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VITAMIN B1, B2, B6 AND B12 LEVELS IN SERUM AND CEREBROSPINAL FLUID OF DROMEDARY CAMELS (*Camelus dromedarius*) AFFECTED WITH NEUROLOGICAL SIGNS

Meligy A.M.A.[1] and El Nahas A.[1]
[1]Department of Clinical Sciences, College of Veterinary Medicine,
King Faisal University, Al-Ahsa, Saudi Arabia

ABSTRACT

This study was designed to evaluate and correlate CSF chemical analysis specially Vitamin B1, B2, B6 and B12 in the diagnosis of neurological manifestations in 13 camels (aging 2-19 years). The studied vitamins in affected animals were compared to healthy. Additionally, the current results of vitamin concentrations of Vitamins B1, B2, B6 and B12 in cerebrospinal fluid and serum samples were determined using HPLC assay. The results revealed that the concentration of those vitamins in the CSF was significantly less than in serum.

Key words: Biochemical, camel, CSF, UPLC–MS/MS, neurological, serum

The vitamin status of the camel is not well known, it needs more studies and the number of references is low, which little work has been done on vitamins in camel specially B vitamins (Faye and Bengoumi, 2018). Usually, B vitamins are synthesised in ruminants by ruminal flora (Mohamed, 2006). There were several methods for the determination of vitamins B including the spectrophotometric method of determination of vitamin B1 in a pharmaceutical formulation was simple, accrued, and rapid but depends on the colorimetric reaction mechanism (Shaopu Liu *et al,* 2002), method of high-performance liquid chromatography (HPLC) (Moreno and Salvado, 2000) and method of liquid chromatography/mass spectrometry (LC-MS/MS) which is an efficient, accurate and stable in the analysis of vitamins, it has a unique advantage of sensitivity, specificity and also the ability to be applied to antibiotics, drugs or metabolite analysis in any biological samples (Geng *et al,* 2017; Roelofsen *et al,* 2017; Nicholas *et al,* 2016).

The determination of normal chemical values of cerebrospinal fluid in different animal species has been documented including cattle, sheep, horses, dogs, cats, and some laboratory animals (Roberta and Simon, 2009; Achaaban *et al,* 2009; Naziji and Maleki, 1998; Arneri and Mousavian, 2007; Stocker *et al*, 2002 and Welles *et al,* 1992). Generally, there is a lack of information on normal CSF constituents and their normal values in camels. Recently Shawaf *et al* (2018) reported some values of CSF constituents from healthy camels in Saudi Arabia. Cerebrospinal fluid may provide a wide range of valuable biochemical and cellular information in the diagnosis of neurological disorders, that helps in the evaluation of the nervous system health of animals (AI-Sagair *et al,* 2005; Frosini *et al,* 2000; Welles *et al,* 1992).

Dubduba syndrome in an emerging viral neurological disease of camels (Al-Dubaib *et al,* 2008). Al-Swailem *et al* (2010) diagnosed cerebral listeriosis in a she camel with neurological signs, i.e. lock of coordination, Parkinson's like tremors of head and lower lip paralysis. Babelhadj *et al* (2018) detected prion disease in camels of Algeria. Authors detected pathgnomonic neurodegeneration and disease specific prion protein (PrP^{sc}) in brain tissues of camels. It is also known as camel spongiform encephalopathy or mad camel disease.

Wernery *et al* (2004) described lock jaw and stiff gait as predominant neurological sign in case of tetanus in a camel.

Neurological disorders present a significant sanitary and economic risk to the animal production industry worldwide (Lecollinet *et al,* 2019). Vitamin B1 (Abbas *et al,* 2008), B6 (Ahmad *et al,* 2013), and B12 (Nijst *et al,* 1990) are closely associated with

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neurological functions. However, Vitamin B1 deficiency has been defined in camels as responsible for polioencephalomalacia (PEM), which is called cerebrocortical necrosis and irritating severe nervous signs (Faye and Bengoumi, 2018). Mohamed (2006) reported data for vitamin B12 in the plasma of a healthy camel. It is important to recognise the relation between levels of vitamin B and neurological disorders in camel because there are indications that some of the diseased camels with neurological disorders may improve after receiving doses of vitamin B (Bhandare, 2009). This study was designed to evaluate the levels of vitamins B1, B2, B6 and B12 in serum and CSF of affected camels by using a unique and accurate technique (ultra-performance liquid chromatography coupled with mass spectrometry) UPLC/MSMS system to help in the diagnosis of neurological manifestations in camels.

Materials and Methods

2.1 Ethical Approval

All experimental procedures and management conditions used in this study regarding blood and CSF sampling in camels were approved by the Ethics Committee at King Faisal University, Saudi Arabia (Permission number KFU-REC/2020-10-06).

2.2 Animals and sampling

Thirteen dromedary camels (aging 2-19 years) with a history of neurological signs were presented to the Veterinary Teaching Hospital, King Faisal University were investigated in this study. The main neurological signs shown by these animals were shivering, tremor, staggering, rotation of the head, slight vision impairment, and progressive worsening general condition. It excluded other diseases through haematological and chemical analysis. Five healthy camels were used for comparison. The animals were examined clinically, and then blood samples were obtained from the jugular vein for serum preparation. After sedation and aseptically preparation of the caudal part of the neck, CFS samples were taken from the Atlanto-occipital articulation according to Shawaf *et al* (2018).

2.3 Vitamins determination

2.3.1 Materials and Reagents

Vitamins B1, B2, B6, and B12 were purchased from ACROS ORGANICS (New Jersey, USA, 1-800-ACROS-01, Geel, Belgium). Pyridoxine hydrochloride (B6, 99%; extra pure, HPLC assay, lot A0310405, code: 150770500). Thiamine hydrochloride (B1, 98.5-101%; extra pure HPLC assay, lot: A0309985, code: 148990100. Riboflavin (B2, 98% extra pure, HPLC assay, lot: A0305365, code: 132350250). Cyanocobalamin (B12, 96% extra pure, HPLC assay, lot: A0304024, code: 405920010). Ethyl acetate, water, ammonium acetate, caffeine, and methanol HPLC grade were purchased from Sigma Aldrich (USA).

2.3.2 Sample Preparation

Serum samples 250 µL, 25 µL (0.5 mM) NaOH and 25 µL Caffeine (Internal standard, analytical grade) (1µg mL^{-1}) were vortexed for a half min, then 2 mL of ethyl acetate solvent (EA) was added and vortexed again for 2 min, followed by refrigerated centrifuge at 4000 rpm for 6 min at 4°C. The supernatant (upper organic layer) was separated and then transferred into another ppt (polypropylene tube), it evaporated under a gentle flow of nitrogen gas to dryness at 37°C. The residue of supernatant was reconstituted with a 0.1 ml mobile phase, and 10µL was injected for analysis according to Geng *et al* (2017).

2.3.3 The procedure of UPLC–MS/MS Conditions

Ultra-performance liquid chromatography coupled with mass spectrometry (UPLC–MS/MS) analysis was done according to Geng *et al* (2017). An ultra-performance liquid chromatography (UPLCTM) system Acquity (Waters, Milford, MA, USA) was interfaced with a triple quadruple mass spectrometer (UPLC/MSMS) (TQDTM, Waters Micro mass, Manchester, UK) using an electrospray interface. Vitamins B1, B2, B6, and B12 and IS in serum were separated, using an Acquity UPLC, C18 analytical column (150 × 4.6 mm, particle size 3 µm, 100A) (Waters). The eluted with the mobile phase of methanol: ammonium acetate 5 mM (60:40, v/v) with a flow rate of 0.5 mL min^{-1} and the temperature of the oven was 30°C. Multiple reaction monitoring (MRM) mode was used for the detection of vitamin B1, B2, B6, B12, and internal standard (IS), with ion transition of m/z 265 → 122.1, 377 → 244, 170 → 152, 678.6 → 359.0, 195.1 → 137.8, respectively. Calibration curves were prepared for serum sample extracts (after reconstitution in mobile phase) were spiked with different aliquots of Vitamins B standard solution to give final concentrations. The calibration samples consisted of six different levels of vitamin B1 (1, 5, 10, 20, 50, 100 ng mL^{-1}), B2 (5, 10, 20, 50,100, 200ng mL^{-1}), B6 (1, 5, 10, 20, 40, 80 ng mL^{-1}) and B12 (5, 10, 20, 30, 40, 50 ng mL^{-1}). Quality control samples of LOD (limit of detection), for vitamin B1 were 1, 2, 50 and 160 ng mL^{-1}, for B2: 5, 10, 20, 35 ng mL^{-1},

for B6: 1, 2, 20, 75 ng mL^{-1}, and for B12: 5, 10, 20, 40, 50 ng mL^{-1}, respectively.The amount of vitamin was calculated as the calibration line y = ax + b. Quality control and quantification purposes were according to Geng *et al* (2017).

2.4 Statistical analysis

Data were recorded in Excel spreadsheets and imported into Stata version 14 (Stata Corp., TX, USA) for further analyses. Descriptive statistics (mean, SEM) were calculated for each parameter. Variation within each parameter was evaluated using the coefficient of variation (CV). The effects were considered significant at $P < 0.05$.

Results

Figures 1, 2, 3, 4 and Table 1 showed serum and CSF levels of Thiamine (B1), Riboflavin (B2), Pyridoxine (B6), and Cobalamin (B12) in healthy and camels with neurological signs. Thiamine (B1) levels showed a significant decrease in the serum of affected camels than healthy ones as well as CSF of affected and healthy. Riboflavin (B2) levels showed a significant decrease in the serum of affected camels than healthy ones but there was no significant difference in CSF of affected and healthy animals; on the other hand, there was a highly significant difference in the serum compared to CSF in healthy animals but with the same significant difference in affected animals. Pyridoxine (B6) levels showed a significant decrease in the serum of affected camels than healthy ones as well as CSF of affected and healthy, Cobalamin (B12) levels showed a significant decrease in the serum of affected camels than healthy ones as well as CSF of affected and healthy, on the other hand, there was a highly significant difference in the serum compared to CSF in healthy animals.

Discussion

Using LC-MS/MS which has higher sensitivity and specificity, is an accurate alternative. However, Electrospray ionisation (ESI) is an effective means of ionising the B vitamins, but it is sensitive to matrix effects which change the relative response between samples and standards due to changes in ionisation efficiency (Geng *et al,* 2017; Roelofsen *et al,* 2017; Nicholas *et al,* 2016). Vitamins are vital for health status, and their lack could affect the animals (Faye and Bengoumi, 2018). Vitamin B1 or thiamine is a coenzyme, which is a needed phase in the synthesis of fatty acids, nucleic acids, aromatic amino acids and steroids, and the precursors to neurotransmitters and bioactive compounds vital for neurological tract function (Kerns *et al,* 2015). Thiamine deficiency has been defined in camels as responsible for polioencephalomalacia (PEM), which is called cerebrocortical necrosis, irritating severe nervous signs Faye and Bengoumi (2018). However, Kiupel *et al* (2005) and Oliveira *et al* (1996) reported that sulfur toxicity is a cause for thiamine deficiency in lama and ruminants, respectively. Mohamed (2006) and Wernery *et al* (2009) reported more levels for thiamine in the serum of healthy animals than in the present study. However, Abbas *et al* (2008) stated that the concentration of thiamin in the serum of a healthy camel calf was less than that reported in the present study. The different results of thiamine in the serum of healthy camels in the present study as compared to previous studies could be attributed to training and feeding type, which affecting microflora by produce thiamin. The results of decreased thiamine in the serum of affected camels in the present study are in agreement with most previous studies (Brent and Bartley, 1984; Faye and Bengoumi, 2018; Kiupel *et al,* 2005; Milad and Ridha, 2009; Nema *et al,* 2014; Rachid *et al,* 2011; Ramos *et al,* 2005). In agreement with our results, Wernery *et al* (2009) reported similar results for thiamine in the serum of healthy camels. However, Abbas *et al* (2008) reported lower results for thiamine (21±10 µg/l) in the serum of affected camels with neurological signs than those reported in the present study. To our best knowledge, there is a lack of information on the concentration of thiamine in cerebrospinal fluid in animals. The decreased thiamine in CSF of

Table 1. The mean concentrations of vitamin B1, B2, B6 and B12 in serum and CSF samples healthy and affected obtained LC–MS/MS methods (mean ± SEM).

	Serum Healthy		Serum Affected		CSF Healthy		CSF Affected	
	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range
B1 (mg/L)	53.56 ± 2.64	40.45-70.56	36.49 ± 1.23	30.37-41.41	48.53 ± 2.92	40.14-70.03	36.9 ± 1.05	30.5-45.13
B2 (mg/L)	350.57 ± 55.05	50.96-650.05	137.01 ± 6.02	100.09-165	55.71 ± 17.64	3.01-330.12	27.61 ± 5.88	3.76-140
B6 (mg/L)	12.63 ± 2.1	5.2-32.2	5.76 ± 0.34	4.9-7.8	4.68 ± 0.28	2.8-6.6	3.28 ± 0.2	2.2-4.9
B12 (ng/100ml)	44.8 ± 1.77	34-59	30.84 ± 0.92	19.6-37	14.21 ± 0.48	9.9-14.1	8.6 ± -55	4.9-13.2

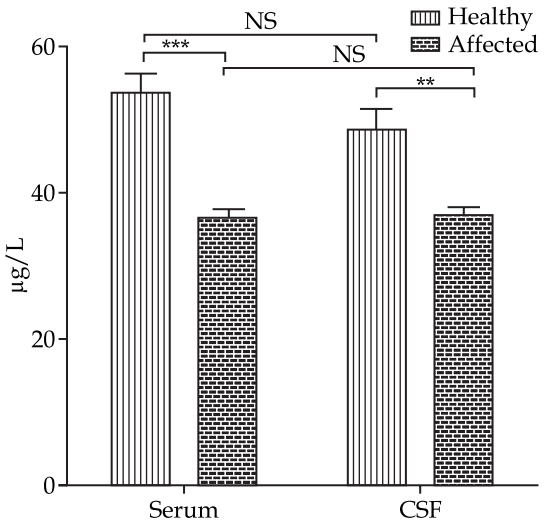

Fig 1. Thiamine (B1) levels (µg/l) in serum and cerebrospinal fluid (CSF) of healthy and camel with neurological disorders. NS>0.05, **<0.01, ***<0.001.

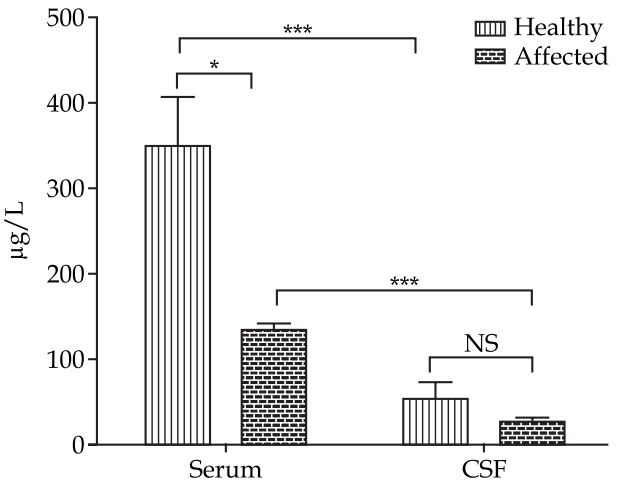

Fig 2. Riboflavin (B2) levels (µg/l) in serum and cerebrospinal fluid (CSF) of healthy and camel with neurological disorders. NS>0.05, *>0.05, **<0.01, ***<0.001.

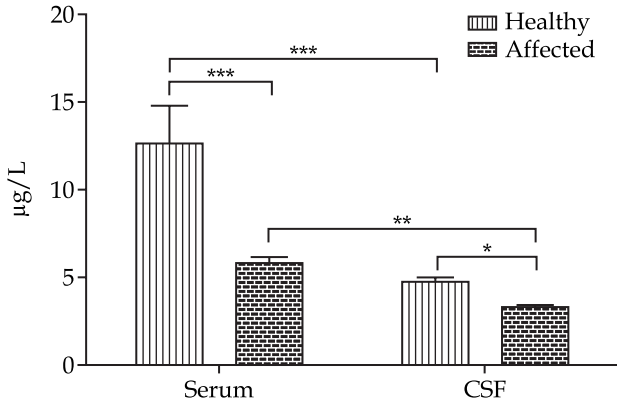

Fig 3. Pyridoxine (B6) levels (µg/l) in serum and cerebrospinal fluid (CSF) of healthy and camel with neurological disorders. *>0.05, **<0.01, ***<0.001.

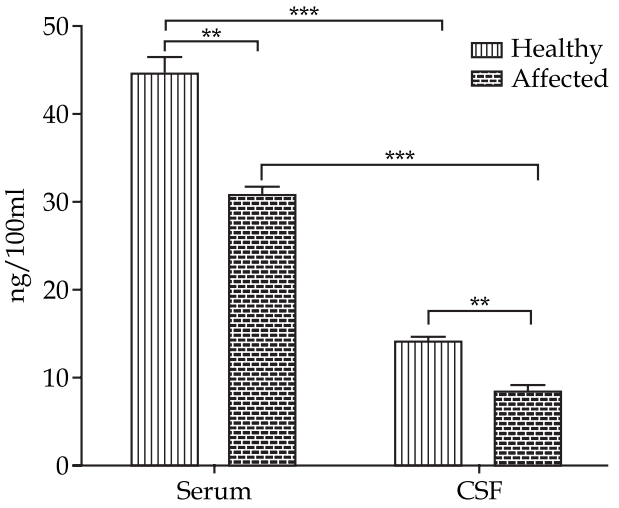

Fig 4. Cobalamin (B12) levels (ng/100ml) in serum and cerebrospinal fluid (CSF) of healthy and camel with neurological disorders. **<0.01, ***<0.001.

healthy camels comparing to its concentration in serum agreed with Spector and Johanson (2007). In agreement with our results, Pedraza and Botez (1992) reported lower levels of thiamin in CSF than those in blood in people affected with ataxia. Similar to our results for thiamine concentration in CSF of healthy and affected animals Jimenez-Jimenez *et al* (1999), found no difference for thiamine levels between healthy and affected people.

There is no data on plasma (Faye and Bengoumi, 2018) or CSF riboflavin concentration in camels. However, it is stated that there are riboflavin entered and transported to CSF at the blood-brain barrier, which can control the riboflavin in the CSF (Spector and Johanson, 2014). The treatments using vitamin B injection including riboflavin in camel affected by hematuria appeared effective (Bhandare, 2009). The deficiency of riboflavin in the current study in affected animals may be attributed to the fact that the diseased animals with neurological symptoms suffered from loss of appetite or difficulty in eating, or there was a decrease in the formation of riboflavin in the digestive system, as a result, some digestive tract disorder leads to neurological symptoms.

Vitamin B6 is closely associated with the function of the nervous system (Ahmad *et al,* 2013). There are no previous studies that reported vitamin B6 concentration in the blood of healthy or diseased

camels (Faye and Bengoumi, 2018). The results of vitamin B6 in the serum of healthy camels in the present study were higher than those reported in people (Spinneker *et al,* 2007). On the other hand Bisp *et al,* (2002); Jungert *et al* (2020) reported lower results for Vitamin B6 in plasma than those reported in the present study. The variation in our results among previous studies in human may be due to the different nature of nutrition between ruminants and humans, as the camel eats plant-based foods while their digestive system synthesises this vitamin (Wernery *et al,* 2009), while humans depend on their animal-based foods with has a higher bioavailability to absorb vitamin B6 (Reynolds, 1988). On the other hand, the method of analysing this compound in the blood, as there are many associated forms of it (Spinneker *et al,* 2007), could affect the variation of its levels among studies (Zhang *et al,* 2018). There is a relationship between B6 levels in serum and CSF (Albersen *et al,* 2015). Spector and Johanson (2007) reported higher results for vitamin B6 in CSF of a rabbit than that reported in the current study. However, Albersen *et al* (2014) reported details for parts of vitamin B6, who stated that pyridoxal phosphate and pyridoxic acid were higher in plasma than in CSF, while pyridoxamine and pyridoxal were higher in CSF than plasma.

Vitamin B12 deficiency was not clinically reported in the camel (Faye and Bengoumi, 2018). Mohamed (2006) reported lower results for cobalamin concentration in plasma (around 25 ng/100 ml) of a healthy camel than that reported in the present study (44.8 ng/100 ml). In contrast to our results for the levels of cobalamin for healthy camels, lower levels were reported in the plasma of healthy sheep (Clark *et al,* 1989). In agreement with the levels of cobalamin in the serum of healthy camels in the present study, Kather *et al* (2020) reported a similar range in dogs. Indeed, higher results for vitamin B12 levels in the serum of healthy people and people affected with neurological diseases were reported comparing to the present results in healthy and diseased camels (Nijst *et al,* 1990). However, Christine *et al,* (2020); Regland *et al* (1992) reported a strong correlation in Vitamin B12 levels between serum and CSF. Higher results were reported for vitamin B12 in CSF of healthy people compared to results in the current study (Nijst *et al,* 1990). Similar studies for higher results of vitamin B12 for serum and CSF in both healthy and affected people were reported (Christine *et al,* 2020; Simpson 1964). The discrepancy of results for B12 in serum and CSF of healthy and affected camels in the current study may be due to several factors, including the pathogenesis of the neurological disorders in camels, which are still not well studied.

Conclusions

We concluded that the affected camel with neurological signs had a deficiency in vitamins B1, B2, B6 and B12.

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