaction monitoring (MRM), were studied in product-ion scan mode in the CID energy range 10–40 eV. Fragmentor values were set experimentally to obtain the highest precursor ion yields (Table 1). Data acquisition and elaboration were performed using the Agilent MassHunter Workstation software package.

Validation parameters

Selectivity and specificity

The absence of endogenous interfering signals was evaluated by analyzing 10 blank urine and blood samples from 10 different adults. Ten urine and blood specimens from abusers of several drugs (such as benzodiazepines, barbiturates, THC, and amphetamines) were analyzed to verify the lack of exogenous interferences.

LOD and LLOQ

The limit of detection (LOD) and the lower limit of quantification (LLOQ) were evaluated on the basis of the signal-to-noise ratio (SNR) for three replicates of blank specimens fortified with decreasing quantities of propofol. The LOD and LLOQ were identified as the concentrations with SNR≈ 3 and SNR≈10.

Linearity, accuracy, and precision

Linearity was estimated for the calibration range 5.0–2000.0 ng/mL for urine and 50.0–20000.0 ng/mL for blood. Five replicates of blank urine and blood fortified at the proper concentrations were analyzed, and the least-squares regression procedure was applied to the data.

Quality control (QC) samples were collected by spiking blank samples with the propofol standard solution at 5.0, 1000.0, and 2000.0 ng/mL for urine, and at 50.0, 10000.0,

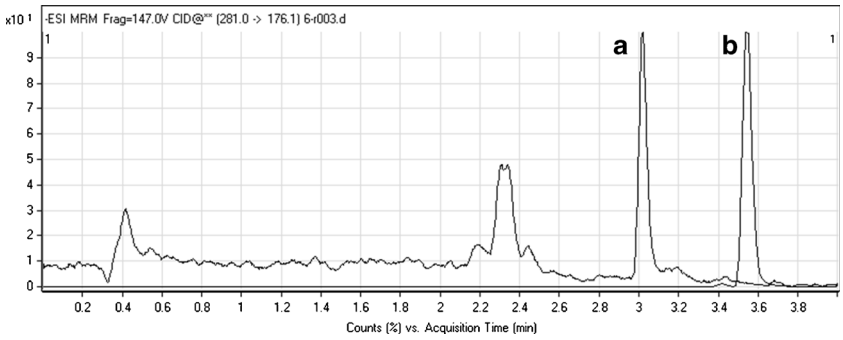

Fig. 2 Comparison between the chromatograms of underivatizated propofol (**a**) and azo-propofol (**b**) obtained in the SIM and MRM modes, respectively

and 20000.0 ng/mL for blood. Accuracy and precision were assessed by analyzing five replicates of each QC sample. Accuracy was expressed as the % mean relative error (%MRE), and the precision as the average of the relative standard deviation (%RSD). Interday precision was calculated based on data obtained on five different days during a month.

Recovery, matrix effect, and stability

Relative recovery was evaluated at the QC concentration levels for propofol and for azo-propofol and at 500 (urine) and 1000 (blood) ng/mL for the IS. The samples were prepared in blank urine and whole blood before extraction, and in post-extraction blank matrices spiked at the same concentration.

To estimate the matrix effect, the slopes from spiked blank matrix solutions and spiked water solutions were compared at the QC concentrations for three replicates.

The stabilities of the derivatized compounds in the treated samples were evaluated by comparing the quantitative results obtained from five replicates of freshly fortified samples (at QC concentration levels) with those obtained from five replicates of the same sample stored at −25 °C and thawed weekly during a month.

Table 1 MRM transitions for each azo compound

Compound	Precursor ion (*m/z*)	Fragmentor (V)	Product ion (*m/z*)	Collision energy (V)
$[M+H]^+$				
Azo-propofol	283	93	**77**/241	21/15
Azo-propofol-d_{17}	299	116	**77**/251	21/13
Azo-phenol	199	86	**77**/93	21/13
$[M-H]^-$				
Propofol	177	134	**177**	0
Azo-propofol	281	147	**176**/161	21/30
Azo-propofol-d_{17}	297	132	**192**/174	21/29
Azo-phenol	197	116	**92**	21

Quantifier transition is shown in bold

Results and discussion

Derivatization

Temperature and pH are the key factors in the formation of ArN_2^+, which is the main step in the reaction. The salt ArN_2^+ is stable within the range 0–5 °C and at acidic pH. Acidic conditions are required to obtain the active diazotizing nitrosyl reagent, but they also lead to the complete protonation of the amino group, making the lone pairs unavailable for the reaction. Nevertheless, the diatotization was successfully carried out using $ArNH_3Cl$, as aniline is insoluble in water. On the other hand, basic conditions are necessary in the AC step in order to increase the concentration of phenolate anions, which are considerably more reactive than phenol itself. Thus, several pH values and dilutions were evaluated, and the optimal conditions were found (these have already been described in previous sections). The molar ratio $ArNH_3Cl$:$NaNO_2$ during diazotization was investigated by analyzing the chromatographic outcomes in terms of signal quality, separation efficiency, and presence of interferences. As expected, any excess increased the amount of byproducts (such as *N*-azo compounds, otherwise known as triazenes) and their interfering signals, albeit without affecting the chromatographic separation efficiency and the derivatization yield. An $ArNH_3Cl$:$NaNO_2$ ratio of 1:1 seemed to be essential. For the AC, an excess of ArN_2^+ was required, starting from a salt:substrate ratio of 2:1. The increase in this ratio didn't lead to significant interferences or reactivity limitations. The reaction time was investigated at 15 min, 30 min, 1 h, and 2 h. The outcomes revealed that the largest peak area appeared at a time of 30 min, and the area then plateaued as the time increased further.

AC derivatization of propofol generates a compound with a molecular composition of $C_{18}H_{22}ON_2$ and a molecular weight of 282 Da. The derivative contains an aryl-azo moiety in the *para* position with respect to the hydroxyl, and the *E*-stereoisomer with respect to the –N=N– bond is predominant (Fig. 1b). This is due to the regioselectivity and stereospecificity of the reaction: the substitution takes place at the *para* position to the activating group, or at the *ortho* position when the *para* position is unavailable. Steric hindrance effects

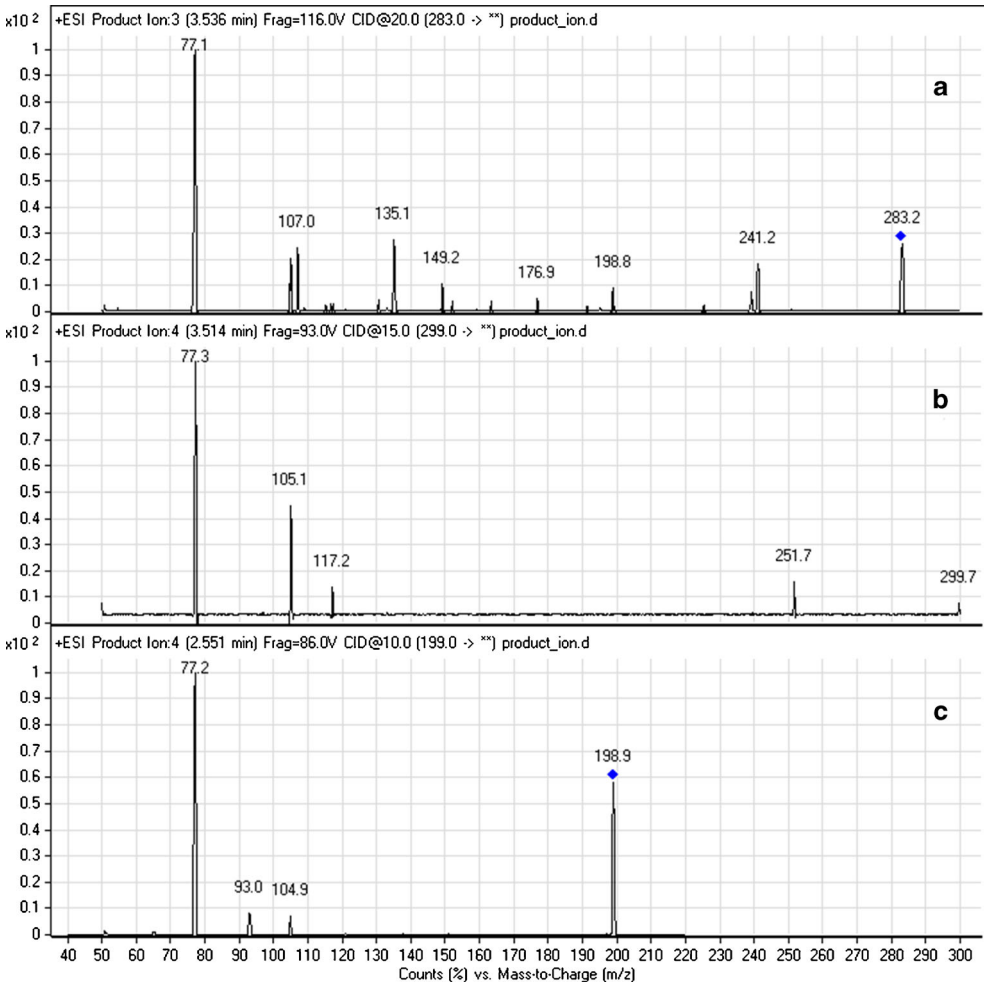

Fig. 3 Comparison of the product ion scan mode spectra of azo-propofol (**a**), azo-propofol-d_{17} (**b**), and azo-phenol (**c**) obtained in the positive ionization mode

ensure that the *E*-stereoisomer is predominant. Isolation of the azo-propofol was achieved by preparative thin-layer chromatography (TLC; PLC silica gel 60, 0.5 mm, 20×20 cm, Merck, Darmstadt, Germany), using a 8:2 (v:v) eluent mixture of Hex/Et_2O (ratio to front=0.5)[1]. The reaction yield was always >85 %. The azo derivative is a yellow-orange noncrystalline solid that is structurally similar to pH colorimetric indicators such as methyl orange, methyl red, and methyl yellow. It appears yellow-orange (λ_{max}=459 nm, water) in acidic solution (pH<5), but changes to yellow (λ_{max}=511 nm, water) under alkaline conditions (pH>8).

PIM MS/MS fragmentation pathway

In order to achieve a complete understanding of the azo-propofol fragmentation pathways, the derivatization was also applied to phenol and to the stable-isotope-labeled standard propofol-d_{17}. The phenol was prepared in our laboratory from the aniline using the diazotization procedure already described. As mentioned, the diazonium salt is unstable above 5 °C and degrades to phenol and nitrogen. Thus, once the salt had been produced at 0 °C, the reaction mixture was warmed to room temperature and maintained under magnetic stirring until the effervescence had disappeared. After that, the AC reaction occurred in the resulting solution without any further processing. The PIM fragmentation spectra of derivatized propofol, propofol-d_{17}, and phenol are shown in Fig. 3. The peak corresponding to $[M+H]^+$ for propofol occurs at *m/z* 283, while the most abundant fragment (used as quantifier) is the phenyl cation (*m/z* 77), which arises from the α-cleavage reaction that takes place between the azo group and the anilinic aromatic ring (Fig. 4). This hypothesis is confirmed by its presence in the spectrum of azo-propofol-d_{17}, with no mass shift due to deuterium atoms. The isotopic standard was also decisive in identifying rearrangements that take place on the propofol ring, as well as in the spectrum of azo-phenol. In particular, this is missing in the spectrum of azo-phenol for the ions at *m/z* 241, 199, 177, 135, and 107, suggesting that the generation of these ions involves the propofol portion of the

[1] Ratio to front (Rf) is a parameter used for the identification or characterization of substances in thin layer chromatography. It is the ratio between the distance traveled by compound and the distance traveled by solvent.

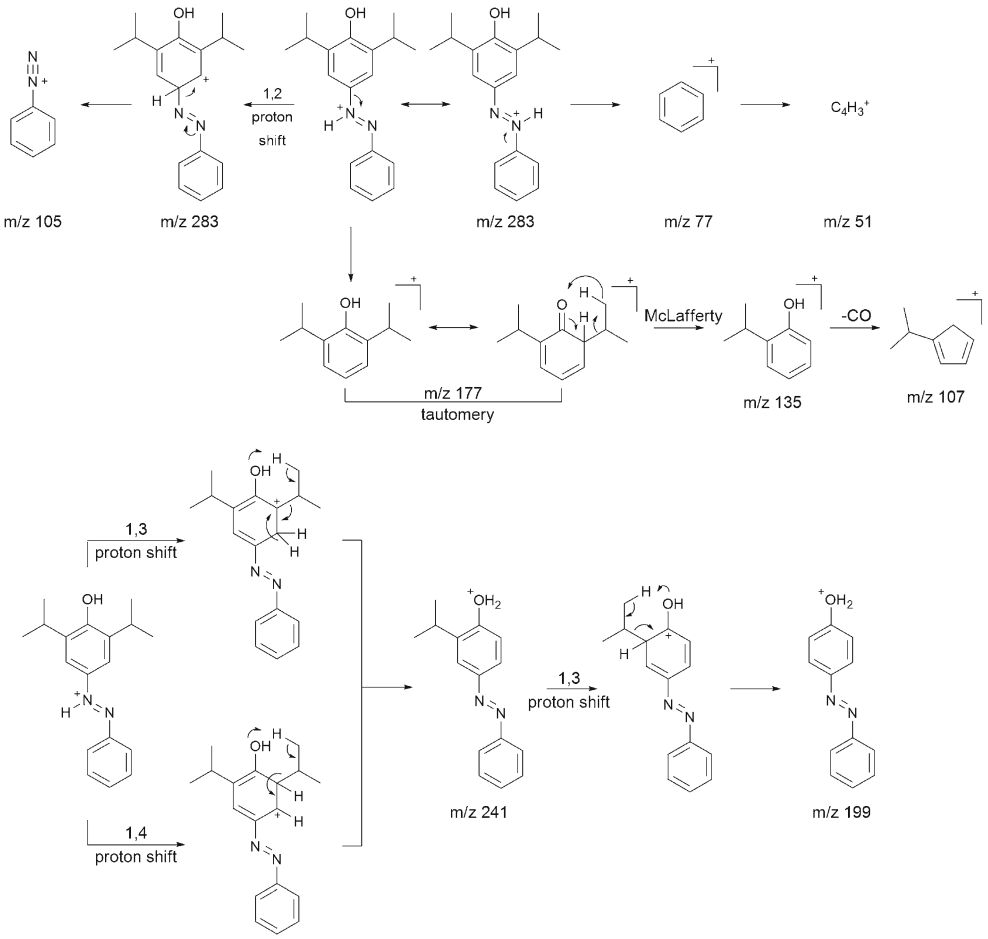

Fig. 4 Proposed mechanism for the generation of the positive MS/MS fragmentation patterns of azo-propofol and azo-propofol-d_{17}

structure and its isopropyl groups. The hypothesized mechanisms are shown in Fig. 4, and they are supported by the fragments at *m/z* 251 and 117 in the spectrum of azo-propofol-d_{17}, as they are the deuterated analogs (+10 shift) of the ions at *m/z* 107 and 241. This latter is the second most abundant ion, and it is generated through 1,3 and/or 1,4 proton shifts followed by rearrangement and the loss of propene (C_3H_6). A McLafferty rearrangement occurs subsequent to the tautomeric rearrangement of the propofol cation (peak at *m/z* 177) to the chetonic form; this still leads to the loss of a propene molecule. Azo-phenol has an analogous ion to the propofol cation at *m/z* 93. The fragment at *m/z* 105 is due to the reverse reaction to AC, which leads to the loss of propofol and the re-establishment of the diazonium cation. Moreover, this fragment is commonly associated with interference, as is that at *m/z* 77. All of this can be accounted for by invoking the formation of azo byproducts that were identified in the chromatograms, as well as in the highly specific fragmentation pattern of the azo-propofol.

NIM MS/MS fragmentation pathway

Fewer ions were obtained in NIM, and there was not the variety of rearrangements seen in PIM (Fig. 5). The peak from azo-propofol $[M\text{-}H]^-$ is at *m/z* 281, whereas the most abundant product ion is at *m/z* 176 (used in quantitative analysis). This was generated by homolytic cleavage of the N–C bond of the phenolic ring. Further homolytic cleavage generates the ion at *m/z* 161 and its deuterated analog at *m/z* 174, with the loss of a methyl radical (Fig. 6). This latter fragmentation scheme is missing from the azo-phenol spectrum, where the ion resulting from the first α-cleavage at *m/z* 92 is the only one visible. As we have just noted, only two fragments were characterized in NIM, leading to a risk of incorrect identification. However, even though there are fewer fragments, they are highly specific and are not shared with any azo side-product or interference. Thus, detection was found to be effective using this approach.

Method validation

Urine

No urine concentrations are reported in the literature because of the extensive metabolism of propofol [21], especially to glucuronide-conjugated form. Acidic hydrolysis is required for urinary samples. The acidic hydrolysis described in the section above was compared with an enzymatic one that is normally used in our laboratory, which involved adding 10 μl

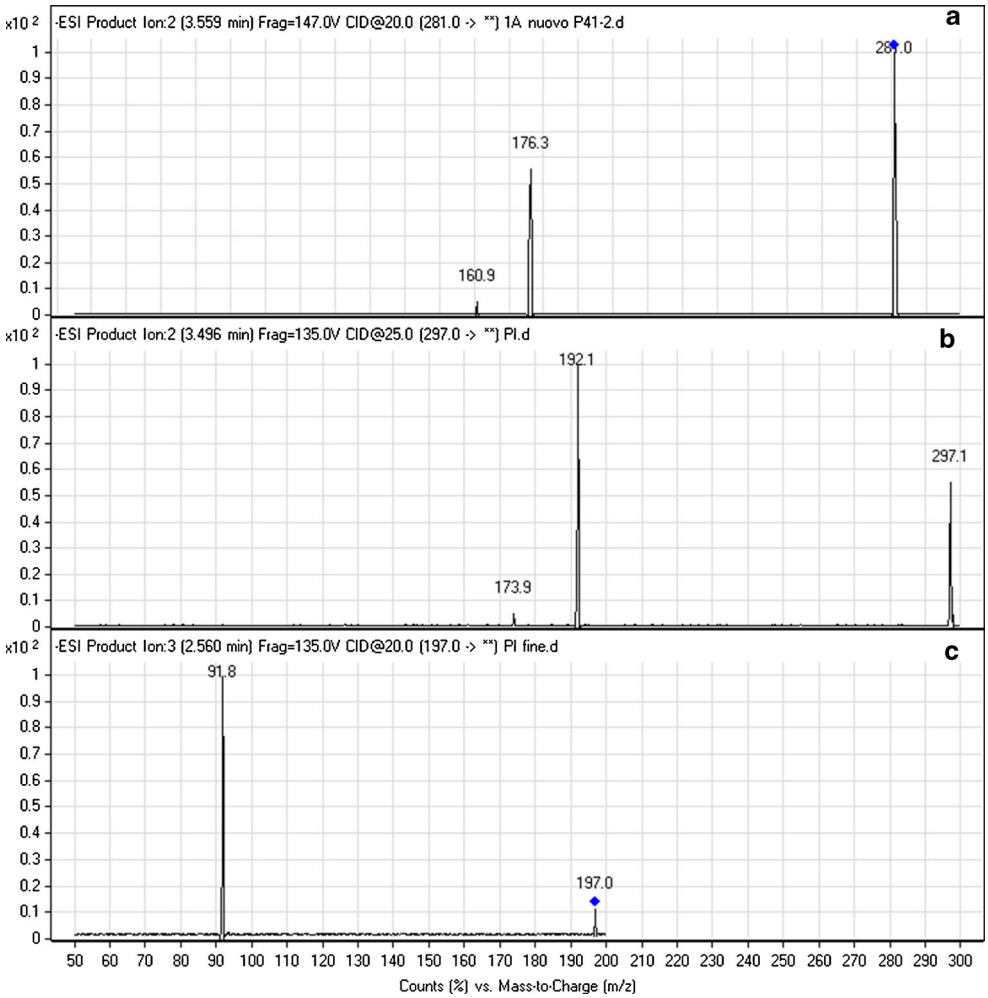

Fig. 5 Comparison of the product ion scan mode spectra of azo-propofol (**a**), azo-propofol-d_{17} (**b**), and azo-phenol (**c**) obtained in negative ionization mode

of β-glucuronidase and 20 μl of a 500 ng/mL solution of IS to 1.0 mL of urine with 1.0 mL of ammonium acetate buffer (pH 4.5) and then incubating this mixture for 18 h at 50 °C [22]. Enzymatic hydrolysis was preferred as it was faster and gave a slightly higher yield of azo-propofol. Selectivity tests did not reveal any interference with the azo-propofol retention time in both ion modes. Minor peaks of protonated interferences were observed at 1.7 min and 3.1 min, but they did not affect the analyte or IS analysis. Validation data for both ionization modes are shown in Table 2. The mean coefficients of determination (R^2) of the calibration curves were 0.9992 (PIM) and 0.9993 (NIM), indicating high linearity. In addition, high sensitivity was observed: LLOQ values were 0.003 (PIM) and 0.0004 (NIM) ng/mL. As the LLOQ values indicate, the NIM analysis was more sensitive than the PIM analysis due to a higher SNR rather than greater signal intensities. Comparing this method (based on the LLOQ) with recent GC-MS (LLOQ: 0.51 ng/mL; LOD: 0.04 ng/mL) and LC-MS/MS (LLOQ: 2.01 ng/mL; LOD: 0.44 ng/mL, restricted to the glucuronide form only) [12] analytical methods, it is notable that our method is about 1000- and 6000-fold more sensitive, respectively.

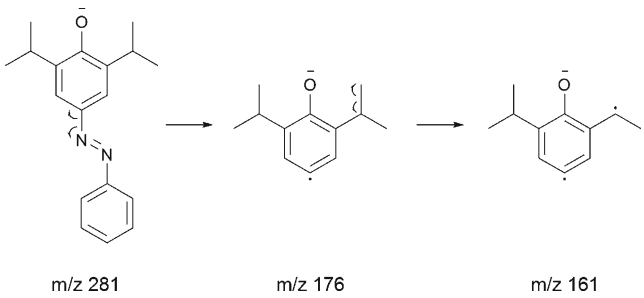

Fig. 6 Proposed mechanism for the generation of negative MS/MS fragmentation patterns for azo-propofol and azo-propofol-d_{17}

Precision and accuracy were within the acceptance criteria (within ≤ 20 % and ± 20 %, respectively, at the first QC level; within ≤ 15 % and ± 15 % for the second QC level, and within ≤ 10 % and ± 10 % for the last QC level): intraday precision was always lower than 7.9 % (PIM) and 2.0 % (NIM); interday precision was below 8.1 % (PIM) and 3.7 % (NIM); %MRE ranges were −4.8 to 1.7 % (PIM) and −1.7 to 5.7 % (NIM).

In general, relative recoveries for the post-hydrolysis and the post-derivatization extractions were always above 85 %

Table 2 Validation results for the detection of propofol in urine

Ion mode	LLOQ (ng/mL)	Accuracy %MRE			Intraday precision %RSD			Interday precision %RSD			Matrix effect
		QC (ng/mL)			QC (ng/mL)			QC (ng/mL)			
		5	1000	2000	5	1000	2000	5	1000	2000	
$[M+H]^+$	0.003	−4.8	1.7	−1.9	7.9	2.9	1.3	8.1	2.1	3.0	+20 %
$[M-H]^-$	0.0004	5.7	1.3	−1.7	1.2	2.1	2.0	3.4	3.7	3.6	+22 %

across the concentration range. A PIM matrix effect was observed, with similar values noted for both ion modes (mean value +21 %). The study of stability showed that the storage of derivatized propofol at −25 °C and the implementation of freeze/thaw cycles led to losses of <8 %.

Blood

The concentration range of propofol in blood is strictly correlated with individual condition [23], but the sedation can be maintained at a serum concentration of 1–4 ng/mL [24]. Propofol detection in blood is strongly conditioned by its binding to proteins and erythrocytes [25], with variations in concentration observed between whole blood, serum, and sedimented cell fractions. Thus, LLE or solid-phase extraction and protein precipitation are the most widely used pretreatment methods for quantifying propofol in whole blood [15, 26, 27]. In this study, several solvents and dilutions were tested, and the procedure described in the section above gave the best recoveries and azo-propofol yields.

Selectivity and specificity studies yielded the same results as those seen for the urine samples. The method showed very good linearity over the tested concentration range of 50.0–20000.0 ng/mL, with mean R^2 values of 0.9994 (PIM) and 0.9995 (NIM), as shown in Table 3. LLOQs were set at 0.50 (PIM) and 0.10 (NIM) ng/mL, which are 200- and 130-fold lower than the LLOQ values of derivatization procedures for dansyl chloride (LLOQ: 20 ng/mL; LOD: unavailable) and *N*-methylpyridinium (LLOQ: 13 ng/mL; LOD: unavailable) that are reported in the literature [14, 15]. For blood analysis, the highest sensitivities were achieved in NIM.

For the intraday measurements, precision values were lower than 2.7 % and 3.2 % (PIM and NIM, respectively), and the accuracies ranged from −3.0 to 9.1 % in PIM and from −5.8 to 9.6 % in NIM. Interday precisions were lower than 3.4 % (PIM) and 2.7 % (NIM). Extraction recoveries were high (>87 %), reproducible, and consistent over the tested concentration levels. Similar to what was seen for urine, an ion enhancement matrix effect was observed, with a mean value of +25 %. Derivatized propofol was stable when stored at −25 °C in treated samples, and the loss due to the implementation of freeze/thaw cycles was below 10 % over the course of a month.

Application to real cases

In the last year, a urine and a whole-blood specimen from two different cases have been analyzed using these methods in order to check for the presence of propofol. Both individuals showed an overall state of sedation with respiratory difficulties and loss of consciousness. The blood specimen was taken from a 56-year-old woman, whereas the urine one was obtained from a 17-year-old girl; the propofol concentrations were 68.6 and 3404.3 ng/μL, respectively.

Conclusions

This study represents the first application of AC as a derivatization method for the detection of propofol using LC/ESI-MS/MS. Several aspects were investigated in order to identify the optimal conditions and gain a substantial improvement in propofol sensitivity. Azo derivatization offers the opportunity to incorporate features that can significantly improve propofol analysis using LC-MS/MS through the introduction of a

Table 3 Validation results for the detection of propofol in blood

Ion mode	LLOQ (ng/mL)	Accuracy %MRE			Intraday precision %RSD			Interday precision %RSD			Matrix effect
		QC (ng/mL)			QC (ng/mL)			QC (ng/mL)			
		50	10000	20000	50	10000	20000	50	10000	20000	
$[M+H]^+$	0.5	−2.0	9.1	−3.0	2.7	1.9	1.8	3.4	2.7	2.5	+23 %
$[M-H]^-$	0.0004	6.4	9.6	−5.8	3.2	1.7	0.4	2.7	1.9	1.8	+27 %

highly ionizable group (–N=N–), which leads to more specific fragmentation pathways in both ionization modes. Furthermore, AC is simple to achieve, as it is a fast reaction that requires only mild conditions and inexpensive reagents. The total run time (from sample preparation to the final result) was estimated to be about 2–4 h. However, the most significant advantage of AC derivatization is the marked improvement in sensitivity that it permits. Two methods can be developed that are both much more sensitive than those reported in the literature. Therefore, the utility of this approach is the excellent sensitivity obtained and its applicability to a biological matrix such as urine. It has no drawbacks. The remarkable sensitivity attained with these methods should prove very useful in forensic chemistry, where it can be necessary to detect propofol at concentrations that are much lower than the therapeutic range. In this context, the next steps are to apply this procedure to other molecules with an aromatic activated ring and to validate this new analytical method with other biological matrices.

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