Assessment of stability of ketamine-xylazine preparations with or without acepromazine using high performance liquid chromatography-mass spectrometry

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Abstract

The objective of this study was to evaluate the stability of 3 distinct preparations of ketamine and xylazine, with or without acepromazine, stored at room temperature or at 4°C for 1, 2, and 3 mo. Drug concentrations were compared to fresh solutions, using a high performance liquid chromatography-mass spectrometry/selected-ion monitoring (HPLC-MS/SIM) assay. The concentrations of ketamine and xylazine, diluted in physiological saline, did not change over time at room temperature or at 4°C. However, acepromazine concentrations decreased over time when stored at room temperature. In contrast, undiluted ketamine-xylazine preparations gradually decreased in concentration when stored at room temperature. All of the drug concentrations remained above 90% of their original concentration when stored at 4°C. In conclusion, when diluted in physiological saline, ketamine-xylazine cocktails can be stored for 3 mo, whereas undiluted cocktails can lose efficacy over 3 mo at room temperature. Storage at 4°C could preserve drug stability.

Résumé

Cette étude vise à évaluer la stabilité de trois préparations de kétamine et xylazine avec ou sans acépromazine gardées à température pièce, ou à 4°C, pour 1, 2 et 3 mois. Les concentrations des drogues ont été comparées à des solutions fraiches, toutes analysées par HPLC-MS/SIM. Les concentrations de kétamine et xylazine, des solutions diluées dans la saline physiologique, sont restées constantes indépendamment du temps et de la température de conservation, par contre la concentration d'acépromazine a diminué dans les préparations gardées à température pièce. En contraste, les concentrations des préparations pures de kétamine et xylazine conservées à température pièce ont diminué avec le temps. En conclusion, la kétamine et la xylazine en cocktail avec du salin peuvent être utilisés pour une période de 3 mois, par contre, conservées à température pièce, les concentrations diminuent progressivement en préparation pure. La conservation des préparations à 4°C favorise la stabilité des drogues.

(Traduit par les auteurs)

Inhaled anesthetic agents are the most commonly used anesthesia method in the field of laboratory animal research. The combination of ketamine and xylazine, with or without acepromazine, is the first choice of injectable drugs for rodent species (1–3). Their use has replaced the previously widely used pentobarbital (4). Besides requiring minimal equipment and training, these drug combinations provide an effective and safe plane of anesthesia. As a dissociative agent, ketamine induces immobilization, analgesia, and hypotension, while xylazine complements these effects with muscle relaxation and further analgesic properties. The effect is further potentiated by adding the tranquilizer acepromazine, which provides sedation and central nervous system depression, thus producing a multimodal anesthesia approach and allowing the use of lower doses of ketamine and xylazine (1–3,5).

Despite this, the combined formulations necessary for administering to rodents are not commercially available. Thus, in-house formulations and dilutions of drugs are prepared in physiological saline or sterile water according to standard operating procedures (SOPs) established by institutional laboratory animal veterinarians and animal health technicians. The expiration dates, or beyond-use dates, of these drug formulations vary widely according to the institution's policies. An Internet survey of SOPs at various universities demonstrates a wide range of recommended expiration dates, from 7 d to 6 mo, with a 1 mo expiration being most commonly used (6). While it might be safer to err on the side of caution and follow the shorter time frames to ensure drug efficacy, too short a usage date can be frustrating for users, as it is costly, time-consuming, and wasteful without proper justification. In fact, very little information is available on the stability of these drugs when combined and/or diluted. It has previously been demonstrated that concentrations of ketamine, xylazine, and acepromazine from both diluted and non-diluted preparations analyzed by high performance liquid chromatography (HPLC) remained stable for at least 6 mo after mixing, if stored at room temperature in a dark place (6). However, efficacy tested in animals decreased progressively with older solutions. The objective of the present study was to evaluate the stability of 3 specific mice or rat formulations over time, when stored in the dark, at room temperature or at 4°C, using a state-of-the-art analytical method based on high performance liquid chromatography-mass spectrometry/selected-ion monitoring (HPLC-MS/SIM).

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Table I. Drug stability results over a period of 3 mo obtained for formulation A [1 mL of ketamine (100 mg/mL), 0.1 mL of xylazine (20 mg/mL), and 8.9 mL of physiological saline]

	Room temperature					
Time	Ketamine			Xylazine		
(mo)	% remaining	SD	%CV	% remaining	SD	%CV
0	100.00	0.26	0.3%	100.00	1.60	1.6%
1	98.56	0.36	0.4%	108.10	7.28	6.7%
2	105.17	1.41	1.3%	111.08	9.68	8.7%
3	106.24	1.21	1.1%	100.16	2.58	2.6%
4°C						
0	100.00	2.42	2.4%	100.00	2.51	2.5%
1	97.75	1.78	1.8%	98.34	4.32	4.4%
2	104.11	2.17	2.1%	97.30	0.76	0.8%
3	100.00	1.46	1.5%	96.83	0.95	1.0%

SD — standard deviation; CV — coefficient of variation.

Table II. Drug stability results over a period of 3 mo obtained for Formulation B [4 mL of ketamine (100 mg/mL) and 1 mL of xylazine (20 mg/mL)]

	Room temperature					
Time	Ketamine			Xylazine		
(mo)	% remaining	SD	%CV	% remaining	SD	%CV
0	100.00	1.66	1.7%	100.00	7.57	7.6%
1	94.72	0.60	0.6%	**89.89***	0.67	0.7%
2	**87.50***	2.04	2.3%	**86.85***	2.24	2.6%
3	**88.73***	0.22	0.2%	**83.19***	3.03	3.7%
4°C						
0	100.00	1.60	1.6%	100.00	4.89	4.9%
1	101.72	1.67	1.6%	107.27	6.50	6.1%
2	105.79	1.00	0.9%	115.17	6.04	5.2%
3	105.45	0.35	0.3%	109.70	4.94	4.5%

* Concentration remaining $< 90\%$ of initial concentration.

$P < 0.0001$ [one-way analysis of variation (ANOVA) with post-hoc Dunnett's test].

SD — standard deviation; CV — coefficient of variation.

Ketamine from Bioniche (Vetalar; Belleville, Ontario), xylazine from Bayer HealthCare (Rompun; Toronto, Ontario), and acepromazine from Boehringer Ingelheim (Atravet 10; Burlington, Ontario) were used for these experiments. The internal standard, d_4-ketamine, was obtained from Cerilliant products (Round Rock, Texas, USA). Other chemicals, including acetonitrile, methanol, and formic acid, were purchased from Fisher Scientific (Ottawa, Ontario).

Three distinct formulations were tested at room temperature and at 4°C for periods of 1, 2, and 3 mo and were then compared to fresh solutions, i.e., initial concentration was normalized to 100% for comparison purposes. Formulation A (typical mouse cocktail) was composed of 1 mL of ketamine (100 mg/mL), 0.1 mL of xylazine (20 mg/mL), and completed with 8.9 mL of physiological saline, for a total of 10 mL. The final concentrations were 10 mg/mL and 0.2 mg/mL for ketamine and xylazine, respectively. Formulation B (typical rat cocktail) was composed of 4 mL of ketamine (100 mg/mL) and 1 mL of xylazine (20 mg/mL), for a total of 5 mL. The final concentrations were 80 mg/mL and 4 mg/mL for ketamine and xylazine, respectively. Formulation C (mouse cocktail with acepromazine) was composed of 1 mL of ketamine (100 mg/mL), 1 mL of xylazine (20 mg/mL), 0.3 mL of acepromazine (10 mg/mL), and 7.7 mL of physiological saline, for a total of 10 mL. The final concentrations were 10 mg/mL, 2 mg/mL, and 0.3 mg/mL for ketamine, xylazine, and acepromazine, respectively. All the solutions were prepared at the same time to avoid possible differences during preparation. Two identical samples of each dilution were prepared and inverted several times to ensure optimal drug mixture. One aliquot was kept at 4°C in a locked refrigerator and 1 aliquot was kept in a safe location at room temperature for the duration of the experiment. The mixtures were tested at selected time points of 1, 2, and 3 mo after initial preparation.

The concentrations of ketamine, xylazine, and acepromazine were determined using an HPLC-MS/SIM assay. Briefly, 10 μL of each formulation (A, B, and C) was mixed with 10.0 mL of d_4-ketamine internal standard solution (10 μg/mL of d_4-ketamine in a mixture of 60:20:20 acetonitrile:methanol:water). The samples were then mixed vigorously and 300 μL of the solution was transferred into an injection vial. Two microliters of each sample was injected using a Thermo Accela HPLC (Thermo Scientific, San José, California, USA) onto a Thermo Hypersil Gold Phenyl 50 × 1 mm column (3 μm) with flow rate of 50 μL/min. The mobile phase consisted of a mixture of acetonitrile, methanol, water, and formic acid at a ratio of 60:20:20:0.4, respectively.

The Thermo LTQ-XL mass spectrometer (Thermo Scientific) was interfaced with the HPLC system using a pneumatic-assisted electrospray ion source. Linear ion trap instruments typically have unit mass resolution throughout the mass range. The instrument was calibrated and the resolution was set at 0.5 to 0.7 Da at full width at half maximum (FWHM). The sheath gas was set at 15 units and the ESI electrode was set at 4000 V in positive mode. The capillary temperature was set at 300°C and the capillary voltage at 15 V. The instrument was operating in selected-ion monitoring (SIM) at m/z 238.1, 221.2, 327.3, and 242.1 for ketamine, xylazine, acepromazine, and d_4-ketamine, respectively. All scan events were acquired with a 10 ms maximum injection time. The calibration lines were constructed from the peak-area ratios for all drugs, as well as the internal standard (d_4-ketamine). Regression analyses were conducted with GraphPad Prism (Version 6.0d) software (La Jolla, California, USA) using a linear curve-fitting module with an estimation of the goodness of fit. The analytical range was 1 to 100 μg/mL for ketamine and 0.1 to 20 μg/mL for xylazine and acepromazine. The sample concentrations were interpolated from the standard curve. The precision percent coefficient of variation (%CV) obtained ranged from 0.2% to 2.7% and the accuracy (%NOMINAL) observed ranged from 97.0% to 102.8% for all 3 drugs. Each analytical value was based on triplicate determination of each sample.

In Formulation A (diluted mouse cocktail), ketamine and xylazine remained stable over the course of 3 mo, both at room temperature and at 4°C (Table I). The concentrations remained relatively close to the original concentrations. In contrast, in Formulation B (ketamine-xylazine rat cocktail), both ketamine

Table III. Drug stability results over 3 mo obtained for formulation C [1 mL of ketamine (100 mg/mL), 1 mL of xylazine (20 mg/mL), 0.3 mL of acepromazine (10 mg/mL), and 7.7 mL of sterile physiological saline]

	Room temperature								
Time	Ketamine			Xylazine			Acepromazine		
(mo)	% remaining	SD	%CV	% remaining	SD	%CV	% remaining	SD	%CV
0	100.00	1.78	1.8%	100.00	2.92	2.9%	100.00	2.92	2.9%
1	103.52	1.08	1.0%	99.53	0.85	0.9%	**93.98***	1.74	1.9%
2	104.66	1.34	1.3%	98.61	0.42	0.4%	**88.06***	0.32	0.4%
3	102.92	2.28	2.2%	100.18	2.86	2.9%	**82.92***	4.23	5.1%
4°C									
0	100.00	6.12	6.1%	100.00	1.01	1.0%	100.00	1.01	1.0%
1	95.63	2.62	2.7%	97.90	1.45	1.5%	95.83	3.20	3.3%
2	94.67	0.74	0.8%	96.38	0.38	0.4%	**93.61***	2.77	3.0%
3	97.52	6.30	6.5%	95.68	2.20	2.3%	**91.47***	2.10	2.3%

* Concentration remaining $<$ 90% of initial concentration.

$P < 0.0001$ [one-way analysis of vaiance (ANOVA) with post-hoc Dunnett's test].

SD — standard deviation; CV — coefficient of variation.

and xylazine gradually decreased in concentration when stored at room temperature (Table II). Xylazine concentration decreased to 83% (± 3) from its original concentration after 3 mo, while ketamine decreased to 88% (± 0.2) in the same period. It was observed that drug concentrations continued to decrease during the 3-month period. Using a 1-phase decay model, the observed half-lives in Formulation B were 15.5 mo (± 0.6) and 11.5 mo (± 0.1) for ketamine and xylazine, respectively. These decreases in drug concentrations were not observed when the solution was stored at 4°C.

Finally, in Formulation C, ketamine, xylazine, and acepromazine diluted in 0.9% saline showed differences in temperature-sensitive stability (Table III). Ketamine and xylazine remained stable over a 3-mo period at both room temperature and at 4°C, which was similar to results obtained for Formulation A. However, acepromazine concentrations decreased significantly at room temperature and at 4°C over a period of 3 mo. At room temperature, the acepromazine concentration decreased to 93% (± 1.7), 88% (± 0.3), and 82% (± 4.2) of the original concentration after 1, 2, and 3 mo, respectively. When Formulation C was stored at 4°C for 3 mo, however, the remaining concentration of acepromazine represents 91% (± 2) of the original concentration. Again, the decrease in drug concentrations was continuously observed during the 3-month period. Using a 1-phase decay model, the observed half-life of acepromizine in Formulation C was 11.1 mo (± 0.7) and 23.6 mo (± 0.6) at room temperature and at 4°C, respectively.

This study evaluated the stability of various preparations of mouse and rat anesthetic cocktails over time and at different storage temperatures with an HPLC-MS/SIM assay. These anesthetic preparations are part of everyday routine in lab animal research and the use of precise beyond-use dates can significantly influence the quality of the anesthesia (7). The advantages of using ketamine combined with xylazine as injectable anesthetics in rodents are multifold and include a reliable dose-dependent loss of consciousness and muscle relaxation, without losing spontaneous ventilation. In addition, the physical compatibility of these drugs and their ability to be combined in a single prepared bottle help to reduce user error. The use of xylazine also provides a reversal possibility with atipamezole or yohimbine, both of which are shown to hasten recovery (8). Acepromazine is often added for longer, more invasive surgical procedures in mice, providing 45 to 60 min of anesthesia.

In the present study, we evaluated drug preparations suitable for use in mice and rats, given that 0.01 mL/g, i.e., volume of solution per body weight, of Formulation A or C can be used in mice and 0.001 mL/g of Formulation B can be used in rats. Most facilities settle on an expiration date that is acceptable to the researchers as well as safe for the animal, but often relies solely on mere historical experience. Prolonging the beyond-use dates of prepared solutions can decrease costs to the researchers as well as decrease the amount of wasted, unused drugs. The disadvantages of using multi-use bottles for longer periods, however, are decreased drug efficacy, as well as increased potential for contamination.

Using the threshold value of 90% of remaining concentration as an acceptable concentration for the anesthetic drug to be used on live animals as stated in a previous report (9), we observed that all of the drug concentrations remained above 90% of their original concentration when kept at 4°C for 3 mo. Interestingly, diluting ketamine and xylazine in 0.9% physiologic sterile saline rendered the solution more stable than the non-diluted anesthetic drug cocktail. Similar results have been seen with ibuprofen diluted in saline (10). It is well-known that technical and instrumental errors are a source of variation that needs to be considered. Some experimental values show concentrations above 100% threshold, but it is important to consider that the analytical variability and the values observed were within the figure of merits generally accepted in bioanalytical chemistry.

When stored at room temperature, undiluted ketamine and xylazine, as well as diluted acepromazine in a ketamine-xylazine formulation, decreased to below 90% of the initial concentrations. These findings suggest that ketamine and xylazine cocktails, when diluted in saline, can be stored for 3 mo at room temperature after preparation and that undiluted ketamine-xylazine as well as ketamine-xylazine-acepromazine cocktails can possibly lose efficacy over 3 mo at room temperature, although storage at 4°C may help

preserve the original concentration of these drug preparations. It may be impractical and difficult to store these drugs at 4°C, however, as ketamine is a controlled substance and must be kept under locked conditions at all times. As locked refrigerators are not often found in institutional facilities, it is more practical and realistic to store these drugs at room temperature. Our results explain previous findings that the efficacy of ketamine-xylazine preparations decreases over time (6).

In conclusion, diluted ketamine-xylazine cocktails can be safely stored at room temperature for 3 mo, whereas undiluted ketamine-xylazine or ketamine-xylazine-acepromazine formulations should be used for only a single month.

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