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APPLICATION NOTE

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# A critical point in chiral chromatography–mass spectrometry analysis of ketamine metabolites

Andrea Barbarossa[1,2] | Anisa Bardhi[1] | Teresa Gazzotti[1,2] | Giampiero Pagliuca[1,2]

[1]Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy

[2]Health Sciences and Technologies-Interdepartmental Centre for Industrial Research (CIRI-SDV), University of Bologna, Bologna, Italy

**Correspondence**
Anisa Bardhi, Department of Veterinary Medical Sciences, Alma Mater Studiorum, University of Bologna, Via Tolara di Sopra 50, Ozzano Emilia (BO), Bologna 40064, Italy.
Email: anisa.bardhi2@unibo.it

## Abstract

Ketamine is a widely used dissociative drug, whose quantification in plasma and urine can be of pharmacological, toxicological, and clinical interest. Although tandem mass spectrometry allows the reliable determination of ketamine and its metabolites in biological matrices, the structural similarity between norketamine (main active metabolite) and dehydronorketamine (a less relevant metabolite) can represent a critical aspect. These compounds differ exclusively in two hydrogen atoms, but the consequent two-unit difference in their mass/charge ratio is partially nullified by the isotopic abundance of the chlorine atom present in their structure. This, along with their similar fragmentation pattern, can result in the incorrect identification of the enantiomers of these ketamine metabolites even with triple quadrupole instruments, if shared transitions are monitored after chiral chromatography. The key to prevent norketamine overestimation is therefore observing analyte-specific MS/MS transitions. Here, we describe in detail how we investigated this issue, during the development of an analytical method for ketamine and norketamine enantiomer determination in plasma.

## 1 | INTRODUCTION

Ketamine (KET, M.W. = 237.73) is a potent dissociative agent, commonly employed in both human and, more recently, veterinary practice. KET is a chiral compound, and the two enantiomeric forms have significantly different pharmacokinetic profiles and pharmacodynamic activities, being the anesthetic potency of *S*-(+)-ketamine 2–4 times higher than that of *R*-(−)-ketamine, and its elimination and recovery from anesthesia sensibly faster.[1,2] The main hepatic first-pass metabolism of KET involves its *N*-demethylation to norketamine (NK), which has similar activity to the parent drug. Other metabolites include multiple products of KET and NK hydroxylation, and dehydronorketamine (DHNK).[3,4] The introduction on the market of pure *S*-ketamine as an alternative to the racemic mixture has increased the interest towards reliable enantioselective quantification of KET and its metabolites in biological matrices, such as plasma and urine. Multiple approaches, including capillary electrophoresis,[5] gas,[6] and liquid[7,8] chromatography–mass spectrometry, have been successfully developed for the purpose in the last decades. In particular, due to its high selectivity, tandem mass spectrometry is often the first choice in this application.

KET and its metabolites can be unambiguously identified by high-resolution single and tandem mass spectrometers with 1-mmu mass discrimination.[5] However, the structural similarity between NK and DHNK, which differ only in two hydrogen atoms, can represent a critical aspect for low resolution instruments. Indeed, the isotopic abundance of the chlorine atom present in both analytes can partially mask the two-unit difference in their respective mass/charge ratios ($[NK + H]^+ = 224\ m/z$, $[DHNK + H]^+ = 222\ m/z$). In addition, NK and DHNK follow mainly the same fragmentation pattern; therefore,

some MS/MS transitions generated by the $^{37}Cl$ isotope (relative abundance = 24.23%) present in DHNK can overlap to those of NK. The consequence is the overestimation of NK levels if such transitions are used, which can lead to incorrect clinical and pharmacological evaluations.

This application note describes this critical issue, which, to our knowledge, has not been previously highlighted in scientific literature, and how it was solved during the development of an analytical method for KET and NK enantiomers determination in plasma.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and reagents

High purity standards of KET, NK, and DHNK, as well as internal standards KET-D4 and NK-D4, were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile, ammonium acetate, and ammonium formate were of liquid chromatography–mass spectrometry (LC–MS) grade and were purchased from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water was freshly prepared in house every day of analysis (Millipore, Darmstadt, Germany).

### 2.2 | Instrumental conditions

The experiment was conducted using a LC–MS/MS by Waters (Milford, MA, USA). Chromatographic separation was obtained with a Waters Acquity UPLC binary pump, equipped with a Phenomenex Lux Cellulose-3 (150 × 2.00 mm, 3.0 μm) column and precolumn (Torrance, CA, USA), kept at 45°C to avoid excessive backpressure. Flow rate was set at 0.450 mL/min. The mobile phase consisted of 20-mM ammonium acetate 0.1% ammonium formate aqueous solution (A) and acetonitrile (B), flowing at 0.450 mL during 9 min under programmed conditions. In details, the chromatographic run started with 85% A for 3 min, changed to 65% A over the following 2 min, then was kept for 1 min before switching back to the initial conditions over 2 min, and finally left equilibrating for 1 min. The LC apparatus was coupled to a Waters Quattro Premier XE triple quadrupole mass spectrometer, operating in positive electrospray ionization mode (ESI +), with capillary voltage set at 3.00 kV and source and desolvation temperatures of 120°C and 400°C, respectively. The instrument operated in selected reaction monitoring (SRM) mode: the transitions observed for each analyte are reported in Table 1, together with the relative cone voltage and collision energy values. The fragment ion at 125 *m*/*z* was then used as specific SRM transition for NK over the range 5–1600 ng/mL during the validation of the method.

### 2.3 | Sample preparation

The extraction procedure was optimized on dog plasma, as previously described.[9,10] Briefly, after the addition of labeled internal standard solutions (20 μL of 500 ng/mL KET-d4 and NK-d4 in methanol), 150 μL of plasma was extracted by addition of 830 μL of methanol, sonication for 2 min, and centrifugation for 5 min at 8000 ×*g*. The supernatant was finally filtered through a 0.2-μm PTFE filter (Phenomenex, CA, USA) before being injected in the LC–MS/MS system.

## 3 | RESULTS AND DISCUSSION

Plasma samples collected during a pharmacokinetic study on the enantiomers of ketamine and its metabolites in dog were first analyzed by capillary electrophoresis in one laboratory[11] and later by LC–MS/MS in another facility.[9,10] However, NK concentrations initially resulted somehow higher when measured by LC–MS/MS. Trying to identify the origin of the issue, we then realized that certain MS/MS transitions might lead to the incorrect quantification of the main active metabolite NK.

Due to the isotopic abundance of the chlorine atom present in its structure, $^{37}Cl$ isotope DHNK (a further metabolite resulting from KET and NK hydroxylation) shares with $^{35}Cl$ isotope NK the 224 mass–charge ratio, which makes the discrimination of the two molecules impossible for a single MS detector with insufficient mass discrimination. In addition, the product ion mass spectra of NK and DHNK show that they follow mainly the same fragmentation pattern; therefore, even with a triple quadrupole (MS/MS) instrument, some

**TABLE 1** Monitored transitions for the different analytes and their relative cone voltage and collision energy values

| Analyte | Transition (*m*/*z*) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|
| KET | 238.0 → 125.0 | 26 | 25 |
| | 238.0 → 179.1 | 26 | 16 |
| | 238.0 → 207.1 | 26 | 12 |
| DHNK | 222.2 → 141.9 | 19 | 25 |
| | 222.2 → 177.1 | 19 | 18 |
| | 222.2 → 205.1 | 19 | 13 |
| NK | 224.2 → 125.0 | 21 | 24 |
| | 224.2 → 179.1 | 21 | 15 |
| | 224.2 → 207.1 | 21 | 11 |

Abbreviations: DHNK, dehydronorketamine; KET, ketamine; NK, norketamine.

transitions generated by DHNK can overlap to those of NK. In particular, $^{37}$Cl DHNK generates the same product ions at 207 and 179 *m/z* as $^{35}$Cl NK, meaning that using one of these transitions during the analysis of real biological samples, where both metabolites are naturally present, would erroneously amplify the detected signal for NK (see Figure 1).

Although NK and DHNK can be efficiently separated by LC,[12] the task is more challenging for the detection of their enantiomers with chiral columns.[13] Moreover, because DHNK is rarely monitored, potentially overlapping chromatographic peaks between the enantiomers of the two metabolites might not be noticed, which could result in the overestimation of NK. When using a low resolution mass spectrometer as detector, monitoring a transition that is exclusive for NK is therefore the only strategy to overcome the problem. As shown in the mass spectra, the product ion at 125 *m/z* is specific for NK, because DHNK fragmentation does not generate a fragment at 123 *m/z*. A further confirmation of this assumption is provided by the chromatograms obtained monitoring simultaneously all the DHNK and NK transitions reported in Table 1 after injection of the standard solution of each analyte in the LC–MS/MS system (Figure 2). As expected, fragments generated by both the 222 and the 224 *m/z* precursor ions can be observed for DHNK standard solution. Signals for the unspecific 224 > 207 and 224 > 179 *m/z* transitions are present, whereas the peak produced by the NK specific 224 > 125 *m/z* transition is missing; in addition, the products of the 222 *m/z* precursor compound fragmentation include also the product ion at 142 *m/z*, which, as previously evidenced, is specific for DHNK. As for NK standard solution, the expected 224 > 125 *m/z* transition is present, and no signals deriving from the 222 *m/z* precursor ion are detected. The importance of selecting this specific transition for NK quantification has not been described previously and, while few studies reported the correct fragment ion at 125 *m/z*,[13–15] others used one of the unspecific product ions.[16–19] We therefore felt that it was worth to discuss and highlight the issue in the present application note. The optimized MS/MS parameters were then applied to the analytical method, which was fully validated in accordance with EMEA/CHMP/EWP/192217/2009 guidelines.[20] The described approach was successfully employed in the analysis of canine plasma samples collected during a pharmacokinetics study on KET and NK metabolites.[9,10]

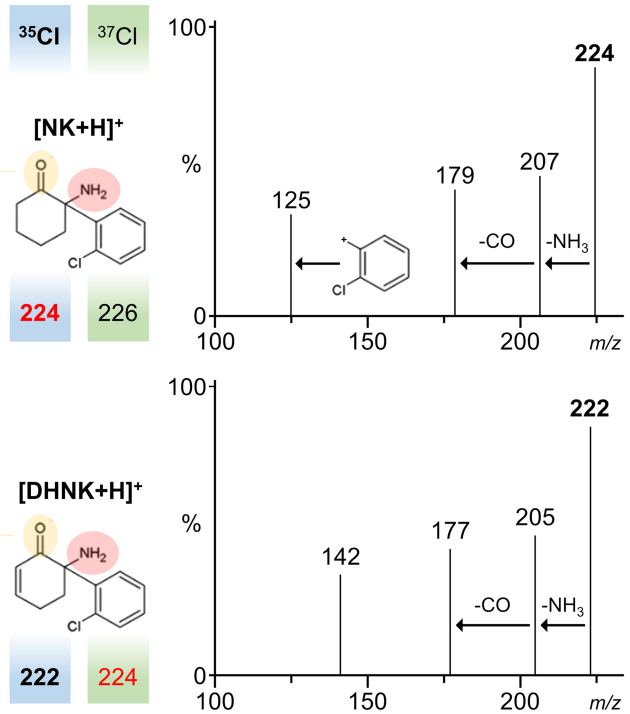


**FIGURE 1** Structural and molecular weight differences between protonated norketamine $[NK + H]^+$ and dehydronorketamine $[DHNK + H]^+$ depending on chlorine isotope abundance (left); product ion mass spectra of $^{35}$Cl NK and DHNK, evidencing the common –NH3 and –CO losses and the compound-specific product ions (right) [Colour figure can be viewed at wileyonlinelibrary.com]

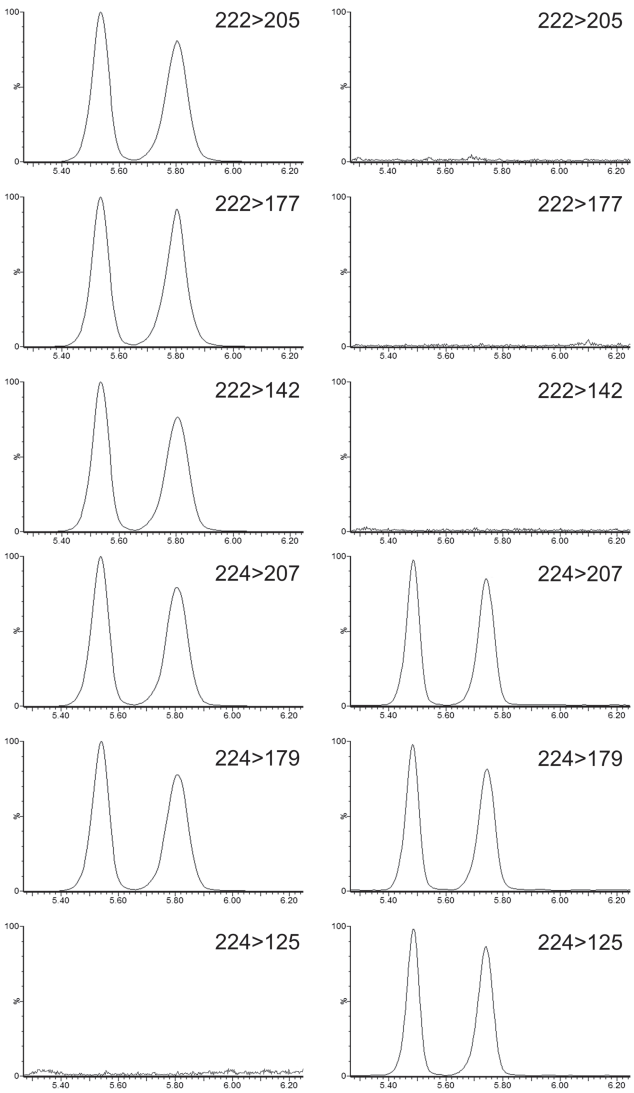


**FIGURE 2** Chromatographic signals obtained after injection of dehydronorketamine (left) and norketamine (right) racemic standard solutions in liquid chromatography–tandem mass spectrometry (LC–MS/MS), simultaneously monitoring all the fragments generated by both analytes. The recorded signals demonstrate that only the 224 > 125 *m/z* transition is specific for norketamine

## 4 | CONCLUSIONS

Thanks to their superior selectivity, triple quadrupole and ion trap mass spectrometers are the gold standard for the analysis of drugs and their metabolites in biological matrices. However, differently from high-resolution mass spectrometry (HRMS), the correct quantification of NK using low-resolution MS/MS strictly depends on the chosen transition, because DHNK can generate overlapping chromatographic signals. The fragment ion at 125 *m/z* is specific for NK, and its signal is sufficiently strong to allow the molecule to be measured from ng/mL to μg/mL level. The critical issue described in the present application note is even more relevant given the fact that NK is the most frequently investigated active ketamine metabolite.

## ORCID

*Andrea Barbarossa* https://orcid.org/0000-0002-7742-4229
*Anisa Bardhi* https://orcid.org/0000-0002-2012-2852

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