

DETERMINATION OF DEXAMETHASONE SODIUM PHOSPHATE AND DEXAMETHASONE BASE IN ONE SAMPLE OF HORSE PLASMA OR/AND SYNOVIAL FLUID USING HIGH RESOLUTION LIQUID CHROMATOGRAPHY. TECHNICAL NOTE

Determinación de la dexametazona fosfato sódica y la dexametazona base en una muestra de plasma o líquido sinovial de equino utilizando la cromatografía líquida de alta resolución. Nota técnica

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ABSTRACT

This trial describes a simple and fast procedure for the determination and quantification of dexamethasone sodium phosphate (DSP) and dexamethasone base (DB) using one small sample of horse synovial fluid. The objective was to use High Resolution Liquid Chromatography (HPLC) for the determination of DSP and DB in synovial fluid. For this purpose, a HPLC method was developed by using standard curves using known concentrations of DSP and DB. The method was further tested using synovial fluid and blood plasma from horses treated with dexamethasone. With the results obtained it was observed that the linearity of the method had a correlation coefficient of 0.990. The minimum detection limit (LD) for synovial fluid was 0.350 µg/mL. The minimum quantification limit (LC) was computed with the formula $LC = LD \text{ volume of Injection/Volume of Sample}$, $0.35 \mu\text{g/mL} * 25 \mu\text{L}/1000 \text{ mL} = 1.4 \mu\text{L}$. In conclusion the described method is adequate for the determination of DSP and DB in one sample of horse plasma and/or synovial fluid. The method proved to be reliable and adequate for the determination of DSP and its DB in blood and synovial fluid of horses treated with the anti inflammatory drug.

Key words: Dexamethasone, horse, plasma, synovial fluid.

Resumen

En este trabajo se describe un método simple y rápido para determinar y cuantificar dexametazona fosfato sódico (DSP) y dexametazona base (DB) empleando solo una muestra pequeña de líquido sinovial equino. Con este objetivo se desarrolló y adaptó un método utilizando la Cromatografía Líquida de Alta Resolución basado en curvas de recuperación estándar en varias pruebas con y sin líquidos biológicos. El método fue validado utilizando sangre y líquido sinovial de un caballo medicado con dexametazona. La linealidad del método presentó un coeficiente de correlación de 0,990. El límite mínimo de detección (LD) en el líquido sinovial fue de 0,350 µg/mL. El límite de cuantificación mínima (LC) fue calculado con la fórmula $LC = LD \text{ volumen de inyección/volumen de muestra}$. 0,350 µg/mL. En conclusión, el método aquí descrito resultó adecuado para la determinación de DSP y de DB en una pequeña muestra de sangre o líquido sinovial de caballos medicados con dexametazona.

Palabras clave: Dexametazona, equinos, plasma, líquido sinovial.

INTRODUCTION

Anti-inflammatory agents such as Corticosteroids are used often in veterinary medicine. Dexamethasone is used as an anti-inflammatory and antialergic agent in horses (*Equus caballus*) [4] because of its powerful anti-inflammatory, anti-ar-

thritic and pain-suppressing effects. Corticosteroids are ideal therapies for muscle diseases. These drugs have been used indiscriminately, which justifies the search for simple and specific methods to detect of these drugs and their metabolites in plasma and synovial fluids. The biological transformation of corticosteroids is well documented. Therefore, it is important to search for a reliable method for the determination and quantification of these compounds. In many clinical cases, the samples in which this determination is carried out are very scarce, which necessitates the search for techniques to identify both the drug and its metabolites in plasma and synovial fluid [2, 5].

Dexamethasone (9-fluoro-11 β , 17, 21-trihydroxy-16 α -methylpregna-1, 4-diene-3, 20-dione) has a basic structure with 21 carbon atoms, and it is 20 to 25 times more potent than the natural corticosteroid cortisol. The synthetic drug is frequently administered to horses by the intramuscular, intravenous and intra-articular routes, and doses range from 2.5 to 25 mg per animal. It is important to note that the identification for the synthetic drugs does not cross-react with cortisol [8, 9, 17].

Intra-articular administration of corticosteroids to horses produces immediate and localized effect, reduce the inflammation process, and stimulates the synovial structures to return to normality. They also reduce the vascular and enzymatic cell responses favoring homeostasis and diminish the secondary effects caused by systemic anti-inflammatory drugs [10, 15, 18].

To compensate the low solubility of dexamethasone base a phosphate ester of the drug is used. The phosphate ester of dexamethasone is rapidly dispersed after administration, and this formulation is called a "prodrug" because to free the active drug (dexamethasone) a direct *in vivo* biotransformation using a hydrolytic path is necessary. This is a limiting factor, because the drug effect depends of the degree of hydrolysis that releases the active drug [6, 7, 14, 16, 19].

Previous studies have shown that to avoid over calculation of the degree of hydrolysis the drug, and/or over estimation of the concentration of dexamethasone sodium phosphate (DSP), *in vitro* hydrolysis of this drug should be prevented by adding Ethylene Diamine Tetra Acetic acid as a stabilizing agent [7].

High Pressure Liquid Chromatography (HPLC) permits the separation, identification and quantification of compounds with similar chemical affinities in mixtures that are difficult to separate using other methods. HPLC permits the analysis, identification and quantification of any drug. Is performed by forming a drug derivative confirm the identify of the drug. The technique described here is based on different rates of passage, different components of a mixture in a moving phase through a stationary phase/column. The result depends on the adequate operation of the HPLC and combination of the different elements involved; (that is, the type, diameter and length of the column and of the moving phase) [19].

The moving phase enables the separation of the compound due to its polarity. Therefore, the polarity of the moving phase can be modified depending on the compounds being studied; this is a great advantage in the separation process of two compounds that is not possible when using other methods [6].

The stationary phase has another advantage in that it can be changed depending on the compounds being studied. When a tested drug is compared to a previously determined 100% HPLC-grade control, it is nearly impossible to have a cross reaction or a large deviation from the standard [9].

There are reports of various extraction techniques for these compounds but none contemplate the possibility of separating and extracting both compounds from the same sample [5-7]. The advantage of HPLC over other techniques (such as immuno electrophoresis, spectrophotometry, gas chromatography and Radio Immuno Analysis (RIA) is that there is no cross-reaction or interference with exogenous and/or endogenous compounds (cortisol) [6, 9, 16].

The purpose of this study was the detection and identification of DSP and DB in one sample of plasma and synovial articular fluid using HPLC.

MATERIALS AND METHODS

Laboratory procedures were carried out at the Toxicology laboratory in Veterinary Faculty of the National Autonomous University of Mexico. Biological samples were obtained from horses stabled at the Large Animal Clinic; of the Veterinary Faculty, National Autonomous University of Mexico Sodium phosphate dexamethasone 99% and DB (98% HPLC grade) were obtained from Sigma Aldrich. HPLC grade methanol (99,98% v/v) from J.T. Baker. Tetrahydrofuran (HPCL grade) was Lichrosolv IV (Merk). Ethyl acetate (99, 9% HPCL grade) was from J.T. Baker. 0,6 M sulfuric acid (v/v) and double distilled, deionized water were used.

A Perkin Elmer Landa 25 spectrophotometer (USA), a Sol Bat J-600, a Perkin Elmer 200 LC Binary injection pump for liquid chromatography, an automatic Perkin Elmer 200 sampler and a Perkin Elmer UV ray detector were used.

Preparation of standards

Dexamethasone base (98%) and DSP (97% HPLC grade) standards were made (0.5 mg/mL) in methanol; and kept refrigerated (Whirlpool, W8TXNFWWT.USA) until use.

Calibration using the standard curve

To obtain standard curves, serial dilutions of DB and its sodium phosphate salt were prepared (0.25 mg/mL, 0.125 mg/mL y 0.0625 mg/mL).

To determine the visible wavelength, an ultraviolet spectrophotometer was used. A scan was performed using both salts, and the outcome of this procedure showed that the maximal absorbance for dexamethasone and its phosphate salt were 273.4 nm and 248.1 nm, respectively.

The settings for HPLC were as follows: Mobil phase 70 mM. Potassium phosphate/methanol /tetrahydrofuran (560; 400; 40). Running time, 6 minutes. Isocratic flux, 1.5 mL/min. Automatic sampler, Injection volume, 25 μ L. Column guard C-18. Silica Ultrasphere C-18 (4.6 mm \times 4.5 cm) column (5 μ m). Wavelength (270) UV. Retention time for dexamethasone sodium phosphate 1.18 minutes. Retention time for dexamethasone base 3.45 minutes. All resulting samples were deep-frozen (Whirlpool, W8TXNWFWT.USA) at -4°C in tubes containing EDTA to avoid in vitro hydrolysis, as described by Blackford et al. [3].

Extraction of the base and phosphate salts of dexamethasone from plasma and synovial fluid

The modified technique of Blackford et al. [2] was used. Samples were thawed at room temperature. 1 mL of synovial fluid and 1 mL of blood plasma and 2 mL of ethyl acetate were transferred to a test tube, which was vortexed (Vortex shaker E-6115 Eberbach USA) for 30 seconds (sec) and centrifuged at 4,500 rpm (3.3 g), on a Cole Palmer centrifuge model EW-17305.00 USA for 5 min. The synovial fluid was sedimented and the ethyl acetate remained in the top layer. Both layers were separated and decanted into clean test tubes. Fifty mL of a 0.6 mM solution of sulfuric acid was added to the synovial sediment which was vortex shaken for 30 sec. 1 mL of ethyl acetate was then added followed, by centrifugation (Cole Palmer centrifuge EW-17305.00) at 4,500 rpm (3.3 g) for 5 min. The synovial fluid was sedimented and the ethyl acetate occupied the top layer, which was decanted into the previous aliquot of ethyl acetate from the first extraction. This final sample was evaporated using nitrogen flux at 50°C . The resulting extract was reconstituted with 500 μ L of the mobile phase and injected into the HPLC.

Once the standards were compared, the diluted samples of the pure solutions of base and phosphate dexamethasone, and all of the variables of the chromatographic technique were assessed.

To transform the height of the chromatograph signal to standard values, a regression was used where R^2 0.999 values an estimate of the concentration of each sample was obtained from the height of the chromatograph using the following formula:

$$y = a + b / x.$$

where a is the intersection, b is the slope and x is value of the signal.

It is important to mention that a strict methodology was carefully followed for the extraction of both the base and the phosphate salt of dexamethasone was used in this work.

Recovery percentage in vitro

To determine the percentage recovery of the known sample, 4 mL of fresh synovial fluid was collected from a healthy horse of the large animal clinic and divided in to 4 units of 1 mL each. Each unit received 50 μ L of $0.25 \text{ mg}\cdot\text{mL}^{-1}$ sodium phosphate and base standards of dexamethasone. Sample 1 was studied and the remainder of the samples were frozen to determine if the frozen time affected the quantity of the detectable drug. For both the first and subsequent samples, the percentage recovery in vitro was 30% with a minimum detection limit of 35 μ L/mL in synovial fluid.

The minimum quantification limit (LC) is the concentration of the drug that can permit accurate analysis and determinations and is computed with the formula:

$$\text{LC} = \text{LD that is: volumen of Injection/volumen of sample, } 0.35 \text{ } \mu\text{g/g/mL}/25 \text{ } \mu\text{L}/1000 \text{ } \mu\text{L} = 1.4 \text{ } \mu\text{g/mL}.$$

Statistical analysis

All synovial fluid samples in which sodium phosphate and water were added were subjected to ANOVA, which enabled a descriptive analysis of repeated observations. Furthermore an inferential analysis was performed using SAS System v.9.2005 [13]. Visual evaluation of the results was performed using Graphs and tables.

Stability of dexamethasone

It was considered important to study the stability of the compounds, because not all of the samples from synovial fluid and plasma were processed on the same day. Rather they were frozen until HPLC determination. For this purpose, 5 mL of fresh horse synovial fluid was collected from three horses of the Large Animal Clinic; of the Veterinary Faculty, National Autonomous University of Mexico, and a known quantity of sodium phosphate and DB was added to 1 mL aliquots of the synovial fluid. One of these five samples was subjected to HPLC analysis and the remaining samples were frozen in a Whirlpool refrigerator model W8TKNWFWT for one week in tubes containing EDTA to study the effect of freezing time on the stability of the dexamethasone compounds. The height of chromatographic curves was found and it was observed that the concentrations of DSP and DB were similar in 99% of the samples.

RESULTS AND DISCUSSION

This procedure demonstrated the usefulness of the analytical method using variables such as linearity, repeatability, precision, accuracy, specificity, minimum limit of detection,

minimum limit of quantification and the stability of the studied compound. To obtain linearity, a calibration curve was constructed using different concentrations of the standard. The correlation coefficient was 0.99, which confirmed and excellent linearity (TABLES I and II).

To corroborate the repeatability of the method, 5 mL of fresh horse synovial fluid was collected and divided into five units of 1 mL each; and known quantities of DSP and DB were added to each unit. One unit of this mixture was analyzed by HPLC, and the remaining four units were deposited in tubes with EDTA and frozen until the final analysis. All samples were analyzed by HPLC after one week. The height of the chromatographic signal was quantified, and it showed that there was 99% confidence in all observations (TABLES I and II).

To determine the precision of the system, repeated injections of 0.25 mg/mL of the DSP and DB standards were injected into the HPLC system, and the signal height and retention

time were studied. It was observed that the standard deviation was below 2%, confirming the precision of the analytic system for both compounds (TABLES III and IV).

The accuracy of the method was evaluated by determining the percentage of recovery of the three standards (0.25 mg.mL⁻¹, 0.125 mg.mL⁻¹ and 0.0625 mg.mL⁻¹). The standard limit was 30.65%, which was out of the 80 to 129% interval, which therefore indicates that the method can be improved. The low figure for the percentage of recovery might be caused by difficulties found during the development of the method. Further studies are recommended to optimize the percentage of extraction (TABLE V).

It is important to corroborate the specificity of the selected method, because of the possibility of interactions with endogenous substances that can create false positives. There was no observed interference with retention times during injections into the system, and the analytes separated adequately.

TABLE I

RESULTS OBTAINED FROM THE STANDARDS OF SODIUM PHOSPHATE DEXAMETAZONE IN THE HPLC CROMATOGRAM

| STD mg/mL | Height | Height | Height | Height | Height | Mean | Corr Coef |
|-----------|----------|----------|----------|----------|----------|----------|-----------|
| 0.25 | 253714.4 | 237859.5 | 255278.1 | 258028.1 | 249401.2 | 250856.3 | 0.99999 |
| 0.125 | 108532 | 126942 | 109309.3 | 131115 | 125597.5 | 120299.2 | |
| 0.061 | 43560.1 | 62760.7 | 46087.6 | 62790.8 | 59250.9 | 54890 | |

STD= Estándar is in miligrams/ mL. Height = Height of the peak. Mean = Mean of 5 observations. Corr Coef= Correlation Coefficient.

TABLE II

CROMATOGRAM RESPONSE TO THE STANDARDS OF DEXAMETASONE BASE

| STD mg/mL | Height | Height | Height | Height | Height | Mean | Corr Coef |
|-----------|----------|----------|----------|----------|----------|----------|-----------|
| 0.25 | 353021.2 | 314937.3 | 335841.9 | 318812.8 | 325633.8 | 329649.4 | 0.99998 |
| 0.125 | 179193.2 | 156217.6 | 169347.8 | 163503.2 | 162317.1 | 166115.8 | |
| 0.061 | 86033.9 | 73666.8 | 82940.7 | 81660.1 | 79497.3 | 80759.8 | |

STD= Standard is in mg/mL. Height = Height of the peak. Mean = Mean of 5 observations. Corr Coef = Correlation Coefficient.

TABLE III

RESULTS OF THE STUDY ABOUT THE PRECISION OF THE DEL SISTEM FOR SODIUM PHOSPHATE DEXAMETAZONE

| Number of Inyections | Height of the Peak | Time of Retention |
|----------------------|--------------------|-------------------|
| 1 | 323021.2 | 1.1 |
| 2 | 314937.3 | 1.09 |
| 3 | 325841.9 | 1.06 |
| 4 | 318812.8 | 1.05 |
| 5 | 325633.8 | 1.08 |
| Mean | 321621.8 | 1.075839834 |
| Standard Deviation | 4701.9 | 0.020736441 |
| Relative Deviation | 1.5 | 1.927465473 |

TABLE IV

RESULTS OF THE STUDY ABOUT THE PRECISION OF THE SISTEM FOR DEXAMETASONE

| Number of Inyections | Height of the Peak | Time of Retention |
|----------------------|--------------------|-------------------|
| 1 | 253714.4 | 3.34 |
| 2 | 247859.5 | 3.36 |
| 3 | 255278.1 | 3.38 |
| 4 | 258028.1 | 3.35 |
| 5 | 249401.2 | 3.4 |
| Mean | 252828.5 | 3.365931192 |
| Standard Deviation | 4190.9 | 0.024083189 |
| Relative Deviation | 1.7 | 0.715498558 |

The minimum detection limit (LD) is the minimal concentration or quantity of the compound that can be reliably detected with a validated method. In this case, the LD for synovial fluid was 0.35 µg/mL.

It is important to determine the stability of standards depending on the drug used and the solution in which it is suspended. In this work, it was corroborated that DSP and DB are stable when dissolved in methanol and frozen for up to one year. It was observed that DSP and DB stability in synovial fluid were also satisfactory.

Results of the statistical study

Means and standard deviations of the data obtained from the processed synovial fluid were compared with basal values for each horse articulation and across individuals. Therefore, the differences between means for each observation had a P value of <0.05.

In addition, all linear regression coefficients had mean values of a <0.5.

Pharmacokinetics

All processed samples were injected into the Channel Local Address Register (CLAR), which transfers the information to the interphase and to the computer, which in turn uses TotalChrom 6,1 (Perkin Elmer Corp. USA) to integrate the information. Therefore, all information is displayed as chromatographs that indicate the concentration of the drug present in the synovial fluid of the horse articulation.

With the chromatographs of the standards and the horse synovial fluid samples, it was decided to consider the height of the curve in the chromatograph, thus the area was not considered, because the resolution at the base did not permit the integration of the entire compound (FIGS. 1-4).

TABLE V
RESULTS OF RECOVERY STUDIES OF DEXAMETASONE USING HPLCA

| Assay Number | Concentration | Amount Recovered | % of Recovery |
|--------------|--------------------|------------------|---------------|
| 1 | 0.25 | 0.075000 | 30% |
| | 0.25 | 0.080000 | 32% |
| | 0.25 | 0.072500 | 29% |
| 2 | 0.125 | 0.040000 | 32% |
| | 0.125 | 0.037500 | 30% |
| | 0.125 | 0.038750 | 31% |
| 3 | 0.06125 | 0.018375 | 30% |
| | 0.06125 | 0.018988 | 31% |
| | 0.06125 | 0.018988 | 31% |
| | Mean | | 30.6% |
| | Standard Deviation | | 1% |
| | Relative Deviation | | 3.262642741 |

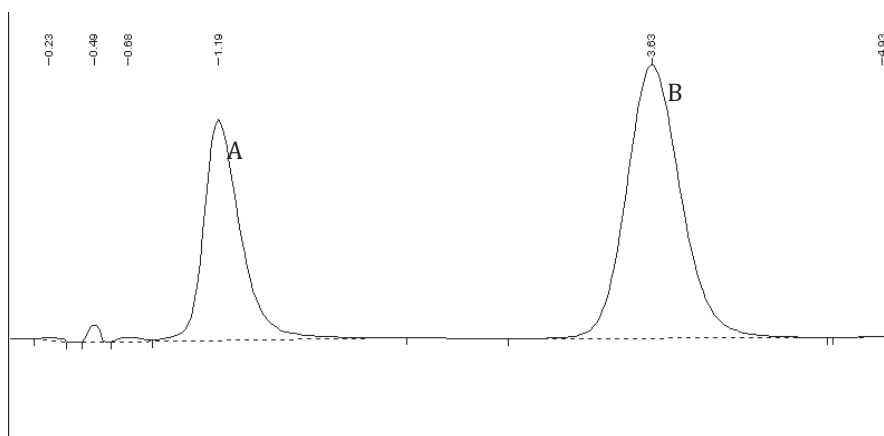


FIGURE 1. CROMATOGRAM OF BOTH THE SODIUM PHOSPHATE AND BASE DEXAMETAZONE STANDARDS A CORRESPONDS TO SODIUME PHOSPHATE DEXAMETHASONE (TR=1.18 min) AND B TO DEXAMETHASONE BASE (TR=3.44 min).

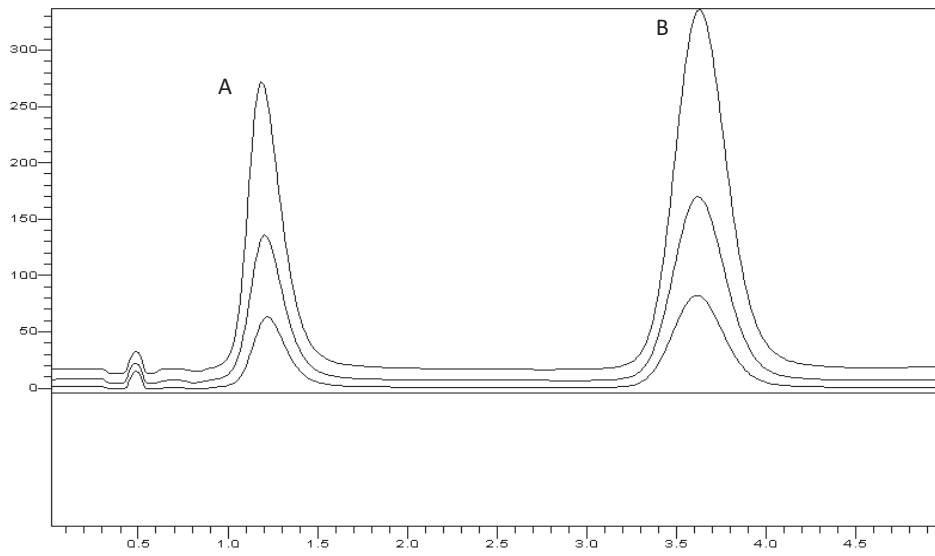


FIGURE 2. CROMATOGRAM OF SODIUM PHOSPATE DEXAMETAZONE STANDARDS (A), (TR=1.19 min) AND OF DEXAMETAZONE BASE (B), (TR=3.44 min) CONCENTRATIONS WERE 0.25, 0.125 AND 0.0625 mg/mL.

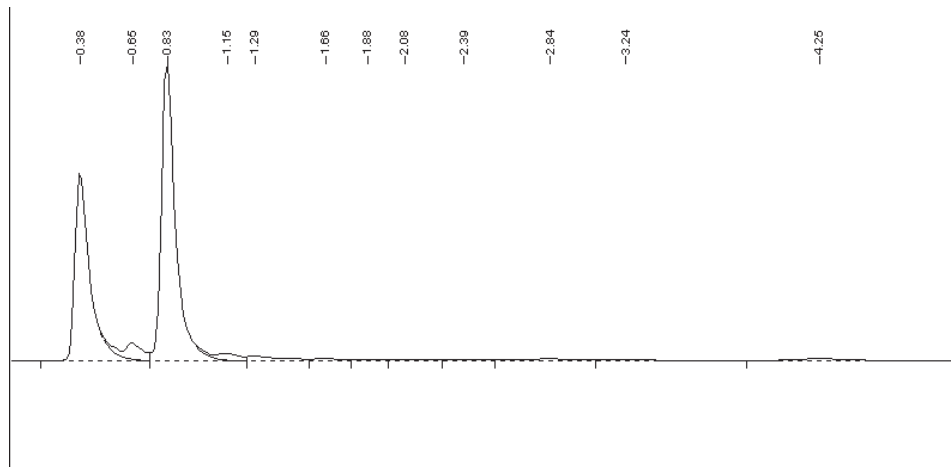


FIGURE 3. CROMATOGRAM OF SODIUM PHOSPATE DEXAMETAZONE AND OF DEXAMETAZONA BASE WITH BOLUS DOSES OF 24 mg AT 0 TIME, WHERE THE DRUG WAS DETECTED.

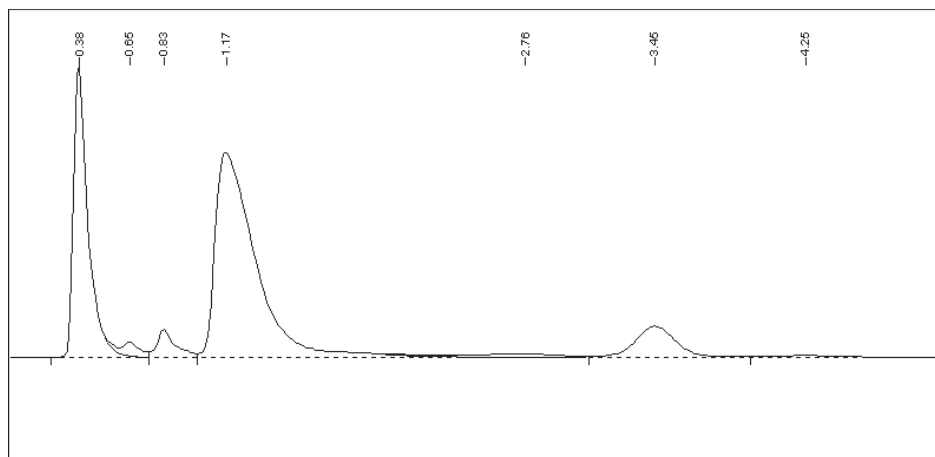


FIGURE 4. CROMATOGRAM OF SODIUM PHOSPATE DEXAMETAZONE AND OF DEXAMETAZONE BASE AT DOSES OF 24 MG VIA INTRAARTICULAR AT TIME 0.25. THE SIGNAL AND THE TIME OF RETENTION TR 1.17 MINUTES CORRESPONDS TO SODIUM PHOSPATE DEXAMETAZONE AND SIGNAL TR 3.45 MINUTES TO DEXAMETAZONE BASE.

To determine the concentration of the drug in the articulation, the height of all of the curves were added as shown in the chromatographs of DSP and DB. These values were then processed using PKA analyst [12] using a two-compartment model with a bolus entrance. The latter indicates the elimination frequency of a drug and should be directly proportional to the concentration of the drug ($P < 0.01$), with a correlation coefficient of 0.99 for the three horses used in this study. The determination coefficient was 0.99 for the horse that received 16 mg of DSP, 0.98 for the horse that received 20 mg of DSP, and 0.99 for the horse that received 24 mg of DSP; R^2 for all three individuals was 0.99, which indicated a strong of association between the variables (time and concentration).

Previous studies have used a simple or serial method for the detection of one compound in each run, which requires multiple samples and the use of different methods and instrumentation [11, 16].

In this work, no interference was observed in the chromatographs, and the separation of the two compounds under study was considered to be satisfactory. Other methods used to determine DBP and DB in plasma and tissues of other species have high degrees of accuracy and precision. However, they also require complex extraction and quantification procedures [1, 5, 6].

CONCLUSIONS

It was concluded that in this work a simple method for the extraction of DBP and DB was carried out with the advantage that minimizes time. Furthermore, the mobile phase and running time are the same even with as little as 0,5 mL of sample. This is advantageous because other methods require a larger volumes for each sample.

It was concluded that metabolites similar to DSB and DB were not detected, which indicates that the method described here is specific for the detection of DSB and/or DB in horse plasma and synovial fluid.

This method would be used for future research that demand a simple and specific test for the determination of DSP and DB in the plasma and synovial fluid of exercising horses.

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