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Determination of ketamine and norketamine in plasma by micro-liquid chromatography–mass spectrometry

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ABSTRACT

A sensitive method of determination of ketamine and norketamine by micro-liquid chromatography/mass spectrometry was developed as part of a clinical trial in a pediatric population. Compounds were extracted from 100 μl plasma samples using solid-phase extraction on Oasis® MCX cartridges. Separation was achieved on a C18 micro-column with a mobile phase composed of formic acid 0.1% and acetonitrile (90/10, v/v). Detection of ketamine, norketamine and their internal standard norketamine D4 was performed using selected ion monitoring at *m/z* 238.2, 224.2 and 228.2, respectively. Under these conditions, no loss of signal due to matrix effect was found and time of analysis did not exceed 10 min. Extracted calibration curves were linear from 5 to 500 ng/ml for each analyte, with correlation coefficients over 0.999. Intra- and inter-day validation studies showed mean recoveries between 98.1 and 101.7% and a relative standard deviation below 1.9%. Extraction recoveries ranged from 84.8 to 89.8% for both ketamine and norketamine. Limit of quantification was 4 ng/ml for each analyte.

1. Introduction

Ketamine (K) is a dissociative anesthetic agent that has been widely used in clinical practice since 1970. At sub-anesthetic doses, ketamine has shown analgesic properties. Ketamine is a NMDA (*N*-methyl-D-aspartate) receptor antagonist: it blocks the NMDA receptor channel by binding to the phencyclidine receptor [1–3].

Ketamine is metabolized in the liver through the cytochrome P450 system (mainly CYP3A4). Its main metabolite, norketamine (NK) is obtained by N-demethylation (Fig. 1) and has shown an anesthetic potency equal to 20–35% that of ketamine, probably contributing to the long-lasting anesthetic effects [4–6].

During the last 15 years a large number of clinical trials have been published to describe the efficacy of low-dose ketamine in the management of acute post-operative pain in adults [7–12]. However, few data on the administration of ketamine in children pain management are available. Thus, the analgesic effect of low-dose ketamine associated with morphine in pediatric patients with chemotherapy-induced mucositis was investigated during a randomized controlled trial versus placebo.

Several analytical methods based on high performance liquid chromatography (HPLC) using ultraviolet (UV) or mass spectrometry (MS) detection have been reported for the analysis of plasma ketamine and norketamine in adults [13–19]. The objective of this study was to develop and validate a sensitive method for the determination of ketamine and norketamine by micro-liquid chromatography–mass spectrometry (LC-MS) in plasma samples collected during this clinical trial.

2. Experimental

2.1. Chemicals and reagent

Solutions of (+/−) ketamine·HCl 1 mg/ml, (+/−) norketamine·HCl 1 mg/ml, (+/−) ketamine D4·HCl 100 μg/ml and (+/−) norketamine D4·HCl 100 μg/ml were obtained from LGC Promochem® (Mohlseim, France). Acetonitrile and methanol (HPLC-grade) were purchased from Prolabo® (Fontenay-sous-bois, France), formic acid and ammoniac 25% from Merck® (Fontenay-sous-bois, France), hydrochloric acid (HCl) 1N from Carlo Erba Reagenti® (Val de Reuil, France), and ammonium carbonate, dibasic sodium phosphate,

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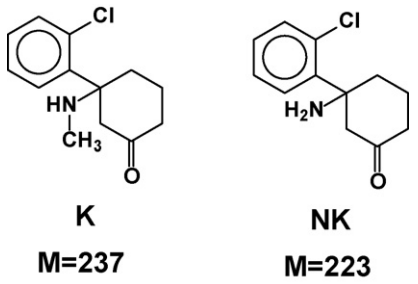

Fig. 1. Chemical structure of ketamine and norketamine.

and monobasic potassium phosphate from Prolabo® (Fontenay-sous-bois, France). Ultra-pure water was obtained using a Milli-Q water purification system (Millipore®, Saint-Quentin-en-Yvelines, France). Drug free human plasma was received from the French blood center EFS (Etablissement Français du Sang). Solutions of drug free plasma used for the method development were obtained by pooling plasma from six donors.

2.2. Apparatus

The chromatographic PerkinElmer series 200 system consisted of two pumps, a vacuum degasser, a column oven, a thermostated autosampler. An IC-400-VAR flow-rate splitter (LC Packings®, Amsterdam, The Netherlands) was used to stabilize the flow rate at 45 μl/min. The mass spectrometer was an API 150 EX (Applied Biosystem®, Courtaboeuf, France) equipped with an electrospray source (ESI) Turbo Ionspray®. Data analysis was performed using Analyst Software 1.4.2.

Solid phase extraction was performed using a vacuum manifold Varian® (Les Ulis, France).

Infusion tests were performed using a syringe pump, Harvard Apparatus® pump 11 (Les Ulis, France) at a flow rate of 10 μl/min.

2.3. Investigation of detection parameters

MS parameters to be optimized were the declustering potential (DP: 0–200 V), the focusing potential (FP: 50–400 V) and the entrance potential (EP: −12 to 1 V), involved in the method sensibility because they are responsible of the ions focusing through the analyzer and the in-source declustering. LC-MS parameters, such as the curtain gas (CUR: 6–15 Arbitrary Unit (AU)), the nebulizer gas (NEB: 8–15 AU), the source temperature (TEM: 0–500 °C) and the auxiliary gas (AUX: 4–8 l/min) were explored.

2.4. Chromatographic conditions

Separation was achieved using a Nucleodur® C18 guard column (5 mm × 1 mm I.D., 3 μm) and a Nucleodur® C18 micro-column (150 mm × 1 mm I.D., 3 μm) purchased from Macherey-Nagel® (Hoerdt, France). The mobile phase consisted of formic acid 0.1% (pH 2.5) and acetonitrile at a flow-rate of 45 μl/min using a flow splitter. The column temperature was kept at 40 °C and the injection volume was 5 μl.

Determination of K and NK was based on the internal standard method, using NKD4 as the internal standard (IS). Mass spectra were recorded in infusion mode using full scan in positive ion mode, with a scan range from *m*/*z* 150 to 250. In liquid chromatography-mass spectrometry (LC-MS) and flow injection analysis (FIA) mode, single ion monitoring (SIM) mode was used to quantitate the target compounds at *m*/*z* 238.2, *m*/*z* 224.2 and *m*/*z* 228.2, respectively, for K, NK and NKD4.

2.5. Preparation of solutions

Stock solutions of K 10 μg/ml, NK 10 μg/ml and NKD4 1 μg/ml were prepared in ultra-pure water. These solutions were divided into 1 ml aliquots and stored at −20 °C.

Calibrator and quality control (QC) solutions were prepared by dilution with an appropriate volume of drug free plasma and were stored in 100 μl aliquots at −20 °C not more than 10 weeks. Indeed, Hijazi et al. [20] reported that K and NK were stable in plasma samples stored at −20 °C for 10 weeks. The six points of the calibration curve were 5, 50, 100, 220, 300, and 500 ng/ml, and the four QC concentrations were 30, 120, 270, 450 ng/ml.

2.6. Extraction procedure

Different extraction cartridges were tested: Sep-Pak® C18 1 ml/50 mg (Waters®, Guyancourt, France), Bond Elut® LMS 1 ml/50 mg (Varian®, Les Ulis, France), Oasis® HLB 1 ml/30 mg (Waters®, Guyancourt, France) and Absolut® Nexus 1 ml/30 mg (Varian®, Les Ulis, France) and Oasis® MCX 1 ml/30 mg (Waters®, Guyancourt, France).

Eluents were evaporated to dryness under a gentle nitrogen stream. Dry residues were redissolved in 100 μl of formic acid 0.1%.

2.7. Validation

Assay specificity was checked by analyzing a pool of six different drug free plasma samples using full scan mode (*m*/*z* 50–1000).

Linearity study was performed in triplicate between 5 and 500 ng/ml. Calibration curves of K and NK were established using peak area ratio (analytes/IS) versus plasma concentration. Slope, intercept and correlation coefficient (r^2) were obtained by linear regression analysis.

Matrix effects were investigated by two different methods. The first one is the post-column infusion method. In practice, a standard solution containing the three analytes (K, NK, NKD4) was infused while an extracted drug free plasma was simultaneously injected into the analytical column. The second method is the post-extraction spike method, which assesses matrix effects by measuring the ionization recovery, determined by the ratio of the peak area of analytes spiked after extraction to the peak area of standards solutions at the same concentration.

Extraction efficiency is first measured by absolute recovery, defined as the ratio of the peak area of analytes spiked before extraction to the peak area of standards solutions at the same concentration. Then extraction recovery was calculated by the ratio of absolute recovery to ionization recovery.

Intra- and inter-day validation study for precision and accuracy was performed using the results obtained with QC samples over six validation days. Mean measured concentrations and their standard deviations (S.D.) at the four QC concentration levels were calculated. Method accuracy was determined by calculating recoveries expressed as [(mean measured concentration)/(nominal concentration) × 100]. Precision was reported as relative standard deviation (R.S.D.) expressed as (S.D./mean) × 100. Limit of quantitation (LOQ) was determined as a signal/noise ratio equal to 10. Acceptance criteria for accuracy and precision were: mean recoveries between 85 and 115% (except 80–120% for LOQ) and R.S.D. lower than 15% (except 20% for LOQ).

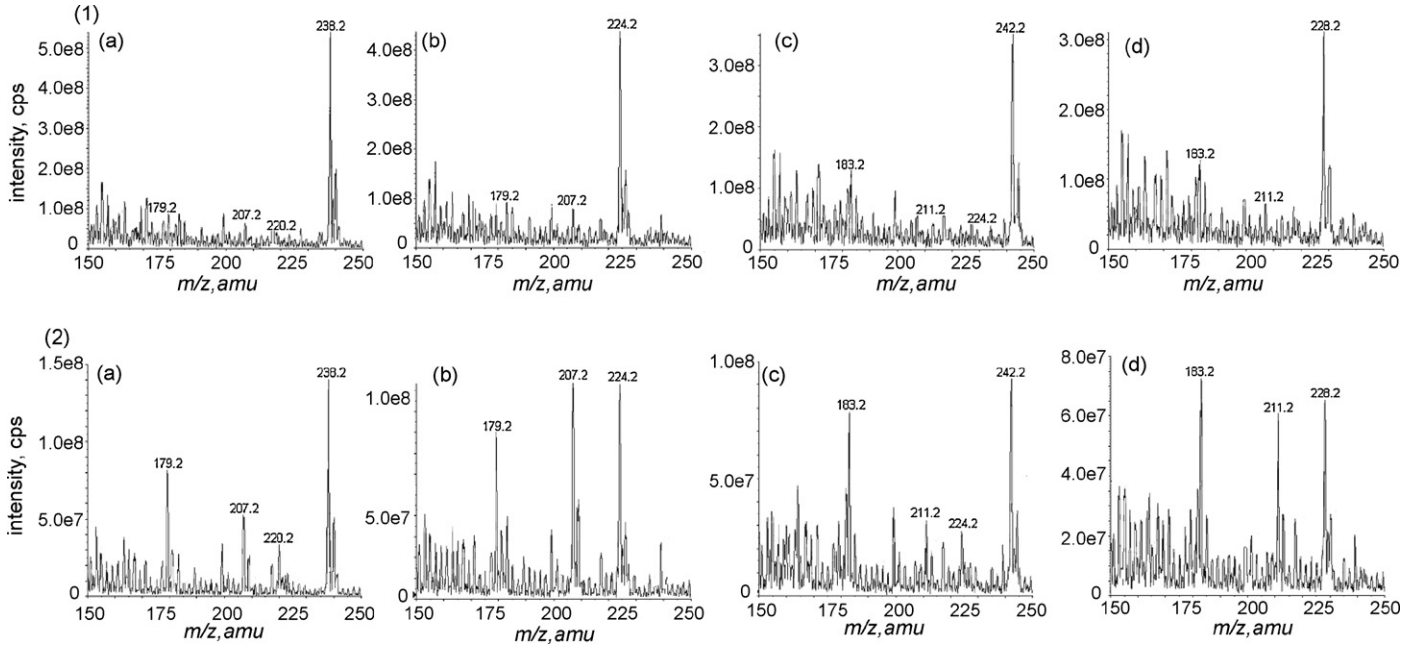

Fig. 2. (1) Mass spectra of (a) K, (b) NK, (c) KD4, and (d) NKD4 (DP = 24 V, FP = 200 V, EP = −5 V). (2) Mass spectra of (a) K, (b) NK, (c) KD4, and (d) NKD4 (DP = 80 V, FP = 200 V, EP = −5 V).

3. Results and discussion

3.1. Detection parameters

3.1.1. MS parameters

Standard solutions of K, NK, KD4 and NKD4 (1 μg/ml) were injected by infusion mode. Signal recording with a simultaneous ramp of DP, EP and FP allowed their optimization. The optimum values based on the maximum signal of ketamine were: DP = 24 V, FP = 200 V, EP = −5 V.

3.1.2. LC/MS parameters

Standard solutions of K, NK, KD4 and NKD4 (100 ng/ml) were injected using the FIA mode. The best values based on the higher signal/noise ratio for K and NK were: TEM = 150 °C, CUR = 10 AU, NEB = 13 AU, AUX = 6 l/min.

3.1.3. Mass spectra

Mass spectra were recorded by infusion of solutions of K, NK, KD4 and NKD4 (1 μg/ml), using full scan (*m*/*z* 150–250) in positive ion mode, with the previously retained conditions (DP = 24 V, FP = 200 V, EP = −5 V). Molecular ions peaks were respectively at *m*/*z* 238.2 for K, 224.2 for NK, 242.2 for KD4 and 228.2 for NKD4 (Fig. 4).

Analysis of the mass spectra showed an in-source declustering. The same mass spectra recorded with a DP = 80 V showed a higher proportion of these fragments at *m*/*z* 220.2, 207.2 and 179.2 for K, 207.2 and 179.2 for NK, 224.2, 211.2, and 183.2 for KD4 and 211.2 and 183.2 for NKD4 (Fig. 2). Those results showed that a KD4 fragment had the same *m*/*z* value as the molecular ion of NK (*m*/*z* 224.2). Consequently, only NKD4 was used as internal standard in order to prevent any interference.

3.2. Chromatographic conditions

3.2.1. Analytical column

In this study, we chose to use a C18 stationary phase, like most techniques for determination of K and NK by HPLC previously reported [7–10]. However, these separations were developed with a conventional column (I.D. 4–4.6 mm) using a high sample volume (0.5–1 ml), which is not adapted to the analysis of pediatric micro-samples [21]. As a consequence, we developed a method by micro-liquid chromatography (I.D. 1 mm). Moreover, the micro-flow rate is more suitable for electrospray detection.

3.2.2. Mobile phase

The aqueous phase was acidified with formic acid 0.1% because of its high compatibility with MS detection. The organic solvent was acetonitrile (AcN). Different proportions of mobile phase were tested to obtain an acceptable resolution (Rs > 1) while keeping a short time of analysis (Table 1).

The best separation between K and NK (Rs = 2.6) was obtained with a mobile phase composed of formic acid 0.1%/AcN 97/3 (v/v). The low proportion of AcN resulted in a long time of analysis (40 min) and was not optimal for the C18 column. Increasing AcN proportion resulted in a decrease of the time of analysis and the resolution. Separation obtained with a mobile phase composed of formic acid 0.1%/AcN 90/10 (v/v) was characterized by the lowest resolution (Rs = 1.2) and by the shortest time of analysis (<10 min). We considered these conditions the best adapted to the stationary phase. For these reasons, the mobile phase consisted of formic acid 0.1%/AcN 90/10 (v/v).

A chromatogram of a standard solution containing 100 ng/ml of K, NK and NKD4 is presented (Fig. 3). Under these conditions, retention times were 7.0 and 7.7 min, respectively for NK and K.

Table 1
Investigation of composition of mobile phase

Composition of mobile phase formic acid 0.1%/AcN (v/v)	tr (min)		Rs
	K	NK	
97/3	36.2	31.3	2.6
95/5	20.9	18.0	1.9
93/7	12.5	11.2	1.8
90/10	7.7	7.0	1.2

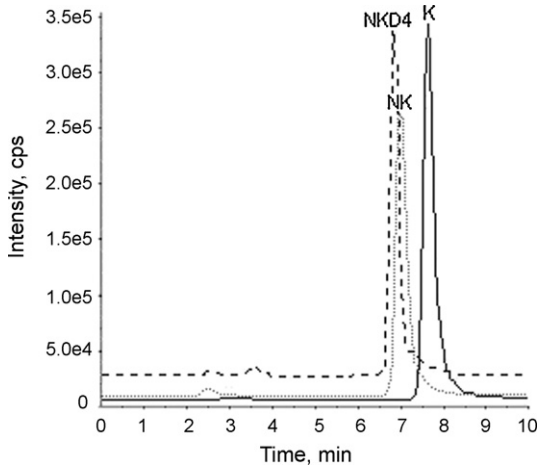

Fig. 3. Chromatogram of a standard solution of K, NK and NKD4 at 100 ng/ml using SIM mode at *m*/*z* 238.2 (K), 224.2 (NK), and 228.2 (NKD4). (—) K; (···) NK; (- - -) NKD4.

3.3. Extraction

Different extraction methods with five SPE cartridges were tested. Extraction efficiency (absolute recovery) of K, NK and NKD4 was measured by three determinations of the four QC concentration levels. Table 2 shows extraction procedures and mean recoveries and their standard deviations. Sep-Pak® C18 (Waters®), Bond Elut® LMS (Varian®), Oasis® HLB (Waters®) and Absolut® Nexus (Varian®) showed low recoveries (<59.0%). The best recoveries, ranging from 86.4 to 87.9%, were obtained using the cation-exchange cartridges Oasis® MCX (Waters®).

3.4. Validation

3.4.1. Specificity

The chromatogram of an extracted drug free plasma obtained using full scan mode (*m*/*z* 50–1000) showed that there were no interfering compounds at the expected retention time of K, NK and NKD4 between 6 and 9 min.

3.4.2. Linearity

Linearity was studied by a triplicate analysis of the six points of the calibration curve. The method was found to be linear over the range 5–500 ng/ml for both analytes. Mean linear regression equations were $y = 0.0118x - 0.0006$, $r^2 = 0.9997$ for K and $y = 0.0086x + 0.0035$, $r^2 = 0.9997$ for NK, where y represents the peak area ratio (analytes/IS) and x plasma concentration in ng/ml. Fischer test showed that slopes were significantly different from zero ($p < 0.001$) whereas Students' t-test showed that intercepts were not significantly different from zero ($\alpha = 5\%$).

3.4.3. Matrix effects

Matrix effects are generally due to co-eluted matrix compounds that may influence the ionization of the target analytes and potentially reduces the analytes signals by ion suppression. Different methods allow determining and quantitating these matrix effects [22,23].

Post-column infusion method is a qualitative way to evaluate matrix effects. Fig. 4 shows a steady signal during the infusion of the standard solution (K, NK, NKD4 at 1 μg/ml) in SIM mode. After injection of an extracted drug free plasma, a signal decrease was observed. This is due to the matrix ion suppression effects caused by extracted plasma sample compounds eluting from the

Table 2
Different solid phase extraction (SPE) procedures tested and absolute recoveries (mean ± S.D.%)

SPE cartridges	Procedures				Absolute recoveries ($n = 3$)		
	Conditioning	Load sample	Washing	Eluting	K	NK	NKD4
Sep-Pak® C18	1 ml methanol, 1 ml ultra-pure water, 1 ml carbonate buffer 10 mM pH 9.2	100 μl spiked plasma, 100 μl carbonate buffer 10 mM pH 9.2	1 ml carbonate buffer 10 mM pH 9.2	1 ml methanol [17,18]	19.3 ± 2.5	24.9 ± 2.4	25.3 ± 3.6
Bond Elut® LMS					39.0 ± 3.1	47.5 ± 2.6	46.8 ± 2.5
Oasis® HLB					44.2 ± 4.5	54.9 ± 5.5	56.4 ± 5.2
Absolut® Nexus	1 ml methanol, 1 ml ultra-pure water, 1 ml phosphate buffer pH 6.6	100 μl spiked plasma, 100 μl phosphate buffer pH 6.6	1 ml phosphate buffer pH 6.6	1 ml methanol	47.0 ± 2.0	57.8 ± 2.2	59.0 ± 0.7
Oasis® MCX	1 ml methanol, 1 ml ultra-pure water	100 μl spiked plasma, 50 μl HCl 0.5N	1 ml HCl 0.1N, 1 ml methanol	1 ml methanol, 5% NH_4OH [19]	87.9 ± 4.2	87.7 ± 3.8	86.4 ± 4.1

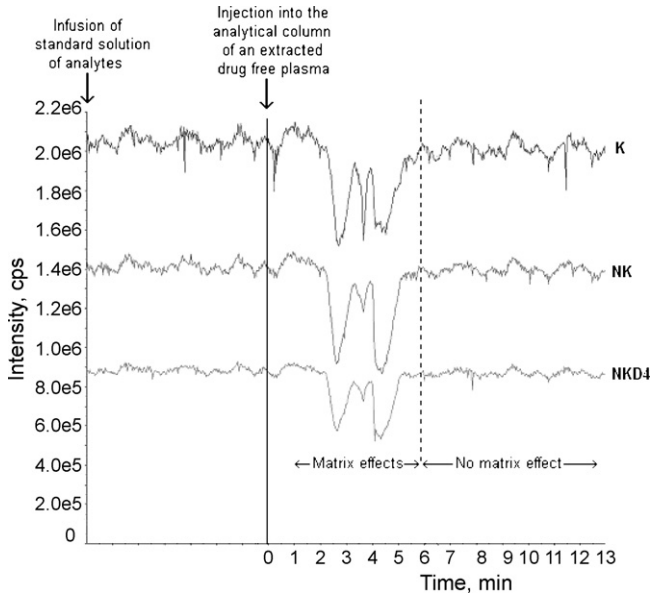

Fig. 4. Signal recording after infusion of a standard solution of K, NK and NKD4 (SIM mode at *m/z* 238.2 (K), *m/z* 224.2 (NK) and *m/z* 228.2 (NKD4)), followed by an injection of an extracted drug free plasma.

column. However, at the expected retention time of K and NK (7–8 min) signal intensity went back up to its initial value, indicating that there was no significant matrix effect observed after 6 min.

Post-extraction spike method quantitatively assesses matrix effects by measuring the ionization efficiency (ratio of the peak area of analytes spiked after extraction to standards solutions at identical concentrations) [23]. Mean ionization recoveries calculated by six determinations of the four QC concentrations were 98.3 ± 2.1% for K, 98.8 ± 1.2% for NK and 98.9 ± 1.3% for NKD4. Students' *t*-test showed that these values were not significantly different from 100% (α = 5%), which confirmed that there was no statistically significant matrix effect.

3.4.4. Extraction efficiency

Absolute recovery (ratio of the peak area of analytes spiked before extraction to standards solution at identical concentrations) calculated by six determinations of the four QC concentrations, ranged from 84.8 to 89.8% (Table 3).

Extraction recovery was determined by the ratio of absolute recovery to ionization recovery. Nevertheless, we previously demonstrated that ionization recoveries were not significantly different from 100% (α = 5%), so extraction recoveries were considered equal to absolute recoveries.

Table 3
Absolute recoveries (mean ± S.D.%) (*n* = 6)

QC concentration (ng/ml)	K	NK	NKD4
30	88.0 ± 4.6	87.6 ± 1.9	84.9 ± 3.2
120	88.3 ± 2.2	87.4 ± 3.9	86.4 ± 3.0
270	89.0 ± 3.8	86.1 ± 2.8	84.8 ± 5.3
450	86.2 ± 5.9	89.8 ± 5.6	89.3 ± 3.5

3.4.5. Intra- and inter-day

A summary of intra- and inter-day precision and accuracy of K and NK is given in Table 4. In this study, intra-day precision was less than 1.9% for both K and NK. Inter-day precision was less than 1.4% for K and 1.2% for NK. The acceptance criteria of accuracy (mean recoveries between 85 and 115%) were met in all cases.

3.4.6. LOQ

This micro-LC/MS method, using a plasma sample volume of 100 μl and an injection volume of 5 μl showed a LOQ of 4 ng/ml for both K and NK. Among the several analytical methods for the determination of plasma K and NK [7,9,10,13], the lowest LOQ reported was 1 ng/ml for both K and NK obtained by LC-MS [13]. However, these methods, developed with a conventional column (I.D. 4–4.6 mm), used a high sample plasma volume (0.5–1 ml) and a high injection volume (from 20 to 70 μl).

4. Application to clinical samples

Forty-five children were included in the randomized controlled trial versus placebo to evaluate the impact of low-dose ketamine (continuous infusion of 1 or 2 $\mu g\,kg^{-1}\,min^{-1}$) on pain in chemotherapy-induced mucositis in children treated with morphine by patient-controlled analgesia (PCA). Blood samples were collected at steady-state and immediately centrifuged and stored at −20 °C until analysis.

One hundred and seventy-seven plasma samples were analyzed in duplicate. In the ketamine group (*n* = 23), plasma concentrations ranged from 8 to over 5000 ng/ml and from 5 to 350 ng/ml for K and NK, respectively. For samples with a ketamine concentration > 500 ng/ml, appropriate dilutions (maximum dilution of 1/10) with drug free plasma were performed. A representative chromatogram with plasma concentrations of 174 and 282 ng/ml for K and NK, respectively is represented in Fig. 5. In the placebo group (*n* = 22) levels of K and NK were undetectable (<4 ng/ml) and no interference was detected at the corresponding retention time.

Table 4
Intra- and inter-day precision and accuracy of K and NK (*n* = 6)

QC nominal concentration (ng/ml)	Intra-day			Inter-day		
	Measured concentration mean ± S.D. (ng/ml)	Mean recovery (%)	R.S.D. (%)	Measured concentration mean ± S.D. (ng/ml)	Mean recovery (%)	R.S.D. (%)
K						
30	29.4 ± 0.6	98.1	1.9	30.5 ± 0.4	101.7	1.4
120	121.2 ± 1.4	101.0	1.2	121.0 ± 0.8	100.8	0.7
270	270.9 ± 1.4	100.3	0.5	270.9 ± 2.2	100.3	0.8
450	452.1 ± 1.2	100.5	0.3	451.1 ± 2.3	100.2	0.5
NK						
30	29.8 ± 0.6	99.5	1.9	30.3 ± 0.4	101.0	1.2
120	119.5 ± 0.9	99.6	0.8	120.3 ± 1.3	100.2	1.1
270	270.0 ± 1.3	100.0	0.5	269.5 ± 1.3	99.8	0.5
450	449.6 ± 1.4	99.9	0.3	451.6 ± 3.3	100.3	0.7

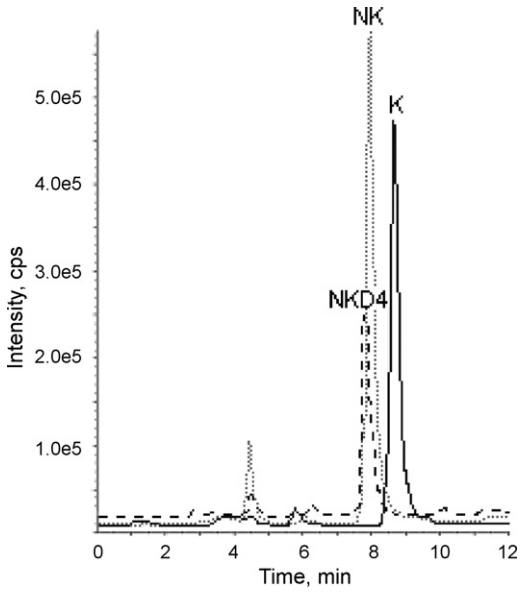

Fig. 5. Chromatogram of a plasma sample from a patient included in the clinical trial (SIM mode at *m*/*z* 238.2 (K), 224.2 (NK), and 228.2 (NKD4)). (—) K; (···) NK; (- - -) NKD4.

5. Conclusion

A simple, specific and sensitive micro-LC/MS method was developed and validated for the determination of K and NK concentrations in pediatric plasma samples. Good linearity, precision and accuracy were achieved. With a micro-column, only 100 μl of plasma were required for analysis, making this method adapted to pediatric patients. One hundred and seventy-seven plasma samples were analyzed and important interindividual variability of drug concentrations was observed. In addition, high ketamine plasma concentrations were measured, compared to the target concentration of 150 ng/ml reported in the literature [8]. Our data will allow to investigate the variability in ketamine pharmacokinetics and evaluate the benefits of low-dose ketamine associated with morphine in the clinical setting.

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