Use of vitamin B_{12} in joint lavage for determination of dilution factors of canine synovial fluid

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Objective—To test a modified saline (0.9% NaCl) solution joint washing (lavage) technique that includes the use of vitamin B_{12} as an internal marker for the evaluation of synovial fluid dilution in lavage samples from canine joints.

Sample Population—9 plasma samples obtained from blood samples of 9 healthy dogs and 9 synovial fluid samples aspirated from stifle joints of 9 cadaveric dogs.

Procedure—Photometric absorbances of 25% vitamin B_{12} solution, canine synovial fluid, and canine plasma were measured in a spectrophotometer to establish an optimal wavelength for analysis. Canine synovial fluid and plasma samples were mixed with the 25% vitamin B_{12} solution to obtain 1%, 3%, 5%, 10%, 20%, and 50% solutions of synovial fluid or plasma. Diluted synovial fluid and plasma samples were used to simulate joint lavage samples and to examine the possible interference of these substances (synovial fluid or plasma) with the absorbance of the 25% vitamin B_{12} solution in photometric analysis.

Results—The optimal wavelength was found to be at 550 nm. Canine synovial fluid and plasma samples did not interfere with the absorbance measurements of the 25% vitamin B_{12} solution up to a 50% dilution of plasma or synovial fluid.

Conclusions and Clinical Relevance—The modified saline solution joint lavage method with the use of a 25% vitamin B_{12} solution as an internal standard provides an accurate and reliable technique for the evaluation of synovial fluid dilution in lavage samples from canine joints. (*Am J Vet Res* 2005;66:1903–1906)

Most canine joints lend themselves to arthrocentesis. The simplicity of the procedure and useful information obtained from analysis of synovial fluid have rendered synovial fluid analysis an almost essential factor in the diagnosis of joint disease.[1] In a study[2] in dogs, measurements of synovial fluid concentrations of molecules or fragments of molecules were made to evaluate disease activity (eg, determination of synovial fluid glycosaminoglycan concentrations for evaluation of cartilage degeneration in dogs with osteoarthritis). However, obtaining a sufficient quantity of synovial fluid from canine joints by arthrocentesis is sometimes difficult. Even from a large joint, such as the stifle joint, it remains difficult to acquire an adequate sample volume when no obvious effusion is found. Alternative methods of obtaining synovial fluid samples have been developed; joint washing (lavage) with saline (0.9% NaCl) solution is 1 method used in human and animal research.[3,4] Hereby, saline solution is injected into the joint cavity and subsequently aspirated.[5-7] This technique frequently yields sufficient sample volume but results in an unknown dilution of synovial fluid, which makes it impossible to make a precise determination of synovial fluid concentrations of any measured substance.[6,7] A modification of the standard joint washing technique to obtain synovial fluid samples in humans has been established by Alstergren et al,[8] with the inclusion of vitamin B_{12} in the saline solution that is used for joint lavage. The use of vitamin B_{12} provides an internal marker of synovial fluid dilution. Vitamin B_{12} (hydroxocobalamin) has a bright red color, making photometric absorbance feasible. It penetrates only biological membranes that have special transport mechanisms; therefore, it remains in the joint cavity during the lavage process.[9-11] Furthermore, it is not pernicious for the surrounding tissues,[10] which is in contrast to other products that can be used for photometric absorbance measurements (eg, methylene blue).[12,13] The purpose of the study reported here was to test a modified saline solution joint washing technique that includes the use of vitamin B_{12} as an internal marker for the evaluation of synovial fluid dilution in lavage samples from canine joints.

Materials and Methods

Test solutions—Vitamin B_{12}[a] was diluted with saline solution[b] to obtain 8% and 25% solutions of vitamin B_{12}. Venous blood samples from 9 healthy 2- to 7-year-old dogs were collected into tubes containing EDTA. Plasma was obtained after centrifugation of blood samples (1,500 × *g* for 10 minutes). Synovial fluid was aspirated from stifle joints of 9 mature cadaveric dogs that were euthanatized for reasons unrelated to the study. Cadaveric dogs lacked any evidence of osteoarthritis or other joint diseases on visual inspection at necropsy.

Photometric comparison—Photometric analyses of vitamin B_{12} solutions as well as for canine synovial fluid and plasma samples were performed at wavelengths of 300 to 650 nm to determine the optimal wavelength for absorbance. Canine synovial fluid and plasma samples were mixed with the 25% vitamin B_{12} solution to obtain 1%, 3%, 5%, 10%,

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20%, and 50% solutions of synovial fluid or plasma. Diluted synovial fluid and plasma samples were used to simulate joint lavage samples and to examine the possible interference of these substances (synovial fluid or plasma) with the absorbance of the 25% vitamin B_{12} solution in photometric analysis.

Sample absorbances were measured by use of a spectrophotometer[c] and 50-µL quartz cuvettes.[d] Detection limit of spectrophotometry is determined by technologic principles of absorptiometry, which are generally accepted to be between an **optical density (OD)** of 0.5 and 2.0. The dilution factor (f) of the vitamin B_{12} solution was calculated as stated by Alstergren et al[14]:

$$f = A/A_o,$$

where A is the sample absorbance value and A_o is the vitamin B_{12} solution standard absorbance value.

The dilution factor can then be used to calculate the true concentration of an endogenous substance in the synovial fluid by use of the following formula:

$$C_s = C_a/(1 - f),$$

where C_s is the synovial fluid concentration of a substance, C_a is the aspirate concentration of a substance, and f is the dilution factor.

Statistical analyses—The Wilcoxon signed rank test was used to compare the absorbance values of canine synovial fluid and plasma samples at a wavelength of 350 versus 550 nm. Pearson correlation coefficients were used to test the significance of correlations. Values of $P < 0.05$ were considered significant.

Results

Absorbance spectra of vitamin B_{12} solutions—Photometric analyses of a 25% vitamin B_{12} solution with 75% saline solution (vol/vol), canine synovial fluid, and plasma samples were performed at wavelengths of 300 to 650 nm (**Figure 1**). Two distinctive peaks were consistently found for the 25% vitamin B_{12} solution. One peak was observed at a wavelength of 350 nm, and a second broader peak was observed at a wavelength of 550 nm. The peak at 350 nm exceeded the upper limit of detection of the spectrophotometer. The second peak at 550 nm did result in an absorbance value within the detection limit. To obtain absorbance values within the detection limits of the spectropho-

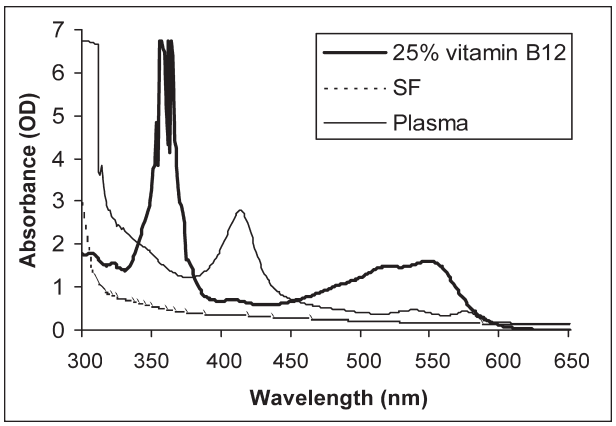

Figure 1—Photometric analysis scans of a 25% vitamin B_{12} solution with 75% saline (0.9% NaCl) solution (vol/vol), canine synovial fluid (SF), and canine plasma performed at wavelengths of 300 to 650 nm. Notice the broad peak for the 25% vitamin B_{12} solution at a wavelength of 550. OD = Optical density.

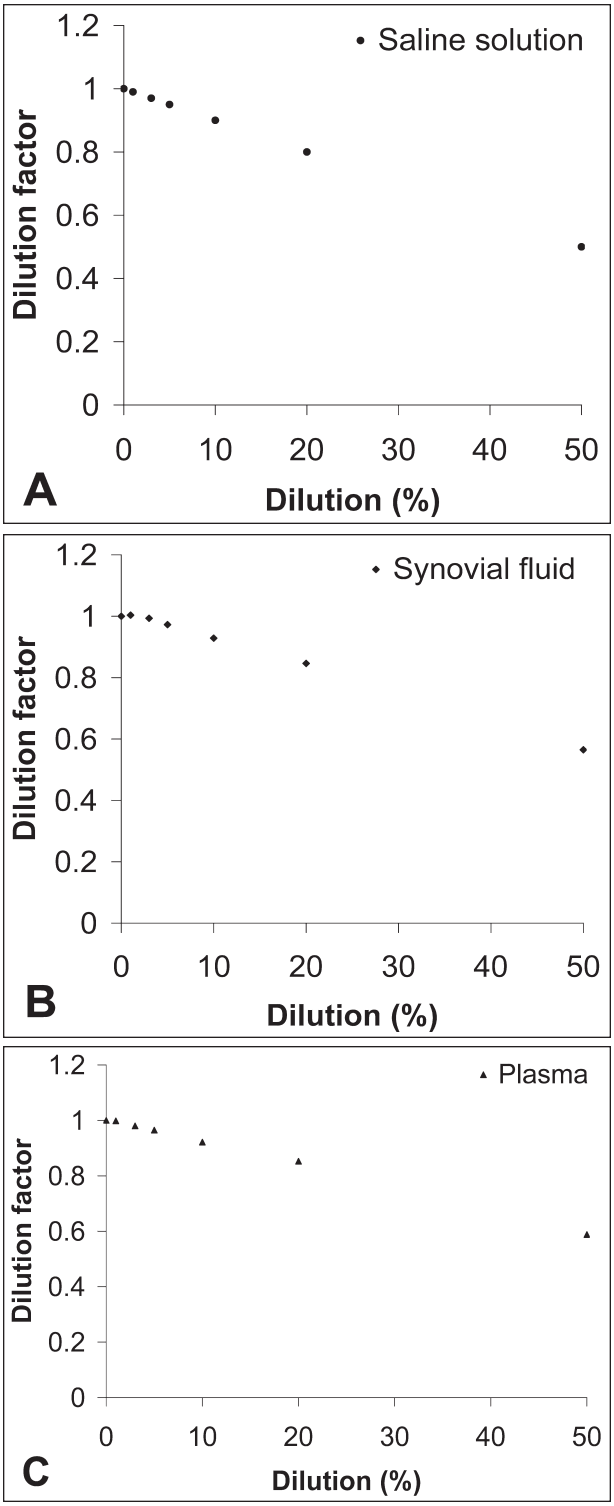

Figure 2—Dilution factors for saline solution, synovial fluid, and plasma mixed with 25% vitamin B_{12} solution to obtain 1%, 3%, 5%, 10%, 20%, and 50% solutions of saline solution (A), synovial fluid (B), or plasma (C). Linear regression equations were as follows: y = –0.01x + 1, y = –0.0089x + 1.0143, and y = 0.0083x + 1.0059 following dilution of saline solution (R^2 = 1), synovial fluid samples (R^2 = 0.998), and plasma samples (R^2 = 0.999) with 25% vitamin B_{12} solution, respectively.

tometer at a wavelength of 350 nm, the 25% vitamin B_{12} solution was diluted with saline solution to an 8% (vol/vol) vitamin B_{12} solution.

Absorbance spectra of canine synovial fluid and plasma samples—The mean ± SD absorbance value of canine synovial fluid samples (n = 9) at a wavelength of 550 nm was an OD of 0.189 ± 0.087, and at a wavelength of 350 nm, it was an OD of 0.59 ± 0.191. The relative contribution of canine synovial fluid to the total absorbance value was significantly less at 550 nm than at 350 nm. The mean ± SD absorbance value of canine plasma samples (n = 9) at a wavelength of 550 nm was an OD of 0.378 ± 0.160, and at a wavelength of 350 nm, it was an OD of 1.694 ± 0.503. The relative contribution of canine plasma to the total absorbance value was significantly less at 550 nm than at 350 nm.

Interference of synovial fluid and plasma with absorbance of vitamin B_{12}—Dilution factors were determined for canine synovial fluid and plasma samples mixed with 25% vitamin B_{12} solution to obtain 1%, 3%, 5%, 10%, 20%, and 50% solutions of synovial fluid or plasma (**Figure 2**). Absorbances were measured at a wavelength of 550 nm. No significant difference was found between calculated dilution factors for canine synovial fluid (R^2 = 0.998) or plasma (R^2 = 0.999) when samples were diluted with the 25% vitamin B_{12} solution.

Discussion

In our study, the use of vitamin B_{12} as an internal standard for the calculation of a dilution factor in canine synovial fluid was evaluated. Vitamin B_{12} has been used in joint lavage of the temporomandibular joint in humans to calculate dilution factors in synovial fluid samples.[7,8,14] Vitamin B_{12}, or hydroxocobalamin, has a bright-red color and is stable in solution, making it suitable for photometric measurements.[15] It does not penetrate biological membranes without special transport mechanisms; therefore, it remains in the joint lumen during lavage.[9-11] Furthermore, vitamin B_{12} is nontoxic, whereas methylene blue is toxic. Methylene blue has been used to quantify bronchoalveolar lavage fluid in humans and in pigs.[12,16] We have successfully tested methylene blue in vitro as an internal marker to calculate the dilution factor in synovial joint fluid. However, complications have been documented after in vivo use of methylene blue in humans (eg, formation of necrotic abscesses after intracutaneous or SC injection, spinal cord necrosis after intrathecal injection, and methylene blue-induced joint effusion).[13,17-20] Because of these alarming complications, we decided not to further evaluate the use of methylene blue but tested the use of vitamin B_{12} instead.

In our study, 2 photometric peaks were consistently found for the 8% and 25% vitamin B_{12} solutions, one at a wavelength of 350 nm and one at a wavelength of 550 nm. Alstergren et al[8,14] have also found 2 distinctive photometric peaks for vitamin B_{12}, one also at 350 nm but the other at a wavelength of 490 nm. The difference in wavelength at the second peak between their study and ours (490 and 550 nm, respectively) may be attributable to the broadness of the peak (Figure 1).

High absorbance values were obtained at a wavelength of 350 nm for the 25% vitamin B_{12} solution (OD, 6.8), which exceeded the upper limit of detection of the spectrophotometer. This is in contrast to Alstergren et al,[8] who also measured 25% solutions of vitamin B_{12} at a wavelength of 350 nm but had absorbance values within the detection limit of their spectrophotometer.[e] We had to dilute the vitamin B_{12} solution to 8% to measure absorbance values at a wavelength of 350 nm. The extreme difference in absorbance values between the 2 studies is hard to explain. The vitamin B_{12} solutions in the 2 studies are composed of 1 mg of hydroxocobalamin/mL of saline solution. The vitamin B_{12} used in our study consists of pure hydroxocobalamin in saline solution. The vitamin B_{12}[f] used by Alstergren et al[7,8,14] consists of hydroxocobalamin with sodium acetate, methyl parahydroxybenzoate, and hydrochloric acid. It is unlikely that these additives resulted in an extreme difference in photometric absorbance between the 2 products because they are not known to have photometric absorbance.

Alstergren et al[14] found that the relative contribution of human plasma to the total absorbance value was lower when measured at a wavelength of 350 nm than at a wavelength of 490 nm. This is important because blood contamination of synovial joint fluid is sometimes unavoidable during lavage and might result in an erroneous interpretation of the dilution factor. In our study, exactly the opposite was found. Namely, the absorbance value of canine plasma measured at a wavelength of 350 nm was approximately 4.5 times the absorbance value measured at a wavelength of 550 nm. This means that the relative contribution of canine plasma to the total absorbance value is substantially higher when measured at a wavelength of 350 nm than at a wavelength of 550 nm. Blood contamination of canine synovial fluid samples is more likely to yield to an erroneous dilution factor when measured at a wavelength of 350 nm. Also, the absorbance value of canine synovial fluid at a wavelength of 350 nm was approximately 3 times the absorbance value at a wavelength of 550 nm. For these reasons, we conclude that 550 nm is the optimal wavelength to use in the determination of absorbance values of canine synovial fluid samples obtained by joint lavage with saline solution that contains 25% vitamin B_{12}.

The extreme difference in mean ± SD absorbance values of human plasma and canine plasma at a wavelength of 350 nm between the study of Alstergren et al[14] and our study (ODs, 0.081 ± 0.028 and 1.694 ± 0.503, respectively) cannot readily be explained. Total protein concentrations are slightly higher in human plasma[21] than in canine plasma[22] (60 to 80 g/L and 54 to 71 g/L, respectively). Therefore, a slightly higher absorbance value would be expected from human plasma. We questioned whether this difference in absorbance could be the result of differences between spectrophotometers. We therefore tested human plasma of 2 volunteers by using the spectrophotometer of our laboratory; no major difference in absorbance was found between human plasma and canine plasma (ODs, 1.525 and 1.694, respectively).

Hemoglobin is a specific protein that can interfere with absorbance values that are measured at a wavelength of 550 nm. The optimal wavelength to measure the absorbance of hemoglobin is 540 nm.[15] Consequently, we advise the exclusion of synovial fluid samples with visible blood contamination or hemolysis from experimental studies.

No significant difference was found between the calculated dilution factors when the 25% vitamin B_{12} solution (as a standard) was mixed with canine synovial fluid or plasma up to a dilution of the 25% vitamin B12 solution of 50%. Dilutions of the 25% vitamin B_{12} solution with synovial fluid up to > 50% can lead to erroneous dilution factors. Consequently, false calculations of the concentrations of endogenous substances in the synovial fluid will be obtained. However, upon in vivo arthrocentesis of canine stifle and shoulder joints, synovial fluid dilution factors ranged from 0.90 to 0.70 (data not shown). This corresponds to 10% to 30% synovial fluid in the joint lavage. The possibility of erroneous dilution factors when this joint lavage technique is used in dogs is unlikely. In addition, joint lavages are needed only if obtaining sufficient sample volume cannot be obtained by direct arthrocentesis.

In conclusion, results of our study indicate that use of a modified saline solution washing (lavage) method with vitamin B_{12} as an internal standard provides an accurate and reliable means to evaluate synovial fluid samples from dogs. The optimal fluid used for lavage contains 25% vitamin B_{12} and 75% saline solution, and the optimal wavelength for photometric analysis of synovial fluid samples is 550 nm.

a. Hydroxocobalamin, 1 mg/mL, Sterop Laboratories, Brussels, Belgium.
b. Plurule, Baxter, Lessines, Belgium.
c. Ultrospec 4000, Pharmacia Biotech, Cambridge, England.
d. Black quartz, 10-mm path length, Pharmacia Biotech, Cambridge, England
e. Shimadzo UV-160A, Shimadzo, Tokyo, Japan.
f. Behepan, Kabi Pharmacia, Uppsala, Sweden.

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