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Validation and clinical application of a spectrophotometric technique for the determination of potassium bromide in canine serum for the control of epilepsy

[Validación y aplicación clínica de una técnica espectrofotométrica para la determinación de bromuro de potasio en suero canino para el control de la epilepsia]

Diego Robaina[1], Victoria Bentancur[1], Gimena Feijóo[1], Juan Pablo Damián[2], Gonzalo Suárez[1]*

[1]Departamento de Clínicas y Hospital Veterinario, Unidad de Farmacología y Terapéutica, Facultad de Veterinaria, Universidad de la República. Alberto Lasplaces 1620, Montevideo, Uruguay.
[2]Departamento de Biociencias Veterinarias, Unidad de Bioquímica, Facultad de Veterinaria, Universidad de la República. Alberto Lasplaces 1620, Montevideo, Uruguay.
*E-mail: gsuarez@fvet.edu.uy, suarezveirano@gmail.com

Abstract

Context: In canine patients with epilepsy, for therapeutic monitoring of seizure control by administration of potassium bromide (KBr), a validated analytical methodology is required for the quantification of serum bromide concentrations (Br^-).

Aims: To validate a spectrophotometric technique for the determination of serum Br^- concentrations and test the applicability in the therapeutic monitoring of epilepsy in canines.

Methods: We started from a serum matrix of 6 companion canines, clinically healthy and with no history of having been medicated with KBr or another drug in the last 6 months. The samples were quantified by spectrophotometry (wavelength 440 nm) at concentrations of 0, 150, 250, 500, 1000, 2000, 3000 and 4000 µg/mL of KBr, in three independent repetitions. The analytical method evaluated the parameters of linearity, accuracy, precision, limit of detection, limit of quantification and absolute recovery.

Results: Linearity was checked for the range of 150 to 3000 µg/mL ($R^2 > 0.99$), with accuracy and precision values of 97.3% and 9.4%, respectively. The detection limit was established at 96 µg/mL and the quantification limit at 150 µg/mL of KBr. The absolute recovery of the test reached 98.4%. The values obtained for the different parameters analyzed validate the technique in the concentration range of 150 µg/mL to 3000 µg/mL, which meet the internationally established acceptance criteria.

Conclusions: The paraclinical results obtained by applying the technique in KBr-medicated epileptic canines support the validation of the technique and the therapeutic monitoring in the control of epilepsy.

Keywords: anticonvulsant; dog; epileptic; monitoring.

Resumen

Contexto: En pacientes caninos con epilepsia, para la monitorización terapéutica del control de las convulsiones mediante la administración de bromuro de potasio (KBr), se requiere una metodología analítica validada para la cuantificación de las concentraciones de bromuro en suero (Br^-).

Objetivos: Validar una técnica espectrofotométrica para la determinación de concentraciones séricas de Br^- y probar la aplicabilidad en la monitorización terapéutica de la epilepsia en caninos.

Métodos: Partimos de una matriz sérica de 6 caninos de compañía, clínicamente sanos y sin antecedentes de haber sido medicados con KBr u otro medicamento en los últimos 6 meses. Las muestras se cuantificaron por espectrofotometría (longitud de onda 440 nm) a concentraciones de 0, 150, 250, 500, 1000, 2000, 3000 y 4000 µg/mL de KBr, en tres repeticiones independientes. El método analítico evaluó los parámetros de linealidad, exactitud, precisión, límite de detección, límite de cuantificación y recuperación absoluta.

Resultados: Se verificó la linealidad para el rango de 150 a 3000 µg/mL ($R^2 > 0,99$), con valores de exactitud y precisión de 97,3% y 9,4%, respectivamente. El límite de detección se estableció a 96 µg/mL y el límite de cuantificación a 150 µg/mL de KBr. La recuperación absoluta de la prueba alcanzó el 98,4%. Los valores obtenidos para los diferentes parámetros analizados validan la técnica en el rango de concentración de 150 µg/mL a 3000 µg/mL, que cumplen con los criterios de aceptación establecidos internacionalmente.

Conclusiones: Los resultados paraclínicos obtenidos al aplicar la técnica en caninos epilépticos medicados con KBr apoyan la validación de la técnica y el monitoreo terapéutico en el control de la epilepsia.

Palabras Clave: anticonvulsivante; epiléptico; monitoreo; perro.

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INTRODUCTION

Epilepsy is a complex and heterogeneous brain disease, due to sudden and abnormal activity of some neural pathways, which develop a wide variety of clinical neurological signs that can become serious consequences for the patient (Berendt et al., 2015; Podell et al., 2016). The prevalence of epilepsy is estimated to be between 0.6 - 0.75% in the general canine population (Berendt et al., 2015). In the Unidad de Neurología of the Hospital Veterinario of the Facultad de Veterinaria (Universidad de la República), the frequency of the reason for consultation for seizures (one of the main symptoms of epilepsy) in canines between 2005 and 2010 fluctuated from 7% to 23% of neurological care per year and 0.4% to 2% of annual hospital care (Feijóo et al., 2011).

The goal of antiepileptic drug therapy (AED) is to achieve a balance between the absence of the manifestation of seizure symptoms and the quality of life of the patient. Eradication of epilepsy seizures is not commonly achieved in canine patients, so a realistic goal would be to decrease the frequency, duration, severity, and the total number of seizures that occur in a given period of time, with limited or acceptable adverse effects for maximizing both the quality of life of the patient and the owner (Bhatti et al., 2015). According to the consensual proposal of the International Veterinary Epilepsy Task Force presented by Bhatti et al. (2015), the clinical approach to the pharmacological treatment of epilepsy should be carried out following the guidelines: establish the criteria to start treatment with AED, select the most appropriate AED for each patient and the dosage, determine when to monitor serum concentrations of AED and adjust the treatment based on the results, identify when it is necessary to add or modify an AED.

Due to its long history of use, wide commercial availability and low cost, phenobarbital (Pb) and KBr have long been the drugs of choice for the treatment of epilepsy in canines (Bhatti et al., 2015). Bromide (Br^-) is an inorganic halide with sedative and anticonvulsant properties, usually administered in the form of KBr, although sodium salt can also be used (Bhatti et al., 2015). Bhatti et al. (2015) report that the bioavailability of Br^- after its administration as an oral solution in canines is 46%, although this value can vary considerably between individuals (Trepanier and Babish, 1995). The elimination half-life in this species is prolonged and can range from 25 to 46 days. Consequently, it can take several months (approximately 3) to reach steady-state concentrations (Trepanier and Babish, 1995; Podell, 1998). The therapeutic ranges reported are approximately 2000 - 3000 µg/mL when used as monotherapy (20 mg/kg every 12 hours) and 1000 - 2000 µg/mL when administered together with Pb (15 mg/kg every 12 hours) (Trepanier et al., 1998).

Serum concentrations at which KBr exhibits therapeutic or toxic effects may differ between individuals, with an overlap between toxic and non-toxic concentrations (Baird-Heinz et al., 2012). Thus, both cases of poisoning can occur in patients with serum levels within the therapeutic range, as well as patients with serum levels above the therapeutic range that do not show clinical signs of intoxication. In view of this situation, the monitoring of serum Br^- concentrations must be accompanied by a clinical evaluation of the patient (March et al., 2002; Baird-Heinz et al., 2012). All subsequent dose adjustments should be made based on the serum concentration of the drug and the clinical signs that appear (Podell, 2008). Using different spectrophotometric techniques, Bhatti et al. (2015) and Trepanier et al. (1998) reported different bioavailability values of Br^-. The validation of an analytical method is the process by which it is established by experimental studies, that the capacity of the method satisfies the requirements for the desired analytical application, based on the determination of various parameters (Díaz de Armas et al., 1998).

The objective of this study is to demonstrate the validation methodology and the applicability of a spectrophotometric technique for the determination of serum Br^- concentrations in the implementation of therapeutic monitoring of epilepsy in canines.

MATERIAL AND METHODS

Substances and reagents

The preparation of a stock solution (SS) of KBr (KBr extra pure 99.9% purity, LUSA Laboratory, batch: K 44231400) was started with a concentration of 40 mg/mL Br^-. From this SS 8 serial dilutions of 0, 1.5, 2.5, 5, 10, 20, 30 and 40 mg/mL of Br^- were prepared (working solutions). The precipitation of macromolecules from the serum was carried out using 10% trichloroacetic acid (Droguería Industrial Uruguaya, batch L130821-03) and the colorimetric reaction using gold chloride (AuCl + H2O, purity >49%, Sigma-Aldrich, batch: BCBH9937V) prepared at a concentration of 0.5%.

Selection of individuals

For the validation protocol, the blood samples of 6 companion canines (named A, B, C, D, E and F), clinically healthy, and without a history of medication with KBr or another drug in the last 6 months were used. In the clinical case study, blood samples from 29 patients were used (10 canines from the Unidad de Neurología of the Hospital Veterinario of the Facultad de Veterinaria, Universidad de la República; and 19 canines from private veterinary clinics).

Clinical trials

All patients included were on KBr-based therapy in the past 6 months, with KBr oral dose range was 10 to 20 mg/kg every 12 hours, and may or may not be combined with an oral dose of Pb in the range of 2.5 to 5 mg/kg every 12 hours.

Trial 1. Stability of the KBr serum concentrations in medicated patients

It was starting from animals that were under treatment with KBr, in combination with Pb (n = 3) or as monodrug (n = 2). Monitoring was performed on two occasions, with an interval of 60 days.

Trial 2. Monitoring of KBr serum concentrations in patients under treatment

The determination of Br^- was carried out in patients (n = 29) who came from the consultation of the Hospital Veterinario of the Facultad de Veterinaria. Data on sex, weight, dose, and co-medication with Pb were collected as covariates in the analysis of the monitoring results.

Obtaining the matrix

The experimental procedures for collect blood from the canines were conducted following the protocol approved by the Comisión Honoraria de Experimentación Animal (CHEA), Universidad de la República, Montevideo, Uruguay (Reg. no. CEUAFVET – 758, 2018).

Blood samples were taken in a dry tube at different times of the day without requiring fasting prior to extraction, storing the samples in a dry tube for subsequent centrifugation (Sigma 2-16KL, Sigma Laborzentrifugen GmbH, Germany)(3000 rpm for 15 min) and serum extraction.

Analytical determination

For the determination of KBr, the spectrophotometric technique proposed by Trepanier and Babish (1995) was used with minor modifications. The technique consisted of fortifying 200 µL of serum with 20 µL of a solution of known concentration of Br^- (working solutions); macromolecules (proteins and nucleic acids) were precipitated from the serum by adding 800 µL of 10% trichloroacetic acid; centrifugation of the samples at 3000 rpm for 15 minutes and extraction of 400 µL of the supernatant to which 80 µL of 0.5% gold chloride were added. After 30 minutes, the spectrophotometric reading of the colorimetric reaction was performed with a wavelength of 440 nm (Shimadzu UV-1800).

Work structure

For the validation of the technique, the work methodology was repeated for three consecutive

days. For each day, 18 samples were prepared and processed.

Linearity

This parameter was demonstrated by quantifying in triplicate (animals A, B and C) the following KBr concentrations: 0, 150, 250, 500, 1000, 2000 and 3000 µg/mL. An absorbance graph (ABS) was constructed as a function of the Br^- concentration for each workday. The linear regression equation, y = ax + b was calculated from the calibration curves using the least-squares adjustment, where *a* is the slope, *b* the intercept, *x* the concentration levels of Br^- and *y* represents the signal of absorbance obtained. Values for the determination coefficient (R^2) were determined for each calibration curve.

Accuracy

It is expressed as the percentage of the recovery. The recommended accuracy varies according to the concentration of the analyte. For concentrations higher than 0.1 µg/mL the recommended accuracy values are in the range of 80% to 110% (Fundación PROSAIA, 2013). The calculation of the accuracy was done by applying equation [1]:

$$\text{Recovery (\%)} = (a / b) \times 100 \quad [1]$$

Where *a*: Recovered bromide concentration (µg/mL) and *b*: Added bromide concentration (µg/mL)

The accuracy was calculated from the fortification levels 150, 250, 1000 and 3000 µg/mL of Br^-, with an n = 6 (animals A, B, C, D, E and F).

Precision

It was expressed as a percentage of the coefficient of variation (CV %) and its recommended value depends on the concentration of the analyte. The accuracy considered as acceptable for this study when concentrations were above 0.1 µg/mL, was up to 15% (Fundación PROSAIA, 2013). Equation [2] was used to calculate the accuracy:

$$\text{Precision (CV \%)} = (\text{Standard deviation} / \text{mean}) \times 100 \quad [2]$$

The accuracy was calculated from the fortification levels 150, 250, 1000 and 3000 µg/mL of Br^-, with an n = 6 (animals A, B, C, D, E and F). Repeatability (intra-day accuracy) and intermediate accuracy (inter-day accuracy) were determined.

Limit of detection (LOD) and limit of quantification (LOQ)

In this work, the procedure proposed by the International Union of Pure and Applied Chemistry, described in Guide No. 2 of the PROSAIA Fundation (2013), was followed. The LOD was calculated for each day as the mean of the results of 4 control samples (animals C, D, E and F) plus 3 times the standard deviation (SD); and the LOQ of each workday as the average of these same results plus 6 times the SD. If the Accuracy and Accuracy in the LOQ meet the validation criteria, then the calculated LOQ was accepted.

Absolute recovery (AR)

The AR percentage was calculated according to equation [3]:

$$\text{Absolute recovery (\%)} = (a / b) \times 100 \quad [3]$$

Where *a*: Bromide concentration in biological matrix (mg/mL) and *b*: Bromide concentration in water (mg/mL)

This parameter (AR) was studied using concentrations of 500 and 1500 µg/mL of Br^- per six-fold, both for the biological matrix and for water. The AR will be obtained as long as the precision is within the acceptance range in all the concentrations analyzed (Fundación PROSAIA, 2013).

Statistical analysis

The data were analyzed by means of descriptive statistical tests, with the application of the least-squares adjustment method in the linear regression model, visualization of residuals and scatter plots. To determine the variation in the monitoring interval of the clinical cases, a paired-samples Student's t-test was applied. The level of significance for the statistical tests was 5%. Statisti-

cal analyzes were performed using R software (R Core Team, 2020).

RESULTS AND DISCUSSION

Method validation

Linearity

The graphs of the absorbance (ABS) as a function of the concentration of Br^- in canine serum constructed for each workday are presented in Fig. 1. According to the reported values (Table 1), the model explains 99% of the variation of ABS data as a function of Br^- concentration ($p<0.05$). In turn, R^2 values are above 0.99 for the 3 days of work, demonstrating linearity in the concentration range of 150 to 3000 µg/mL of Br^-. The ABS results obtained for the concentration of 4000 µg/mL were discarded in the construction of the graphs since the inclusion of that point decreases the R^2, removing the linearity method, which would be indicative of a tendency to saturation of the reaction (plateau of the graph). Given that the therapeutic ranges reported in the literature for Br^- vary from 1000 - 2000 µg/mL in combination with Pb and from 2000 - 3000 µg/mL in monotherapy (Trepanier et al., 1998), the linearity of the method is validated in the ranges of clinical interest.

Through the use of similar spectrophotometric techniques used to determine the concentration of Br^- in the serum of different species, wide ranges of linearity have been obtained. Quast et al. (2015) reported linearity ranges between 25 and 5000 µg/mL of Br^- for sheep, while Raidal and Edwards (2005) indicated a range of 5 to 5000 µg/mL of Br^- in equines. The relative concentration of gold chloride and the species could explain the difference in amplitude for the linearity ranges we achieved. Regarding gold chloride, the reagent used in the present study indicated a purity level greater than 49% (Sigma-Aldrich, batch: BCBH9937V), a higher amplitude in the linearity range would possibly be reached if the reagent were higher purity, ensuring a greater number of gold molecules available for the colorimetric reaction to occur. A deficit in the number of gold molecules could be the cause of deviations in linearity when reaching high levels of Br^- (4000 µg/mL) (data not shown).

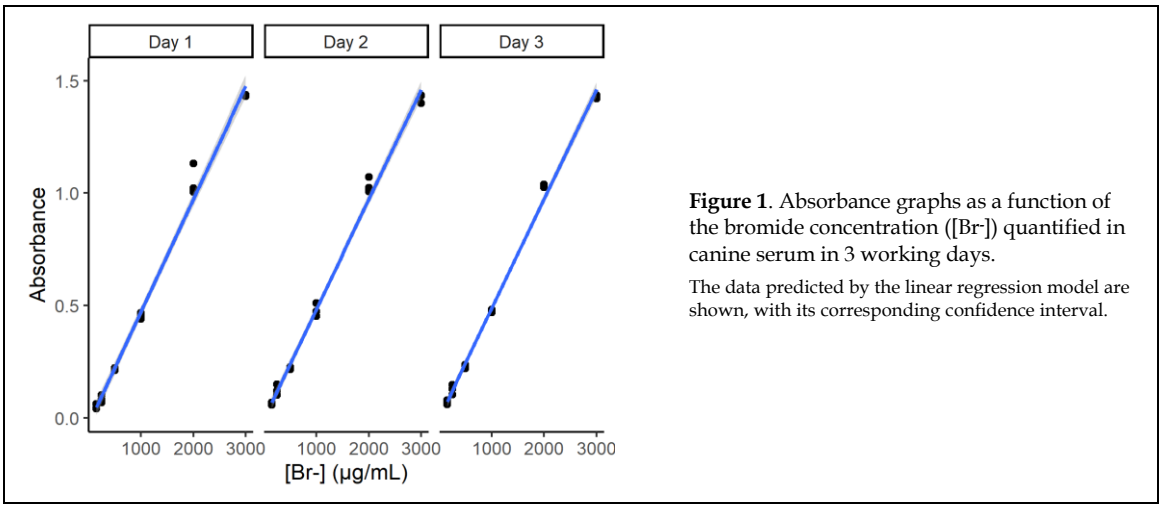

Figure 1. Absorbance graphs as a function of the bromide concentration ([Br-]) quantified in canine serum in 3 working days.

The data predicted by the linear regression model are shown, with its corresponding confidence interval.

Table 1. Linear regression analysis for the quantification of the bromide concentration in canine serum for 3 working days.

	Day 1	Day 2	Day 3
Concentration range (µg/mL)	150 - 3000	150 - 3000	150 - 3000
Slope (CI90%)	0.001* (0.0005, 0.001)	0.0005* (0.0005, 0.001)	0.0005* (0.0005, 0.0005)
Intercept (CI90%)	-0.029 (-0.057, -0.002)	-0.005 (-0.027, 0.017)	-0.002 (-0.020, 0.016)
n[c]	18	18	18
R^{2}[a]	0.993	0.995	0.997
Residual Standard Error (df[b]: 16)	0.048	0.038	0.032
F-statistic (df: 1 - 16)	2,145,144*	3,131,579*	4,599,015*

[a]R^2: Determination coefficient; [b]df: degrees of freedom; [c]n: number of samples; *p<0.05.

Accuracy and precision

Tables 2 and 3 present the intra-day and inter-day accuracy and precision results by concentration level. All the values obtained are included in the acceptance range accepted by PROSAIA (Fundación PROSAIA, 2013) for these parameters.

Limit of detection and limit of quantification

Given the amplitude of these ranges for both parameters, instead of using a global value of 3 days, the LOD and LOQ are defined based on the most conservative scenario, that is, the highest values are considered. LOQ is considered at the lower range of linearity in agreement with validation criteria for accuracy and precision (Fundación PROSAIA, 2013). In summary, the assay presents a LOD of 96 µg/mL and a LOQ of 150 µg/mL of Br^-. Pharmacokinetic studies in equines and sheep report LOQs of 5 µg/mL (Raidal and Edwards, 2005) and 25 µg/mL (Quast et al. 2015), respectively. Trepanier and Babish (1995) in canines, only define the LOD at 20 µg/mL, without reporting the LOQ. In all cases, the reported values are lower than those calculated in this work. However, none of these articles specify the calculation methodology used for the calculation of LOD and LOQ, nor is the spectrophotometer model used as a measurement instrument specified in the work methodology. These introduce the possibility that the differences could be attributed to higher sensitivity, explaining the differences in LOD and LOQ. For example, parallel studies in our laboratory, carried out with other equipment (Spectronic 21, Metrolab 1600), demonstrated lower levels of analytical sensitivity against the same concentration range (LOQ >500 µg/mL) (data not shown).

Absolute recovery

The results obtained for the AR tests at the fortification levels of 500 and 1500 µg/mL were 100.3% and 96.6%, respectively, and the overall AR of the test is 98.4%. With the global AR value obtained, and because the accuracy of the method is 9.4%, it is shown that the extraction of Br^- from canine serum is almost total, and the AR is accepted. These obtained values allow the matrix exchange between canine serum and distilled water for routine use in the laboratory, for example, at the time of performing the control points of the calibration curves in the implementation of the analytical routine.

Trial 1. Stability of serum concentration in medicated patients

Br^- was detected in all blood samples analyzed from animals medicated with KBr, which provides practical value to the technique. Fig. 2 shows the data obtained for each patient individually and grouped by sampling day, not reporting significant differences at the beginning and end of a 60-day treatment period (p = 0.14). At the time of sampling, all the patients received KBr for a period

of more than 6 months; therefore, it is expected that the Br^- concentration was already in steady-state, and therefore there was no significant variation between the values of both groups. The results obtained by using the technique in KBr-medicated patients coincide with the therapeutic range and the stability of blood drug levels with long-term treatments reported by Trepanier et al. (1998), Trepanier and Babish (1995) and Podell (1998) and thus endorsing the validation performed.

Table 2. Intra-day accuracy and repeatability by level and global obtained in the three days of work for the validation of a technique to quantify the bromide concentration in canine serum.

Day	Bromide concentration (µg/mL)	By level (Intra-day)			Global (Intra-day)		
		SD[a]	Accuracy (%)	Precision (CV %)[b]	DS[a]	Accuracy (%)	Precision (CV %)[b]
1	150	13	107	12	10	98	10
	250	9	88	10			
	1000	4	97	4			
	3000	2	97	2			
2	150	9	94	10	8	97	9
	250	15	98	15			
	1000	5	97	5			
	3000	1	98	1			
3	150	12	87	14	9	96	9
	250	14	99	14			
	1000	3	96	3			
	3000	1	98	1			

[a]SD: Standard deviation; [b]CV %: Coefficient of variation.

Table 3. Accuracy and intermediate precision (inter-day) by level and global obtained for the validation of a technique to quantify the bromide concentration in canine serum.

Bromide concentration (µg/mL)	By level (inter-day)			Global (inter-day)		
	SD[a]	Accuracy (%)	CV %[b]	SD[a]	Accuracy (%)	CV %[b]
150	13	96	14	9	97	9
250	13	95	14			
1000	4	96	4			
3000	1	98	1			

[a]SD: Standard deviation; [b]CV %: Coefficient of variation.

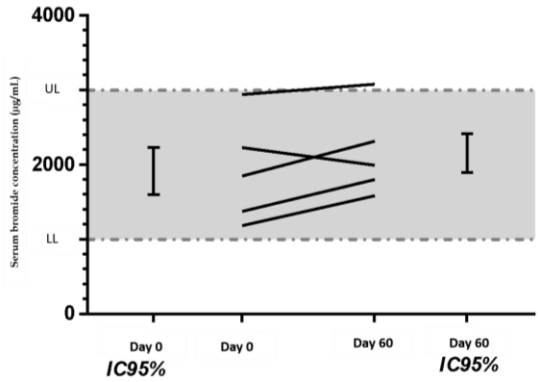

Figure 2. Serum bromide concentrations quantified in five canine patients on antiepileptic treatment with potassium bromide (KBr).

LL and UL represent, respectively, the lower and upper limit of the total therapeutic range (KBr as monodrug or in combination with phenobarbital). [Br-]: Bromide concentration.

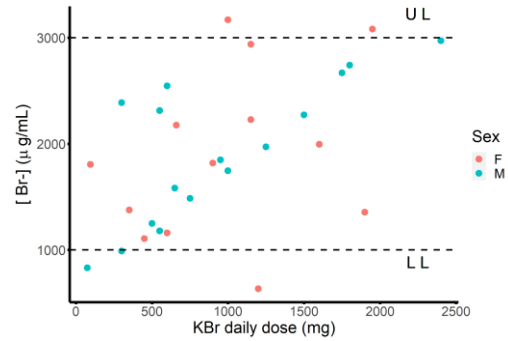

Figure 3. Serum bromide concentrations quantified in twenty-nine canine patients receiving antiepileptic treatment with potassium bromide (KBr).

LL and UP represent, respectively, the lower and upper limit of the total therapeutic range (KBr as monodrug or in combination with phenobarbital). [Br-]: Bromide concentration.

Trial 2. Monitoring of serum concentrations in clinical cases

Fig. 3 shows the relationship between the daily dose of KBr administered and the Br^- serum concentrations, stratifying by the sex of the patients. Said graph responds to the need to visualize the existence of dose-proportionality for Br^- in canines. There are no differences between sexes according to the daily dose administered and the serum concentrations observed; with a correlation coefficient ($r = 0.528$; $p = 0.003$) between the levels of Br^- and the increase in the dose, which highlights the importance of jointly evaluating the clinic with monitoring when administering this drug and adjusting the individual daily dose. Therapeutic monitoring, together with the clinical evaluation of the patient's response, are decisive to demonstrate the implication in the individual variability when a therapeutic dosage is established, as evidenced by the reported concentrations outside the therapeutic range, but without toxicological or subtherapeutic implications.

Study limitations

No limitations were detected as the validation process followed strict guidelines on analytical method validation. Future studies should focus on population pharmacokinetics for potassium bromide in canine patients.

CONCLUSIONS

The present study demonstrates the validity of the spectrophotometric technique for the quantification of Br^- in canine serum and the applicability in the therapeutic monitoring of patients medicated with KBr.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Robaina D	Bentancur V	Feijóo G	Damian JP	Suárez G
Concepts or ideas	x	x	x	x	x
Design	x				x
Definition of intellectual content	x	x			x
Literature search	x	x			
Clinical trial	x	x	x		
Experimental studies	x	x			
Data acquisition	x	x			
Data analysis	x	x			x
Statistical analysis					x
Manuscript preparation	x			x	x
Manuscript editing	x			x	x
Manuscript review	x	x	x	x	x

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