Determination of temporomandibular joint fluid concentrations using vitamin B₁₂ as an internal standard

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The aim of the investigation was to test the reproducibility and accuracy of a new method to measure temporomandibular joint (TMJ) fluid concentrations of various substances by saline washing, using exogenous B₁₂ as a marker. An in vitro test was first performed with glucose as a test substance. The difference between a B₁₂-calculated and known standard concentration of glucose was very small. Saline washing of the TMJ was performed on 13 patients having signs of TMJ arthritis, and the aspirates obtained were analyzed for neuropeptide Y-like immunoreactivity (NPY-LI) and interleukin-1β (IL-1β). Vitamin B₁₂ was mixed with the saline immediately before injection, and a sample of the aspirate was later compared photometrically with the injection solution. There were positive correlations between saline aspirate and joint fluid concentrations for NPY-LI and IL-1β, and the correlations were stronger for saline aspirates with high joint fluid content. This study shows that the method is reliable for measurement of joint fluid concentrations of various substances, such as NPY-LI and IL-1β.

There is ample reason to assume that neuropeptides and cytokines contribute to the local development of joint inflammation (1, 2). The severity of joint inflammation has been correlated to intra-articular release of substance P (SP) (3). In the arthritic temporomandibular joint (TMJ), SP, neuropeptide Y (NPY) and calcitonin gene-related peptide have been found in levels high above plasma levels (4). Also, a strong co-variation has been found between TMJ fluid concentrations of NPY and other neuropeptides (5). Interleukin-1β (IL-1β) is an important mediator of pathophysiological events in rheumatoid arthritis and other forms of acute or chronic inflammatory disease (6), and cytokines in joint fluid are generally held to be important in the mechanism of tissue injury (7). At present, very little is known about the interaction of interleukin-1β and neuropeptides. The ability to measure the exact joint fluid concentration of these substances, as well as others, is important to understand the development of the local inflammatory process in the joint.

In a large joint such as the knee, a joint fluid sample with a volume sufficient for analysis is relatively easy to obtain by direct aspiration. However, in a small joint like the TMJ, it is more difficult to obtain a sufficient sample volume, and sometimes the sample volume is too small even for analysis of one single substance. There are several possible methods to obtain a joint fluid sample, e.g. by direct aspiration through a needle, inserted into the joint cavity, by washing the joint cavity with saline through a needle or by sampling during an arthroscopic or surgical procedure. All these techniques have disadvantages. Direct aspiration seldom yields the joint fluid volume or the amount of substance required for the analyses, and washing of the joint with saline results in a dilution of the joint fluid, which makes it impossible to make a precise determination of the joint fluid concentration.
Determination of TMJ fluid concentrations

The use of arthroscopy or surgical procedures of the joint is not justified for this purpose, and they are, besides, likely to cause a greater alteration in the release of neuropeptides, cytokines and other transmitters than a simple puncture. The method of saline washing of the TMJ, on the other hand, has certain advantages, i.e. smaller risk of trauma to the joint tissues and a sample volume sufficient for analysis of several substances. The aim of this study was to test a new modification of the saline washing method for measuring TM joint fluid concentration of various substances by using exogenous B12 as a marker of joint fluid dilution.

Material and methods

In vitro test

An in vitro test was first performed to investigate the reproducibility and accuracy of the method. Two concentrations (5.35 and 2.80 mM) and two volumes (100 and 250 μl) of glucose standard solutions were used (Table 1). A 1 ml B12-saline solution from a mixture of 0.9 ml Behepan® (vitamin B12, 1 mg/ml; Kabi Pharmacia, Uppsala, Sweden) and a 4.1-ml saline solution was added to each standard glucose solution and 200 μl were taken from each of these solutions for analysis. In order to calculate the glucose concentration in the standard solutions from the B12-glucose samples, the absorbances of the samples and the B12-saline solutions were photometrically compared (λ=490 nm; Microplate EL-311 spectrophotometer, BioTek Instruments, Winooski, MO, USA). A dilution factor, f (=standard solution absorbance/sample absorbance, or clinically, injection solution absorbance/aspirate sample absorbance), was calculated for each sample. The formula below was used to calculate the standard glucose concentrations and joint fluid concentrations.

\[
C_T = \frac{C_S}{(1 - \frac{1}{f})}
\]

where \(C_T\) = true concentration and \(C_S\) = sample concentration. Finally, the glucose concentrations of the standard solutions and samples were analyzed by an enzymatic method described below.

Patients

This study also comprised 13 patients, 11 females and 2 males, with a mean age of 53 yr having signs and symptoms of TMJ arthritis. The mean duration of TMJ disease was 3.9 yr and systemic disease 16.5 yr. Three of the patients had rheumatoid arthritis, one had psoriatic arthritis, three had ankylosing spondylitis, one had osteoarthritis, two had chronic unspecific polyarthritis, and two had common variable immunodeficiency. Three of the patients were examined at two different occasions. All their values were included in the analysis, since the scope of this study was to investigate the accuracy and reproducibility of the method, and not primarily to determine TMJ fluid concentrations. We therefore consider the repeated measurements to be independent.

Joint fluid sampling

TMJ anesthesia was achieved by blocking the auriculotemporal nerve with 1.0–2.0 ml 2% Lidocain (Xylocain®, Astra, Södertälje, Sweden). The TMJ was punctured with a standard disposable needle (diameter=0.65 mm), which was inserted into the posterior part of the upper joint compartment. Saline was injected into the TMJ with a push-and-pull technique. One ml saline was injected from a syringe into the joint through a three-way stopcock, whereafter as much as possible of the injected saline was aspirated back into a second syringe (Fig. 1). The injection was repeated four times, making a total of 4.0 ml saline injected. The following method was used to determine the distribution of the joint fluid and saline in the aspirate. A volume of 0.9 ml Behepan was mixed with 4.1 ml saline immediately before injection and the mixture was kept in darkness. A small volume of the joint aspirate (0.25 μl) was then compared photometrically with a sample from the initial injection solution to calculate the dilution factor (f). This factor was then used to calculate the true joint fluid concentration with the formula given above.

<table>
<thead>
<tr>
<th>Sample volume μl</th>
<th>Glucose</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample mM</td>
<td>Calculated mM</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.10</td>
<td>0.44</td>
</tr>
<tr>
<td>SEM</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.28</td>
<td>1.13</td>
</tr>
<tr>
<td>SEM</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.25</td>
<td>0.55</td>
</tr>
<tr>
<td>SEM</td>
<td>0.02</td>
<td>0.00</td>
</tr>
</tbody>
</table>
To compensate for infrequent hemolysis in the samples, the content of hemoglobin was measured with the HiCN-method as standardized by ICSH (The International Committee for Standardization in Haematology) and a standard curve of hemolysis absorbance was made. The total absorbance of the sample was in this way compensated for the additional absorbance due to hemolysis.

The samples with a dilution factor \( f \) greater than 1.1, which means more than 10% joint fluid in the aspirate, were investigated separately in order to find out whether the amount of joint fluid in the aspirate has any significance for the measurement.

Analyses

The glucose concentrations were determined with a commercially available kit (Sigma Chemical Co., St. Louis, MO, USA). The joint fluid samples were analyzed for neuropeptide Y-like immunoreactivity (NPY-LI). Samples were extracted using a reverse-phase C18 cartridge (Waters Sep-Pak; Millipore, Milford, MA, USA) and analyzed by a competitive radi immunoassay (8–10). The concentrations of IL-1\( \beta \) in the samples were determined with an ACETM Human Interleukin-1\( \beta \) ELISA (Enzymelinked immunosorbent assay) kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Statistics

The statistical significance of the correlation between the joint fluid and saline aspirate concentrations was calculated by Pearson’s product-moment correlation coefficient. The probability level of \( P<0.05 \) was considered significant. The variability of the ratio between aspirate and joint fluid concentrations as well as between calculated and standard glucose concentrations was estimated by the standard error of the mean (SEM).

Results

In vitro

The results of the in vitro experiments are shown in Table 1. The coefficient of correlation between the calculated and standard concentrations was for all samples: \( r=0.84 \) \( (P<0.001, n=15) \) and for sample volume 250 \( \mu l \): \( r=0.99 \) \( (P<0.001, n=10) \). In the combination of a sample volume of 100 \( \mu l \) and a standard concentration of 2.80 mmol/l the concentration of glucose was below the detection limit for the method used.

Patients

The mean saline aspirate and joint fluid concentrations of NPY-LI and IL-1 are shown in Table 2. The concentration of NPY-LI in the saline aspirates was positively correlated to the calculated joint fluid concentrations \( r=0.73, P<0.01, n=20 \). When \( f>1.1 \) the corresponding values were \( r=0.90, P<0.001 \) and \( n=15 \) (Fig. 2). The concentration of IL-1\( \beta \) in the saline aspirates was also positively correlated to the calculated joint fluid values \( r=0.66, P<0.001, n=25 \). When \( f>1.1 \) the corresponding values were \( r=0.95, P<0.001 \) and \( n=13 \) (Fig. 3).

Discussion

Vitamin B\( _{12} \) has important characteristics for the use as an internal standard to calculate the dilution factor. Firstly, it has a suitable optical absorbance spectrum for measurement, with a peak at 490 nm. Secondly, it does not penetrate biological barriers.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Neuropeptide Y</th>
<th>Interleukin-1( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aspirate</td>
<td>Joint fluid</td>
</tr>
<tr>
<td>Total</td>
<td>261</td>
<td>2426</td>
</tr>
<tr>
<td>( n )</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>SEM</td>
<td>35</td>
<td>958</td>
</tr>
<tr>
<td>( f&gt;1.1 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n )</td>
<td>262</td>
<td>1222</td>
</tr>
<tr>
<td>SEM</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

- Table 2 shows the saline aspirate and joint fluid concentrations (pM) of neuropeptide Y and interleukin-1\( \beta \). \( f \)-dilution factor (B\( _{12} \)-glucose standard absorbance/sample absorbance), SEM=standard error of the mean. Number of samples in each test=5.
membranes without special transport mechanisms and therefore remains in the joint cavity during saline washings. Especially the latter condition is important, since most other molecules would diffuse through the synovial membrane into the capsular tissue and become out-of-reach for aspiration. Vitamin B₁₂ has not been used as a marker for this purpose, but has been used autologously as a marker for measurement of in vitro clearance and elimination during hemofiltration (11) and in intestinal disease research (12, 13). Interference in measurement procedures due to the B₁₂ complex is very infrequent (14). No reports of interferences in immunoassays of regulatory peptides have been reported.

In the in vitro part of this study, the dilution factors calculated (1.10 and 1.28/1.25) were very close to the true values (1.10 and 1.25, respectively), demonstrating an accurate determination of the dilution. The small SEM of the dilution factors shows that this method has a high degree of reproducibility. The calculated concentration of any substance with this method relies upon two factors, i.e. the dilution factor and the determination of sample concentration, and its accuracy and reproducibility will depend on both. However, the accuracy and reproducibility of the determination of the dilution factor by B₁₂ are independent of any biochemical analyzing method.

IL-1β and NPY-LI showed relatively strong correlations between their joint fluid and saline aspirate concentrations. This implies that the aspirate concentrations can be used for relative assessment of the joint fluid concentrations provided the procedure is performed with this standardized technique. It is quite clear from our results that the ratio between the true joint fluid concentration and the saline aspirate concentration differ between samples and patients. However, the SEM of the mean ratios was low, if dilution factors greater than 1.1 are considered. It therefore seems that the dilution factor, i.e. the joint fluid concentration of the aspirate, influences the accuracy of the clinical measurements, and only aspirates with a dilution above a certain limit should be used for quantification. Accordingly, the aspirate should preferably contain at least 10% joint fluid.

The presence of hemoglobin in the aspirates was compensated for in the clinical measurements if there were signs of hemolysis in the sample. A small bleeding occurs occasionally upon puncture of joints, and more likely in joints with arthritis. The higher the degree of inflammation and hyperemia, the higher the risk for an intra-articular bleeding. The main reason for the compensation is that hemoglobin and vitamin B₁₂ are quite close in the optical absorbance spectrum. Hemoglobin has its absorbance maximum at a wavelength of 540 nm, while B₁₂ has its maximum at 490 nm. Hemolysis of erythrocytes, and subsequent release of hemoglobin, increases the absorbance at 490 nm and renders an erroneously low dilution factor.

In conclusion, this study shows that the present method is reliable for measurement of joint fluid concentrations of various substances, such as NPY-LI and interleukin-1β.

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References


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