REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 26, No.1 (2025)
http://www.veterinaria.org
Article Received: Revised: Published:

Validation Of Combur-Test10 (Roche) Urinary Dipsticks And Study Of The Effect Of Storage Time And Temperature On Ph, Density, Protein, And Crystal Formation In Equine Urine Samples.

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ABSTRACT
Urinalysis is a diagnostic test routinely performed in veterinary medicine. This test is extremely important in the early diagnosis of kidney disease and diseases of the lower urinary tract, as well as other systemic diseases. It should be performed as soon as possible after collection, as changes in its composition, such as crystal formation, may occur. There are some factors that can influence the formation of these crystals in vitro, including temperature and storage time. If it is not possible to analyze urine immediately, samples should be refrigerated between 2ºC and 8°C in order to preserve some physical and chemical properties of urine, however refrigeration may increase the likelihood of in vitro crystal formation. Routine analysis of urine biochemistry is performed using reactive urine strips. The objective of this study was to validate the Combur-test10 (Roche) urinary dipsticks and to study the effect of storage time and temperature on the parameters: pH, urine density, amount of protein and crystal formation in equine urine samples. Combur-test10 (Roche) urinary dipsticks were found to be unreliable for correctly estimating urinary protein, urine density and pH in horses. Storage time and temperature had no significant effect on the parameters pH, urine density, amount of protein and the number of calcium carbonate crystals during the 24 hours of the study. As for calcium oxalate crystals, their number was stable only up to 12 hours of the study and an increase in their formation was observed over time at both temperatures.

Keywords: crystals, equine, urinary dipsticks, urinary pH, urinary protein, urine specific gravity.

Introduction

The urinalysis is a laboratory test that is easy to perform and can be readily conducted in veterinary clinical practice. Urinalysis serves as a valuable indicator for various diseases, making it a procedure of great importance in clinical laboratories (1, 2).

In the laboratory routine, it is crucial to avoid pre-analytical errors (3). Knowledge and reduction of these pre-analytical errors are essential to achieve more reliable and consistent results (4, 5). The result of urinalysis depends largely on the collection method, which can influence result interpretation (6). The most common collection techniques include spontaneous voiding, catheterization, and cystocentesis (7, 8).

Urine constitutes an unstable mixture in which, following collection, in vitro alterations promptly take place (9,10). Sample analysis should be conducted promptly after collection, preferably within the first two hours thereafter (11, 12). Some authors also recommend analysis between 30, and 60 minutes post-collection (2, 8, 10, 13). Parrah and colleagues (2013) further indicate that urine should be analyzed within 30 minutes post-collection.

In clinical practice, it's not always possible to analyze the sample promptly within the first few hours of collection. In such cases, the sample should be refrigerated between 2ºC and 8°C (10). However, refrigeration also has some drawbacks, including increased urine density and inhibition of certain enzymatic reactions on urine reagent strips (14). Refrigeration can also lead to the formation of struvite and calcium oxalate crystals. Therefore, it's important that refrigerated urine be brought to room temperature, approximately 20 minutes, before analysis (10, 14, 15). Freezing the sample causes destruction of urinary casts and other cellular components. In cases where immediate analysis after collection is not possible, urine should be refrigerated for a maximum period of 6 hours (9) to prevent pre-analytical errors (16).

A complete urinalysis starts by urine macroscopic characteristics evaluation, such as color, turbidity, and urine specific gravity (USG). It also includes a semi-quantitative measurement of chemical parameters using a urine dipstick (2, 17, 18), and a microscopic urinary sediment analysis (8, 19, 20). Urine color and transparency are assessed by observing the sample container against a white background in a well-lit area (21). Normal urine is transparent, yellow, or amber, depending on the urochromes concentration and urobilin, or the animal's pathology (4, 22). Equine´s urine has a wide range of colors, from nearly colorless to brownish-yellow, due to turbidity and mucinous content (23, 24). In equine´s,

urine is typically cloudy and viscous due to the presence of mucus and calcium carbonate crystals (15, 23, 25), as in horses, the kidney is the primary excretory organ for calcium. Mucus is a viscous substance composed of a mixture of glycoproteins and proteoglycans (26). It can also alter the appearance of urine, making it thicker and cloudy (27). Turbidity should be observed immediately after urine collection (28).

Urine specific gravity is determined using a refractometer and requires only a drop of urine (14, 15). It should be measured in the supernatant of urine after centrifugation and not in uncentrifuged urine (as suspended particles may scatter light) (29). The presence of large amounts of protein and glucose can increase the specific gravity of urine (30). Adult horses should be able to concentrate their urine to a specific gravity greater than 1.030 (31), although the reference range is between 1.025 and 1.050. For foals, the reference range varies between 1.001 and 1.025 (32). Routine urine chemical examination typically involves the use of urinary dipsticks (33). These dipsticks are designed for human use, and their results in animal samples should be verified and interpreted carefully. Several studies have investigated the performance of human dipsticks in domestic animals, including dogs, cats, cattle and sheep (34). Urinary pH can be measured using a pH meter, or more easily, through urinary dipsticks (35). The pH meter provides a more precise measurement. Herbivores, with a more alkaline diet, typically exhibit physiological urinary pH between 7.0 and 8.5 due to the presence of soluble calcium bicarbonate (26). Like pH, USG can also be assessed on urinary dipsticks, which detects the concentration of ions in the urine.

Evaluation of urinary sediment allows for the identification of cells, crystals, casts, live organisms such as bacteria or parasites, and other artifacts in urine (3, 36).

Horses often present a varied number of crystals in their urine. Since equine urine is alkaline, many calcium carbonate crystals and calcium oxalate crystals (both mono and dihydrate) are found. Although characteristic of acidic urine, calcium oxalate crystals can also be found in alkaline urine (37). The presence of these crystals in equine urine has no clinical significance (38, 39). In healthy animals, calcium phosphate crystals can also be observed (40). As many crystals form after storage, urinary sediment should be microscopically observed soon after collection to increase the likelihood that the sample represents changes present in the patient (19). In urinary sediment, besides crystals, many other elements can be observed. Mucus threads, which do not have any pathological significance, can also be observed and are seen as irregular structures with a filamentous, long, and thin shape (41).

Although there are very few reports on the validation of the use of human dipsticks in equids (42), to the best of our knowledge, there are no reports on the effect of storage time and temperature on pH, density, protein, and crystal formation in equine urine samples The aim of this work is to validate the Combur-teste10 urinary dipsticks (Roche) and to study storage time and temperature effects on the parameters: pH, density, amount of protein and the formation of crystals in urine samples from equids.

Methods

Urine samples from 16 animals were used, including 12 horses and 4 donkeys. Among the 12 horses, 10 were males and 2 were females, while all donkeys were males. All samples were collected via spontaneous voiding.

Analysis were performed at the Clinical Pathology Laboratory of Veterinary Hospital of the University of Trás-os-Montes and Alto Douro (LPC-HVUTAD). The samples were evaluated by two experienced laboratory technicians independently. First, urine samples were screened for their physical properties (color and turbidity). Urine specific gravity was measured before and after centrifugation using a portable refractometer calibrated daily. Urinary pH was measured with a pH meter calibrated using two buffers. The samples were assessed for pH, USG, and proteins using Combur-test10 (Roche) urinary dipsticks. Urinary sediment was microscopically examined to identify crystals and other constituents. The concentration of urinary protein was determined using a standard colorimetric method on the clinical biochemistry analyzer Respons®920 (Diasys). The method used for measuring urinary protein is spectrophotometry with pyrogallol red method.

For the assessment of the effect of time and temperature, each sample was divided into fourteen aliquots. One aliquot was analyzed immediately after collection (baseline sample), while others were stored at room temperature or refrigerated for 6, 12, and 24 hours. The remaining aliquots were frozen for later analysis of urinary protein. Refrigerated samples were kept for up to 30 minutes after collection, with analyses performed 30 minutes after the specified storage period. Ambient and refrigeration temperatures were recorded daily during the study (figure 1).

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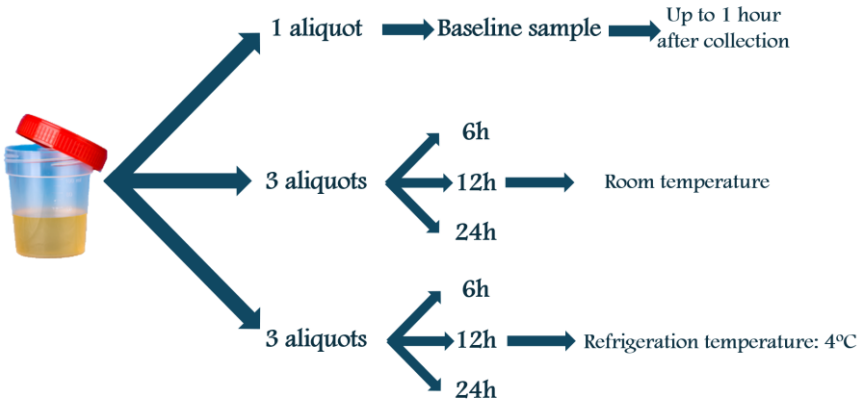

Figure 1 - Schematic representation of the experimental design for the evaluation of time and temperature effects on equine urine analysis.

Data Analysis

The correlation between the semiquantitative dipstick analysis and the reference methods was determined using Spearman's correlation. Correlations were classified based on the proposal of Papasouliotis and colleagues (2006) (i.e., rS = 0.93–1 as excellent correlation, rS = 0.80–0.92 as good, rS = 0.59–0.79 as fair, and rS < 0.59 as poor correlation). For assessing time and temperature effects, a simple analysis of variance (ANOVA) was performed. The urinary protein with the dipstick, since it is read qualitatively, was categorized; a negative result would be 0 mg/dL, 1+ would be 30 mg/dL, 2+ would be 100 mg/dL, and 3+ would be 500 mg/dL.

Statistical analysis was performed with the statistical software SPSS, version 27. Differences were considered statistically significant at $P < 0.05$.

Results

Regarding the macroscopic aspects of urine, colour and turbidity, it was found that, according to the colour and turbidity classification scale, all samples (n=16) presented normal colour at the time of collection, ranging from pale yellow to darker yellow. Regarding turbidity, approximately 75% (n=12) of the samples were turbid, while 25% (n=4) were clear.

It was also observed that in samples stored at room temperature, the colour changed over time, becoming darker, a more brownish-yellow hue. In samples that were refrigerated, there was no change in colour. Turbidity remained the same throughout the study period, at both storage temperatures.

As for the evaluation of USG in total urine or urinary supernatant, there was agreement of results in all tested samples (n=16). All the USG values obtained with refractometer were within the upper detection limit of 1.050.

For validation of the use of Combur-test10 (Roche), urinary dipsticks, the performance result was compared with the results obtained through standard methods, namely potentiometry, refractometry, and spectrophotometry for the parameters pH, USG and urinary protein. Regarding pH, a fair correlation was obtained between the results obtained in the urinary dipsticks and the standard method (rS=0.593, p<0.001). The results differed in relation to USG measured in the urinary dipsticks and the standard method (rS=0.368, p<0.001), obtaining a poor correlation. Regarding protein, there was also a poor correlation (rS=0.357, p<0.001).

The results of the effect of time and temperature as well as the presence of crystals are represented in tables 1 and 2. Overall, the crystals observed were calcium carbonate ($CaCO_3$) and calcium oxalate (CaC_2O_4).

Table 1 - Stability of equine urine parameters in samples stored at room temperature (n=16).

Parameters	**0h** **Mean** **(95% CI)**	**6h** **Mean** **(95% CI)**	**12h** **Mean** **(95% CI)**	**24h** **Mean** **(95% CI)**	**Stability limit (h)**
pH dipstick	8 [7.9 ; 8.4]	8 [8.2 ; 8.7]	9 [8.3 ; 8.7]	9 [8.3 ; 8.7]	24
pH *standard*	7.72 [7.40 ; 8.03]	7.80 [7.48 ; 8.11]	7.92 [7.60 ; 8.23]	7.98 [7.66 ; 8.29]	24
USG dipstick	1.014 [1.010 ; 1.018]	1.014 [1.010 ; 1.018]	1.014 [1.010 ; 1.018]	1.014 [1.010 ; 1.018]	24
USG *standard*	1.024 [1.018 ; 1.030]	1.024 [1.018 ; 1.030]	1.024 [1.018 ; 1.030]	1.024 [1.018 ; 1.030]	24

Protein dipstick (mg/dL)	26.25 [21.13 ; 31.37]	26.25 [21.13 ; 31.37]	26.25 [21.13 ; 31.37]	26.25 [21.13 ; 31.37]	24
Protein *standard* (mg/dL)	19.74 [11.78 ; 27.70]	23.44 [15.48 ; 31.40]	19.13 [11.17 ; 27.09]	20.84 [12.89 ; 28.80]	24
$CaCO_3$ crystals (unit)	11.99 [8.89 ; 15.08]	11.93 [8.83 ; 15.02]	11.86 [8.76 ; 14.95]	10.85 [7.75 ; 13.95]	24
CaC_2O_4 crystals (unit)	1.29^{b} [-0.60 ; 3.19]	3.31^{ab} [1.42 ; 5.20]	3.87^{ab} [1.98 ; 5.76]	5.21^{a} [3.31 ; 7.10]	12

CI - confidence interval; h - hours; USG - urine specific gravity; $CaCO_3$ - calcium carbonate; CaC_2O_4 - calcium oxalate. Different letters among parameters indicate statistical significance (P<0.05).

Table 2 - Stability of parameters for equine urine samples stored under refrigerated temperature (n=16).

Parameters	**0h Mean (95% CI)**	**6h Mean (95% CI)**	**12h Mean (95% CI)**	**24h Mean (95% CI)**	**Stability limit (h)**
pH dipstick	8 [8.0 ; 8.4]	8 [8.2 ; 8.7]	8 [8.2 ; 8.7]	9 [8.3 ; 8.7]	24
pH *standard*	7.72 [7.43 ; 8.01]	7.81 [7.52 ; 8.10]	7,93 [7.64 ; 8.22]	7.14 [7.85 ; 8.43]	24
USG dipstick	1.014 [1.010 ; 1.018]	1.014 [1.010 ; 1.018]	1.014 [1.010 ; 1.018]	1.014 [1.010 ; 1.018]	24
USG *standard*	1.024 [1.018 ; 1.030]	1.024 [1.018 ; 1.030]	1.024 [1.018 ; 1.030]	1.024 [1.018 ; 1.030]	24
Protein dipstick (mg/dL)	26.25 [21.13 ; 31.37]	26.25 [21.13 ; 31.37]	26.25 [21.13 ; 31.37]	26.25 [21.13 ; 31.37]	24
Protein *standard* (mg/dL)	19.74 [12.45 ; 27.03]	19.01 [11.72 ; 26.31]	21.14 [13.85 ; 28.44]	21.41 [14.11 ; 28.70]	24
$CaCO_3$ Crystals (unit)	11.99 [8.93 ; 15.04]	11.31 [8.26 ; 14.37]	11.98 [8.93 ; 15.04]	13.21 [10.15 ; 16.26]	24
CaC_2O_4 Crystals (unit)	1.29^{b} [-0.26 ; 2.84]	2.75^{b} [1.20 ; 4.30]	3.58^{ab} [2.03 ; 5.13]	6.02^{a} [4.47 ; 7.57]	12

CI - confidence interval; h - hours; USG - urine specific gravity; $CaCO_3$ - calcium carbonate; CaC_2O_4 - calcium oxalate. Different letters among parameters indicate statistical significance (P<0.05).

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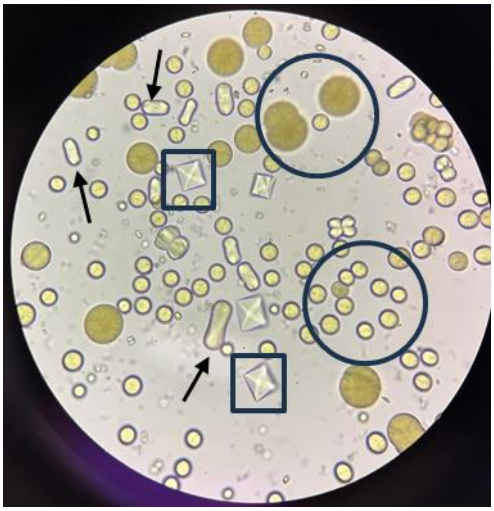

Figure 2 – Microscopic examination of urinary sediment (400x); Calcium carbonate crystals (circle), calcium oxalate monohydrate (arrows), and dihydrate (square). Species: Equus caballus.

From the observation of urinary sediment from different samples, besides calcium carbonate and calcium oxalate dihydrate and monohydrate crystals (figure 2), other elements such as mucus, also typical of equine urine, and some unclassified crystals were also observed.

Discussion

The European guidelines for urinalysis were first published in 2000 to ensure accurate analysis by providing clear instructions on sample storage, time, and other important concepts for conducting procedures. These guidelines recommend analysis within 2 hours after collection (16). However, different authors recommend different storage times: urine samples analysed within 30 minutes after collection, or refrigerated and examined as soon as possible (44); urine samples evaluated up to 60 minutes after collection to minimize the effect of temperature and time on in vitro crystal formation (45). Thus, there is no consensus on the time that can elapse between collection, and urine analysis without altering the results. In veterinary medicine, some studies discuss the effect of temperature and/or time on urinalysis (13, 45, 46, 47, 48), but as far as we know, there are no published studies discussing the stability of equines urine samples. Urinary dipsticks were initially designed for human medicine. Previous studies have shown that some urinary dipsticks used in human medicine are not reliable for certain parameters in horse urine (42). Therefore, care should be taken to use them, as some parameters are not useful or reliable for use in veterinary medicine (34).

Thus, the aim of this study was to determine the effect of storage time and temperature on parameters pH, urine density, protein quantity, crystal formation and to validate the use of Combur-test10 (Roche) urinary dipsticks in equine´s urine samples that met LPC-HVUTAD standards.

According to literature, macroscopic aspects of urine (colour and turbidity) should be evaluated at the time of collection (25), as changes may occur when the sample is exposed to room temperature. In this study, we observed that all samples, had a normal colour at the time of collection, ranging from pale yellow to darker yellow. Regarding turbidity, most of the samples were turbid. Samples that were refrigerated maintained their initial colour, while samples left at room temperature became more brownish over time. This information is not consistent with the results of Neumann and colleagues (2020), who reported that the colour and turbidity of the samples did not change during the 48-hour study period. Sharkey (2017), however, warns that in vitro oxidation of some plant compounds can lead to colour changes, thus emphasizing the importance of conducting analysis immediately after collection and possibly justifying the changes observed in our study.

According to our results, the USG parameter remained constant over the 24-hour study period, consistent with the findings of Neumann and colleagues (2020). Also, measuring USG in the supernatant did not show advantages over measuring it in the total urine. When we compared the USG results obtained by the urinary dipstick with the reference method, a poor correlation was found, demonstrating that, in horses, as previously described for dogs and cats (33), the urinary dipstick is not a reliable method, and the refractometry should be used instead. According to Schott & Esser (2020), USG should not be measured using urinary dipsticks. Preference is thus given to use a refractometer for USG measurement (49), provided it has a wide range (1.000 to 1.060) to avoid result extrapolation in more concentrated urines. It is also recommended that the most accurate method for evaluating density is osmolality assessment because glucose or proteins can bias the results (50). In the presence of glucose and protein, Osborne and colleagues (1995) recommend making corrections to density readings, as they increase urinary density when measured by refractometry.

Urinary pH is a valuable and easily measurable biochemical marker and a part of a complete urine examination, as it is essential for the chemical interpretation of urine and urinary sediment (51, 52, 53, 54). When evaluating urinary pH,

attention should be paid to the pre-analytical phase because if the urine container is exposed to air, and room temperature (leading to CO_2 loss) or contaminated with detergents or disinfectants, urinary pH may increase (13, 55). In this study, the pH remained constant over the 24-hour period, consistent with the results of Neumann and colleagues (2020). When comparing the results obtained by the urinary dipstick with those of the reference method, the correlation was reasonable. It is therefore suggested that urinary dipstick be used only to estimate pH in routine urinalysis, but not recommended when more precise pH measurement is needed for diagnosis, prevention, and treatment of a disease. In such cases, pH should be determined by a pH meter (33, 53, 54, 55).

The pH meter is considered the standard method for measuring this parameter; however, it is used infrequently in veterinary medicine (54). The reasons for its less frequent use are the need for calibration and periodic maintenance with test solutions, as well as user training (53). Unlike pH meters, urinary dipsticks are single-use and can measure a variety of parameters besides pH; moreover, they are more economical and do not require as much user practice (54). One disadvantage of urinary dipsticks is the inter- observer variation in evaluating colour variations, and individual visual acuity may also vary (51, 56).

In this study, the urinary protein also remained constant over the 24-hour study period, consistent with Neumann and colleagues' findings (2020), where this parameter remained stable for 48 hours. However, when comparing the results obtained by the urinary dipstick with those of the reference method, the correlation obtained was poor, so the use of the strip for detecting proteinuria in equines is not recommended. The proteinuria is an important marker of kidney disease (57). The presence of hematuria can affect the diagnosis of proteinuria (15), so it is important to rule out the presence of extra-renal causes of proteinuria. Alkaline or highly concentrated urine can also cause false-positive results (19, 25).

Equines urine is typically characterized by the presence of crystals, especially those containing calcium in their composition (40). Crystal formation depends on various factors such as species, breed, urine pH, hydration status, and underlying disease. Crystals form more easily in concentrated urine and, in many cases, are not associated with pathology (14, 15). Calcium oxalate dihydrate crystals can form when foods rich in oxalate are ingested (14, 15). The detection and identification of urinary crystals in veterinary medicine are done by observing urinary sediment (58). The observation of crystal formation in urine stored at room temperature or refrigeration is not consistent. In a study by Albasan and colleagues (2003), there was an increase in struvite and calcium oxalate crystals in the urinary sediment of dogs and cats both at 6 and 24 hours after collection, in both refrigerated and room temperature samples. Another study concluded that dog urine samples should be evaluated within 60 minutes after collection to minimize the effect of temperature and time on in vitro crystal formation (45). Neumann and colleagues (2020) found that the number of calcium oxalate crystals remained stable only up to 12 hours, both in refrigeration and at room temperature. In this study, and in accordance with Neumann and colleagues' findings (2020), there was a significant increase in the number of calcium oxalate crystals at 24 hours after collection, at both storage temperatures. As far as we know, there are no previous studies that examine crystal formation in horse urine over time. In addition to crystal formation, according to Schott & Esser (2020), urinary sediment should be evaluated within 30-60 minutes after sample collection, as cellular element degradation and bacterial overgrowth can occur, as reported by Sharkey (2017).

Many of the evaluated parameters depend on the time between sample collection and laboratory analysis (59). Thus, sample preservation conditions are an important step in the delayed analysis of urine samples, where refrigeration plays a crucial role. Refrigeration is the most commonly used method for preserving urine samples in veterinary medicine, although the literature also mentions the use of formaldehyde or ethylenediaminetetraacetic acid (EDTA) for preserving urinary sediment (14, 21). However, refrigeration is the most commonly used method for preserving urine samples and is preferable to the use of preservatives because it has less impact on the biochemical results of urinary dipsticks (13). There is no defined time for refrigerating urine to maintain its preservation because it depends on the presence of different elements in each sample (60). Although refrigeration of urine samples is recommended to reduce cellular degradation and bacterial growth, it has been reported that refrigeration leads to the rupture of some cells in urine. Additionally, refrigeration and prolonged storage have been associated with a significant increase in crystal formation (45). To avoid some of the undesirable effects, refrigerated samples should be brought to room temperature before analysis, which can be achieved by leaving the sample at room temperature for about 15 minutes or by holding the sample in the hand for some time (46).

Regarding the addition of preservatives, although not recommended for routine urine analysis, the use of this method may be necessary for samples that take longer to transport and cannot be refrigerated. The choice of preservative is important so as not to alter the urine composition or interfere with laboratory analyses (14). Traditionally, preservatives have been used in samples for microbial analysis, as the use of these compounds typically prevents metabolic changes and bacterial growth within the sample (60). Formalin is beneficial in preserving sediment, and sodium fluoride inhibits glycolysis; however, both preservatives interfere with urinary dipstick (14) and should therefore be avoided. Ethanol is described to preserve cellular constituents (60).

Studies in human medicine have shown that urine can be satisfactorily preserved (chemically and microscopically) at room temperature using test tubes containing 0.4% chlorhexidine, 5.6% ethylparaben, and 94% sodium propionate as a preservative solution until the time of analysis. The same authors used these preservatives in a dog urine study and observed that the number of crystals in the sediment correlated better with baseline samples than with refrigerated samples but showed less correlation with leukocyte and erythrocyte count parameters (45).

In this study, we evaluated the preservation of equines urine at room temperature, and refrigeration at three different storage times without the use of preservatives, and for the parameters pH, urinary density, and protein, it was found that the samples remained stable up to 24 hours after collection, both at room temperature and refrigeration.
On the other hand, pre-analytical conditions can also affect the use of urinary dipsticks. If the analysis is not performed promptly and according to handling recommendations, changes in urine will occur, and the results will not be as intended (61, 62). Most of the time, when analysing urine with a urinary dipstick, there are many causes of error that can affect the laboratory procedure, such as: the technician's subjectivity regarding the color found on the reagent strip (63); sample quality; pigmented or turbid urine; the validity of the dipstick; strips exposed to light and air; exceeding the strip reading time; reading the strip in the vertical position; sample contamination with disinfectants; removing excess urine from the strip with paper; putting a finger on the test area. Highly pigmented urine samples are a characteristic that leads to many false-positive results in various parameters of urinary dipsticks, including nitrites, protein, ketone bodies, urobilinogen, and bilirubin (14).
To reduce the subjectivity of urinary dipsticks reading by the human eye, automatic readers have been created. In many cases, there are differences between manual reading and automatic reading. The observed differences can have consequences in clinical practice leading to poor decisions, so it is important to use automated urine strip reading devices to eliminate the inherent subjectivity of manual reading (64) and further reduce the overall procedure time (65).
This study is limited by the low number of samples, as well as the low number of samples per species, which did not allow us to draw species-specific conclusions. Thus, all results obtained should be interpreted with caution, although most of them are consistent with existing literature.

Conclusion

With this study, it was possible to verify that the Combur-test10 (Roche) urinary dipsticks are not a reliable method for accurately estimate urinary protein, USG, and pH in equines. The sample storage time and temperature did not have a significant effect on pH, USG, protein measurement, and the number of calcium carbonate crystals during the 24-hour study period. Unlike calcium carbonate crystals, the number of calcium oxalate crystals remained stable only up to 12 hours of the study.

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