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ORIGINAL ARTICLE

Evaluation of Automated Determination of Bilirubin and Oxyhemoglobin in Cerebrospinal Fluid Using the DrugLog© Instrument

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SUMMARY

Background: **In a heterogenous group of patients with acute headache it is important to diagnose subarachnoid hemorrhage (SAH), a potentially lethal but treatable condition, with short turnaround time and high precision. Spectrophotometry of cerebrospinal fluid (CSF) is an essential part in the investigation of patients with suspected SAH but the analysis is slow and operator dependent.**
Methods: **We have evaluated a new instrument for ultraviolet and visible light (UV-VIS) spectroscopy (DrugLog©, Pharmacolog, Uppsala, Sweden) for automatic determination of oxyhemoglobin and bilirubin in CSF samples. The instrument incorporates software for calculating the absorbance values thus eliminating operator bias. Bilirubin and oxyhemoglobin in CSF was analyzed both with DrugLog© and traditional spectrophotometry at 415 and 476 nanometers (A415 and A476) using patient samples containing varying amounts of bilirubin and oxyhemoglobin.**
Results: **The DrugLog© method showed a strong correlation both for bilirubin (Pearson's r = 0.996) and oxyhemoglobin (Pearson's r = 0.993). The DrugLog© method had good linearity and precision, offering an automated determination of bilirubin and oxyhemoglobin, eliminating operator bias.**
Conclusions: **The DrugLog© instrument has a short assay time and showed good agreement with traditional spectrophotometry.**
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INTRODUCTION

Acute subarachnoid hemorrhage (SAH) is a severe disease with a mortality of approximately 60% [1]. Many of the patients surviving will have sequelae such as neurological and cognitive deficits as a consequence of the SAH [2]. The annual incidence is estimated to be approximately 6 - 7 cases per 100,000 persons [3].
Since it is a potentially treatable condition, early and correct diagnosis is vitally important to minimize morbidity and mortality due to SAH [4]. The investigation

of patients with SAH includes computer tomography (CT) [5]. However, CT scans are negative in a subgroup of patients, and lumbar puncture (LP) followed by cerebrospinal fluid (CSF) spectrophotometry is therefore considered an essential investigation in these patients [6]. If the time between the SAH and the CT scan is long there is an increased risk of negative results. Also the risk of negative findings is also higher if the SAH was small, as in a warning leak [6]. In both groups spectroscopic analysis of the CSF is indicated. It is important to identify patients with warning leaks since cerebral aneurysms, potentially amenable to intervention, can be found and treated before severe SAH occur. There are a number of different methods to analyze CSF from patients with suspected SAH. After CSF hemorrhage has occurred, the red blood cells undergo lysis and phagocytosis. The released oxyhemoglobin is converted *in vivo* into bilirubin, and sometimes methemoglobin [7]. Only bilirubin arises solely from *in vivo* conversion and is thus considered the most specific marker. The CSF investigation includes the analysis of oxyhemoglobin and bilirubin. Some laboratories perform visual inspection of the CSF, but it has previously been demonstrated that spectrophotometry has increased sensitivity over visual inspection [8].

Most European laboratories seem to follow the UK guidelines [8]. In the guidelines, the preferred method for detection is scanning by spectrophotometry. Spectrophotometric scanning for the diagnosis of SAH is time consuming, relatively expensive and often requires interpretation to define baselines. The results are thus often influenced by the operator and it may be difficult to have trained staff available outside office hours.

Considering the drawbacks of spectrophotometric scanning, we wanted to develop an automated evaluation of the scanning spectrophotometry that was operator independent and provided similar results at all hours of the day. Consequently, in a prospective study we evaluated the UV-VIS spectroscopy on the DrugLog© spectrophotometer and accompanying software for determination of bilirubin and oxyhemoglobin by comparing the results with results obtained by a standard spectrophotometer.

MATERIALS AND METHODS

The CSF samples used were routine requests sent to the Department of Clinical Chemistry and Pharmacology, Uppsala University Hospital, Uppsala. The Uppsala University ethical committee approved the method comparison study (01-367). The ethical permit limits the patient information to age and gender and the samples had to be surplus samples without patient identity. As the samples were surplus samples without any patient identity it was not possible or required to obtain informed consent from the patients. The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

The CSF samples were spiked with hemoglobin and bilirubin. Hemoglobin was prepared by washing human red blood cells three times with phosphate buffered saline (PBS, 0.02 M Na_2HPO_4, 0.15 M NaCl, pH 7.2) and then lysing the red blood cells with 3 parts distilled water. Bilirubin was purchased from Merck (No. 2011, Merck, Darmstadt, Germany). The bilirubin was used to prepare a bilirubin stock solution.

UV-VIS spectroscopy (DrugLog©, Uppsala, Sweden) was used for all spectrophotometric scans. DrugLog© collects transmittance spectra which can easily be translated into absorption spectra. PBS was used as blank. For method comparison the samples were also analyzed on a Shimadzu® UV 1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Five CSF samples spiked with different amounts hemoglobin and bilirubin were measured over a period of five days. At day one, the five samples were brought to room temperature and vortexed. For each sample, 600 µL was transferred to a cuvette, Brand UV-cuvette micro and four consecutive spectrophotometric scans were collected with DrugLog©. For each sample, four aliquots of 600 µL were stored in 1.5 mL safe lock Eppendorf tubes at -20°C for later measurements. The following four days, one aliquot of each sample per day was brought to room temperature, vortexed, and transferred to a cuvette before four spectrophotometric scans were collected. Prior to the first scan of the day, a cuvette with PBS was prepared and scanned to be used as a reference. Total CVs at 415 and 476 nm were calculated. The total CV of one sample monitored for five days was determined as the standard deviation divided by the mean absorbance value. The standard deviation and mean were calculated in Excel using the stdev.s and mean functions. As arguments for the functions, the mean values for each day were used.

A dilution series of hemoglobin spiked CSF samples was prepared by mixing the sample with PBS in the range from 100 to 0%. Ten samples in the series were prepared. The 100% sample was measured with DrugLog© in triplicate to get assigned values. The expected value of each sample in the series was then calculated from the assigned values and dilution factors. All samples were measured in duplicates at 415 nm. The deviation from expected values was calculated.

Absorbance at 415 nm on 76 CSF samples spiked with hemoglobin were measured on both Shimadzu UV 1800 and DrugLog©. Absorbance at 476 nm on 73 CSF samples spiked with bilirubin were measured on both Shimadzu UV 1800 and DrugLog©. The results were evaluated using R studio.

Five samples of native cerebrospinal fluid samples spiked with hemoglobin and bilirubin distributed in the Swedish external quality assessment (EQA) program for the diagnosis of SAH as arranged by Equalis (Uppsala, Sweden) 2017 - 2018, and thereafter stored in -80°C, were spectrophotometrically scanned with DrugLog© and compared with consensus mean absorbance values in the EQA program. The total number of partici-

Table 1. The daily means, total means, total standard deviations (SD) and total CVs for five cerebrospinal fluid samples from the precision study with UV-VIS spectroscopy.

Day	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Day 1	0.72	0.36	0.14	0.12	0.05
Day 2	0.74	0.35	0.13	0.12	0.05
Day 3	0.73	0.36	0.14	0.13	0.06
Day 4	0.73	0.36	0.14	0.13	0.07
Day 5	0.72	0.36	0.15	0.17	0.06
Total mean	0.73	0.36	0.14	0.13	0.06
SD	0.009	0.005	0.008	0.020	0.009
Total CV (%)	1.30	1.30	5.40	15.28	15.27

Table 2. Linear dilution of a cerebrospinal fluid sample to study linearity. The expected values were calculated from dilution factors and the assigned values to the 100% sample.

% sample	Expected absorbance values	Observed absorbance values	Recovery (%)
100	0.721	0.720	99.88
80	0.577	0569	98.63
60	0.433	0.435	100.68
40	0.288	0.281	97.49
20	0.144	0.141	97.71
10	0.072	0.069	95.05
5	0.036	0.034	94.06
2.5	0.018	0.022	121.97
1.25	0.009	0.009	96.06
0	0.000	0.001	100.00

pants in the program has been 15 - 20.

RESULTS

The 5-day precision study showed CVs lower than 16% for all samples with absorbance values ranging from 0.05 up to 0.73. Data are presented in Table 1.

The stability was tested daily up to 5 days storage at -20°C. The observation of the measured samples over time are shown in Figure 1.

The total CV for all samples was less than 16%. Linear dilution of a cerebrospinal fluid sample to study linearity. There was very good linearity with $R^2 = 0.9994$ and a high correlation between observed and expected absorbance values at 415 nm with a slope close to 1.0. See Figure 2. The same linearity study is also presented in Table 2.

The method comparison data shows very high correlation coefficients of absorbance values between Shimadzu® UV 1800 and DrugLog© at both 415 nm and 476 nm, see Figure 3, Figure 4, and Table 3.

The results obtained with the DrugLog© were compared with results produced by other laboratories using external quality assurance (EQA) provided by the Swedish EQA organization Equalis. The DrugLog© method provided slightly higher results than the consensus mean value for the participants in the EQA program but there was good agreement between the results (Figure 5 and Table 4).

DISCUSSION

Identification of blood in CSF with UV-VIS spectroscopy can be done with high precision, repeatability, and with excellent agreement with spectroscopy methods. UV-VIS spectroscopy on DrugLog© provides rapid, point of care CSF analysis and the software helps the clinician to identify bilirubin and oxyhemoglobin in the

Table 3. Absorbance measurements at 476 nm using Shimadzu® UV 1800 and DrugLog©.

Shimadzu® UV 1800	DrugLog©
0	-0.009 *
0	-0.007 *
0.007	0.069
0.008	0.073
0.009	0.161
0.010	0.064
0.012	0.182
0.013	0.164
0.036	0.141
0.039	0.122
0.044	0.192
0.045	0.170
0.045	0.169
0.046	0.132
0.046	0.150
0.047	0.194
0.047	0.167
0.050	0.150
0.051	0.123
0.053	0.164
0.067	0.148
0.069	0.144
0.088	0.250
0.141	0.157
0.196	0.384
0.197	0.322
0.208	0.354
0.208	0.409
0.210	0.298
0.210	0.358
0.335	0.288
0.338	0.434
0.373	0.298
0.376	0.548
0.728	0.823
0.861	0.962
0.862	0.938
0.867	0.954
0.866	0.945
1.701	1.700
0.000	-0.005 *
0.000	-0.002 *
0.096	0.205

Table 3. Absorbance measurements at 476 nm using Shimadzu® UV 1800 and DrugLog© (continued).

Shimadzu® UV 1800	DrugLog©
0.044	0.220
0.046	0.082
0.047	0.129
0.048	0.114
0.050	0.112
0.060	0.132
0.070	0.134
0.070	0.102
0.070	0.171
0.072	0.143
0.089	0.191
0.093	0.210
0.107	0.218
0.108	0.296
0.109	0.211
0.110	0.193
0.115	0.124
0.200	0.337
0.206	0.312
0.206	0.287
0.371	0.502
0.374	0.461
0.376	0.470
0.378	0.481
0.563	0.573
0.571	0.585
0.583	0.662
0.728	0.838
0.730	0.821
0.737	0.815
0.872	0.950
0.928	0.962
0.934	0.998
0.943	0.998
1.695	1.723
1.698	1.686
1.702	1.731

*** Samples not included in Figure 4 since negative values not allowed in the Passing Bablok function used**

CSF samples.

SAH is an acute condition that may occur at any time and the test should be performed without unnecessary

Table 4. Comparison of DrugLog© with participant consensus mean value for samples used in external quality assurance (EQA).

Equlis material	Number of Equalis measurements	Mean absorb Equalis	Standard deviation Equalis	CV Equalis	Absorbance value DrugLog©
2017.01A NBA	3	0.0008	0.004	48.2	0.0013
2017.01A NOA	12	0.0571	0.002	4.3	0.0667
2017.01A 415 nm	19	0.0827	0.005	5.5	0.0963
2017.01A 455 nm	12	0.0173	0.003	15.3	0.0215
2017.01B NBA	13	0.0354	0.002	4.4	0.0416
2017.01B NOA	12	0.0625	0.003	4	0.0738
2017.01B 415 nm	18	0.1044	0.005	4.6	0.1198
2017.01B 455 nm	13	0.0696	0.004	5.2	0.0781
2018.01A NBA	3	0.0005	0.0004	75.5	0.0022
2018.01A NOA	12	0.0841	0.0041	4.9	0.0920
2018.01A 415 nm	19	0.1147	0.0037	3.3	0.1257
2018.01A 455 nm	12	0.0200	0.0015	7.4	0.0253
2018.01B NBA	13	0.0503	0.0029	5.8	0.0576
2018.01B NOA	12	0.0506	0.001	2.1	0.0541
2018.01B 415 nm	19	0.0849	0.0026	3.1	0.0959
2018.01B 455 nm	12	0.0812	0.0021	2.6	0.0903
2018.02A 415 nm	17	0.0155	0.002	12.9	0.0204
2018.02A 455 nm	11	0.0100	0.0017	16.6	0.0145

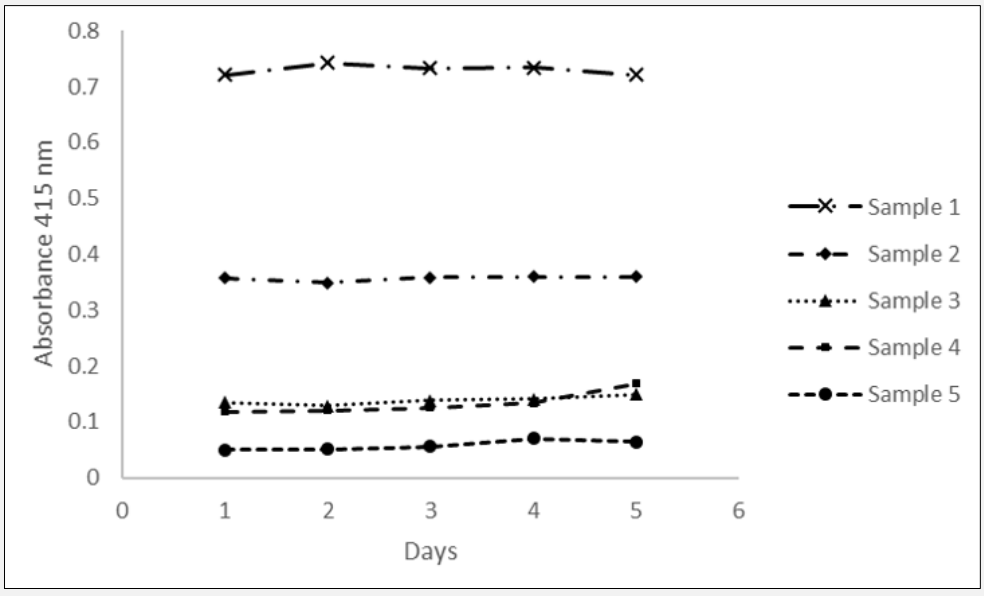

Figure 1. Five cerebrospinal fluid samples with varying hemoglobin contents were stored for up to 5 days at -20°C and were tested each day to investigate sample stability.

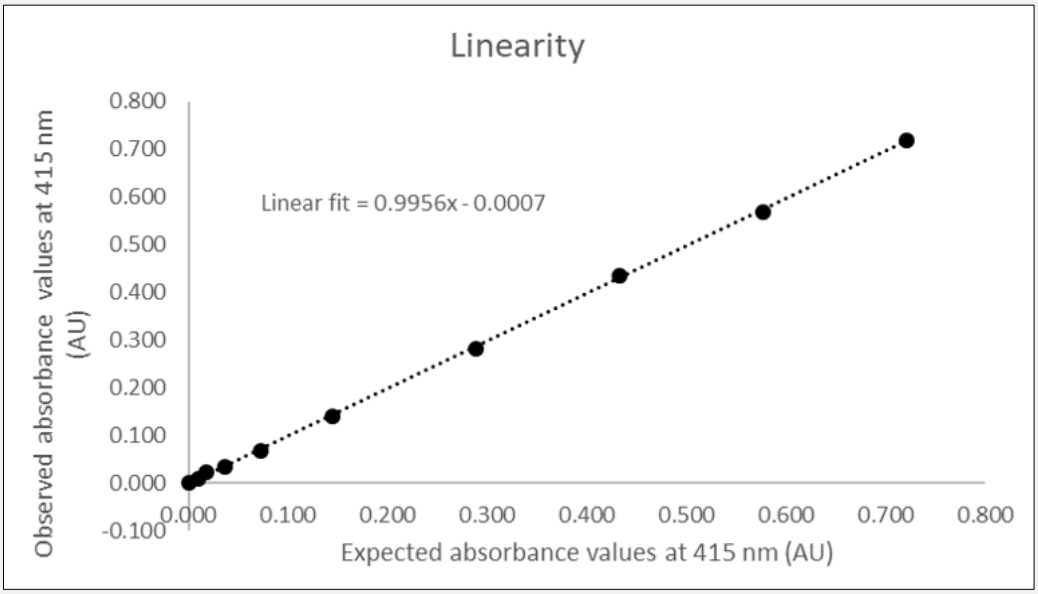

Figure 2. Dilution of a cerebrospinal fluid sample to investigate linearity of the DrugLog© instrument at 415 nm.

The observed absorbance values are on the y-axis and the expected absorbance values on the x-axis. The expected values were calculated from dilution factors and the assigned values to the 100% sample.

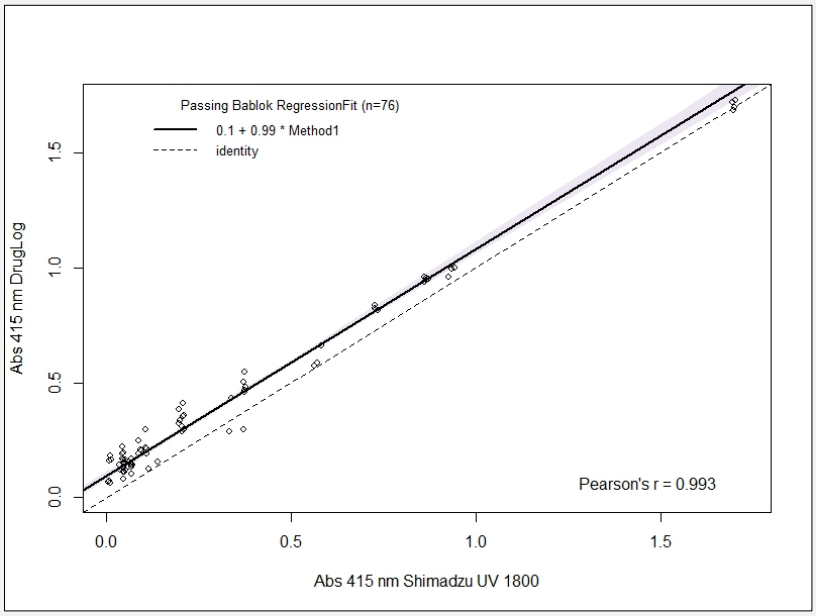

Figure 3. Passing Bablok method comparison between hemoglobin measurements at 415 nm with Shimadzu UV 1800 (x-axis) and DrugLog© (y-axis).

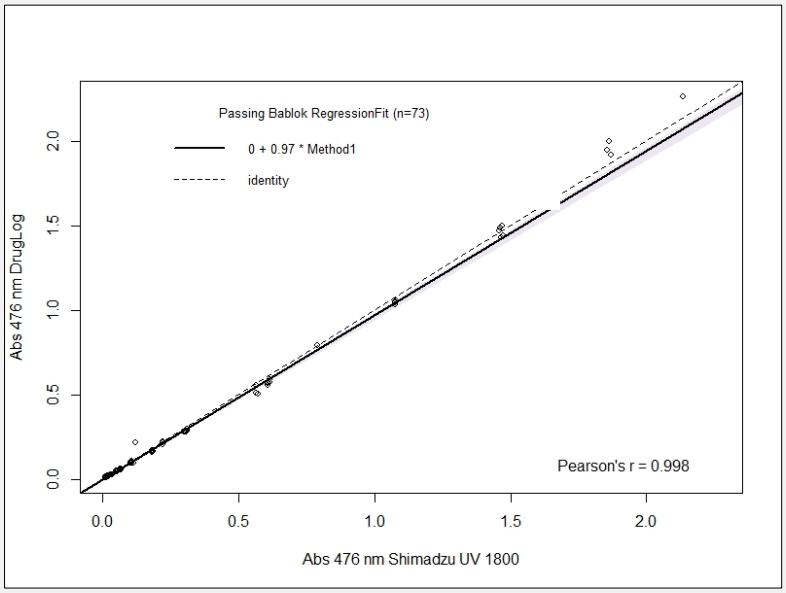

Figure 4. Passing Bablok method comparison between bilirubin measurements at 476 nm on DrugLog© (y-axis) and Shimadzu® UV 1800 (x-axis).

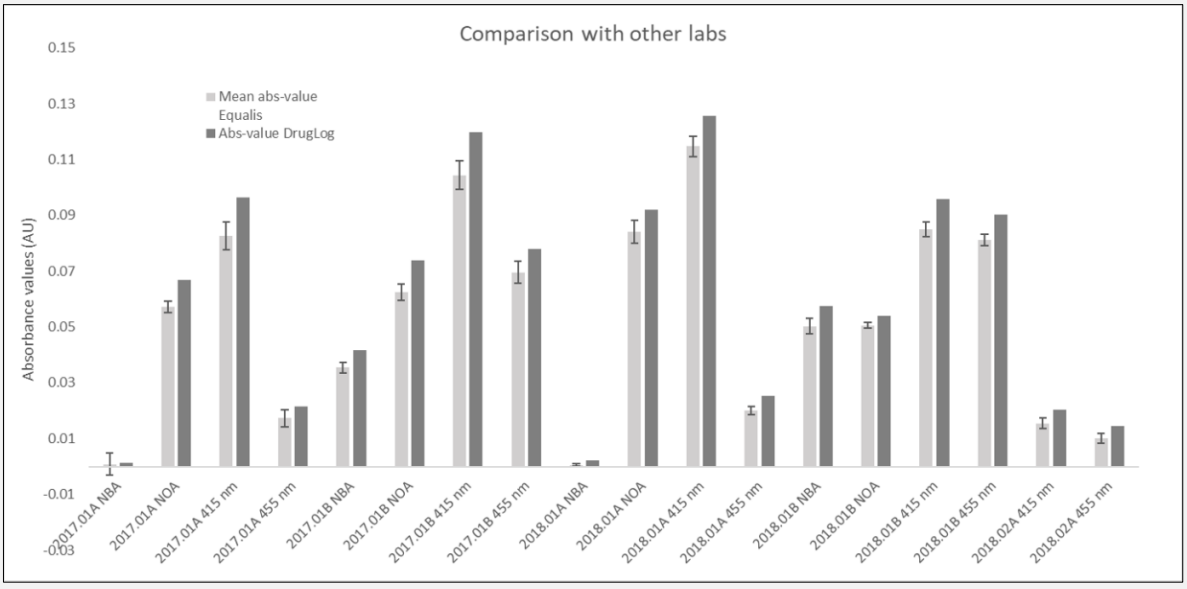

Figure 5. Comparison of DrugLog© with participant consensus mean value for samples used in external quality assurance (EQA).

The names of the distributed EQA materials, including the year they were sent out are presented on the X-axis. The consensus mean value for the participants in the EQA program and the DrugLog© values are presented on the y-axis. The error bars for the EQA material represent one standard deviation for the consensus mean.

delays [9]. Although an imperfect test [10,11], the CSF spectrophotometry is held as the gold standard in the diagnosis of SAH, and when combined with CT has been shown to detect SAH reliably [12]. This means that the laboratory measurements often will be performed by staff that do not perform the assay regularly with limited support from the rest of the laboratory staff. When an assay is rarely performed, this will most likely increase the inter-operator variability for analyzing the samples e.g., how the baseline is drawn. The DrugLog© instrument draws the baseline automatically based on UK guidelines [8] and will thus minimize inter-operator variability. The difference between no bleeding and SAH carries important clinical consequences. This may put additional stress on the operator when performing the assay manually. Replacing the manual technique with an automated measurement will make the measurements more reproducible and hopefully reduce the stress for the operator. The start-up time for the instrument and the assay time is less than 2 minutes and the assay can thus be performed very rapidly, contributing to a short test turnaround time.

There was very good correlation between traditional spectroscopy and the DrugLog© results in the present study showing that DrugLog© will give similar results as the traditional spectroscopy for both oxyhemoglobin and bilirubin. The agreement was also verified with external quality assurance (EQA) materials provided by the Swedish external quality assurance program organized by Equalis (Uppsala, Sweden). The DrugLog© gave slightly higher values than the consensus mean values for the EQA materials. The method had a low total coefficient of variation and good linearity.

In the future, prospective studies in patient cohorts with suspected SAH should be performed with the DrugLog© instrument to evaluate the performance of the UV-VIS spectroscopy in diagnostics of SAH in clinical practice.

CONCLUSION

The DrugLog© instrument offers a rapid, objective method for analyzing bilirubin and oxyhemoglobin in cerebrospinal fluid samples.

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Declaration of Interest:
Hans Dahlin and Magdalena Andersson were employed by Pharmacolog at the time of the study. The other authors declare no conflict of interest. The funding organizations played no role in study design; the collection, analysis and interpretation of data; the writing of the report; or the decision to submit the report for publication.

References:

1. Steiner T, Juvela S, Unterberg A, et al. European Stroke Organization guidelines for the management of intracranial aneurysms and subarachnoid haemorrhage. Cerebrovasc Dis. 2013;35:93-112 (PMID: 23406828).

2. Swartz RH, Bayley M, Lanctôt KL, et al. Post-stroke depression, obstructive sleep apnea, and cognitive impairment: Rationale for, and barriers to, routine screening. Int J Stroke. 2016;11:509-18 (PMID: 27073189).

3. de Rooij NK, Linn FH, van der Plas JA, Algra A, Rinkel GJ. Incidence of subarachnoid haemorrhage: a systematic review with emphasis on region, age, gender and time trends. J Neurol Neurosurg psychiatry. 2007;78:1365-72 (PMID: 17470467).

4. Muehlschlegel S. Subarachnoid Hemorrhage. Continuum (Minneap Minn). 2018;24:1623-57 (PMID: 30516599).

5. Long B, Koyfman A, Runyon MS. Subarachnoid Hemorrhage: Updates in Diagnosis and Management. Emerg Med Clin North America. 2017;35:803-24 (PMID: 28987430).

6. de Falco FA. Sentinel headache. Neurol Sci. 2004;25 Suppl 3: S215-7 (PMID: 15549540).

7. Marlet JM, Barreto Fonseca Jde P. Experimental determination of time of intracranial hemorrhage by spectrophotometric analysis of cerebrospinal fluid. J Forensic Sci. 1982;27:880-8 (PMID: 7175468).

8. Cruickshank A, Auld P, Beetham R, et al. Revised national guidelines for analysis of cerebrospinal fluid for bilirubin in suspected subarachnoid haemorrhage. Ann Clin Biochem. 2008;45: 238-44 (PMID: 18482910).

9. Ogunlaja OI, Cowan R. Subarachnoid Hemorrhage and Headache. Curr Pain Headache Rep. 2019;23:44 (PMID: 31123920).

10. Ditta M, Galea J, Holland J, Patel HC. Lumbar puncture and the diagnosis of CT negative subarachnoid haemorrhage: time for a new approach? Br J Neurosurg 2013;27:599–602 (PMID: 23448246).

11. Sayer D, Bloom B, Fernando K, et al. An observational study of 2,248 patients presenting with headache, suggestive of subarachnoid hemorrhage, who received lumbar punctures following normal computed tomography of the head. Acad Emerg Med. 2015;22:1267–73 (PMID: 26480290).

12. Vermeulen M, Hasan D, Blijenberg BG, Hijdra A, van Gijn J. Xanthochromia after subarachnoid haemorrhage needs no revisitation. J Neurol Neurosurg Psychiatry. 1989;52:826-8 (PMID: 2769274).