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Experimental Determination of Time of Intracranial Hemorrhage by Spectrophotometric Analysis of Cerebrospinal Fluid

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ABSTRACT: A method for determining the time elapsed after a cerebrovascular accident by examination of the cerebrospinal fluid (CSF) is described. Hemorrhagic intracranial trauma was simulated in dogs by injecting blood into the subarachnoid space. Daily samples of cerebrospinal fluid were gathered during the subsequent 14 days, and the concentrations of oxyhemoglobin and bilirubin were measured using spectrophotometric methods. The hemoglobin coefficient (HC), defined as the ratio of the oxyhemoglobin concentration to the sum of the concentrations of the cerebrospinal fluid pigments, and the hemoglobin-bilirubin index (HBI), the ratio of the oxyhemoglobin and the bilirubin concentrations, are found to permit accurate calculation of the time elapsed after the hemorrhage.

KEYWORDS: pathology and biology, hemorrhage, cerebrospinal fluid, hemoglobin, bilirubin, spectrophotometry

It is common practice in forensic medicine to deal with patients having cranial trauma with meningeal hemorrhage resulting from an accident, injury, or trauma of unknown cause. It could be necessary, in a medical examination, to indicate, as precisely as possible, the moment such a hemorrhage occurred. After intracranial hemorrhage, the cerebrospinal fluid (CSF) exhibits yellow-reddish spots resulting from contamination by blood pigments. This coloring is known as xanthochromia, and the blood pigments involved are hemoglobin, methemoglobin, and bilirubin. Because the two last-named pigments derive from the first (the cells of the reticuloendothelial system transform the hemoglobin from hemolyzed erythrocytes into bilirubin), it is obvious that as their concentrations increase the hemoglobin concentration must decrease. Thus, an indication of the time elapsed from the beginning of the hemorrhage till the moment of examination can be obtained from the relationship between the amounts of the two pigments.

The concentrations of those pigments can be measured by either chemical or physical techniques. The latter are usually simpler to perform, yet they yield fully satisfactory results. One of the simplest yet most powerful physical techniques is spectrophotometry. It is espe-

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cially applicable to the problem being discussed here, since the pigments responsible for xanthochromy absorb maximally in the visible portion of the electromagnetic spectrum.

In the region between 400 to 500 nm, the maxima of absorption of the two principal hemoglobin pigments are located at the following wavelengths: 412 to 415 nm for oxyhemoglobin and 430 to 480 nm for bilirubin. Figures 1 and 2 indicate that the positions of these absorption maxima are constant, independent of the pigment concentration. The mixture of pigments, however, has an additive effect on the spectral absorption curve, that is, the absorbance of the mixture is equal to the sum of the absorbances of each of the components of the mixture. This is illustrated in Fig. 3.

Since the pioneer works of Froin (1903), cited by Scully [*1*], and Nonne (1910 and 1914), cited by Robinson and Miller [*2*] and Scully [*1*], the physiological mechanism responsible for xanthochromy of the cerebrospinal fluid (CSF) and its interpretation have been the object of intense study. Merritt and Fremont-Smith [*3*] state that xanthochromy begins 4 h after the start of meningeal hemorrhage, increases in intensity up to the seventh day, and disappears after approximately 20 days. Barrows et al [*4*] concluded that oxyhemoglobin is responsible for the initial coloring of the CSF. Its concentration was found to reach its highest level a few days after hemorrhage and then gradually disappear; the bilirubin concentration, on the other hand, increased progressively to its maximum level and then decreased, disappearing in two or three weeks. Kronholm and Lintrup [*5,6*] and Kronholm [*7,8*] made a detailed study of the spectral absorption curves of the chromatic components of the xanthochromic cerebrospinal fluid. Levere et al [*9*] discussed new spectrophotometric techniques for the measurement of hemoglobin.

Method

Dogs were used for two reasons: their large size and their availability. The 50 healthy dogs selected were all nearly the same size and weight (10 kg). No special selection as to sex was made. Only 21 of the animals were evaluated for three reasons: (1) some died during the experiment, (2) several samples of CSF were lost because of technical difficulties at the time of puncture, and (3) two dogs developed meningitis and had to be killed.

For 14 days after anesthesia by intravenous injection of thiamylal (30 mg/kg body weight),

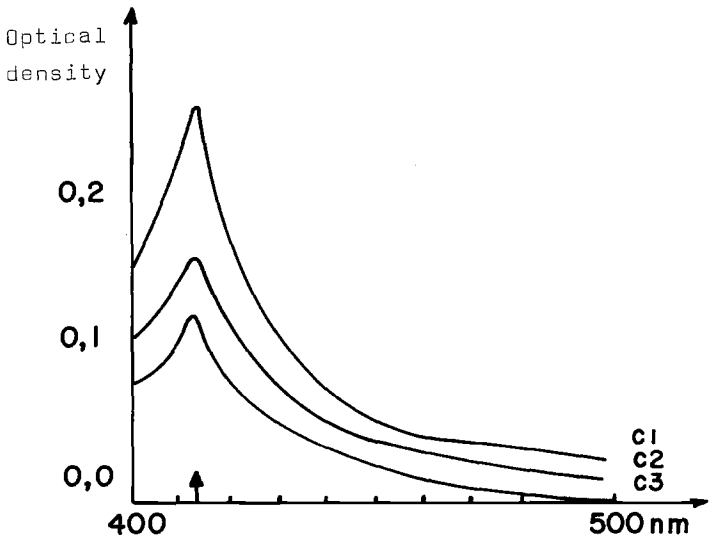

FIG. 1—*Spectral absorption curves of pure hemoglobin solutions of three concentrations (C1 > C2 > C3).*

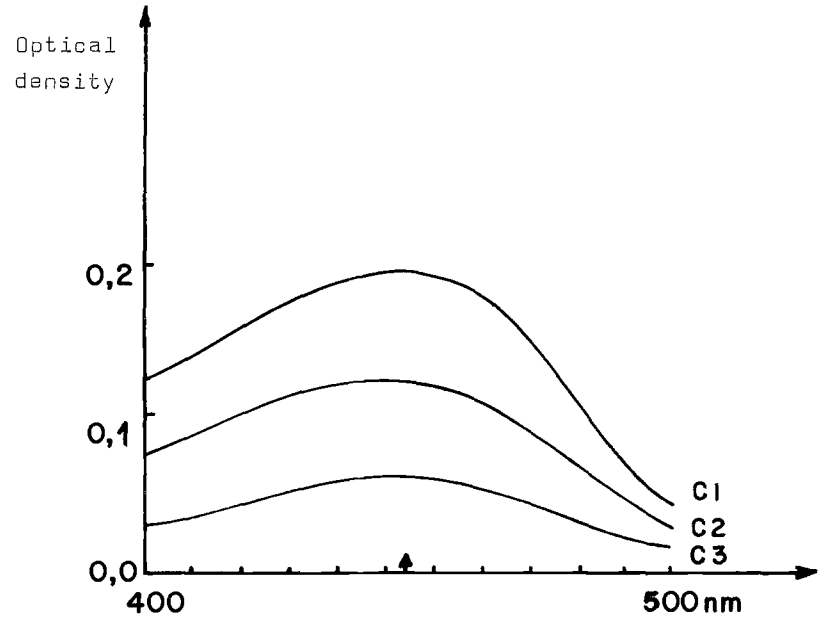

FIG. 2—*Spectral absorption curves of pure bilirubin solutions of three concentrations (C1 > C2 > C3).*

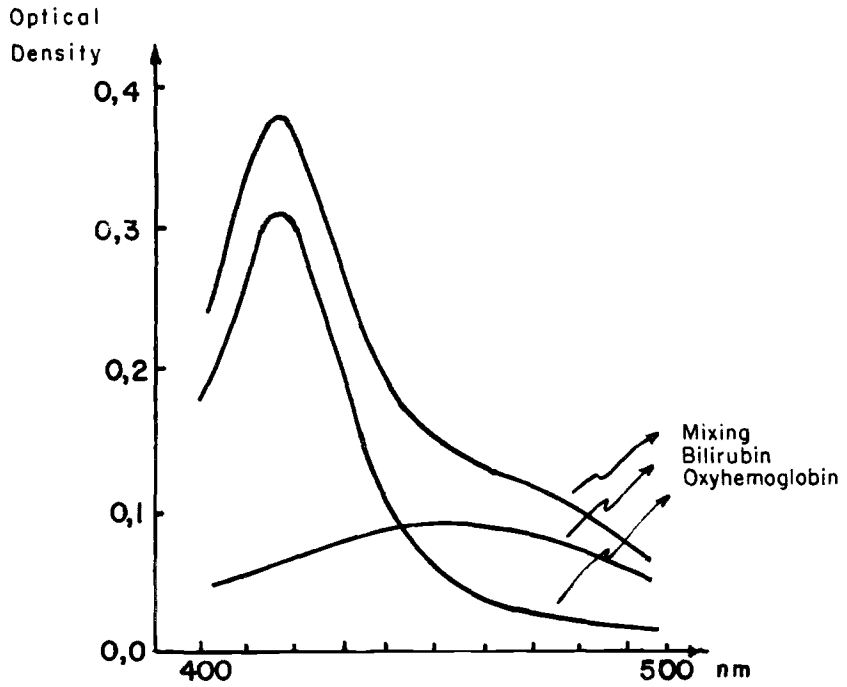

FIG. 3—*Spectral absorption curves of pure solutions of hemoglobin and bilirubin and of a mixture of the two.*

each dog was subjected daily to one suboccipital puncture. On the first day after obtaining a cerebrospinal fluid sample, we injected 1 mL of the animal's own blood into the subarachnoid space, simulating hemorrhage.

During the next 14 days, we collected daily cerebrospinal fluid samples. Within one half hour of the collection, we centrifuged the samples at nearly 2000 rpm for 10 min. This was done to avoid artifacts caused by a possible hemorrhage at the moment of puncture. The extinction coefficient at 415 nm was determined for duplicate samples of supernatant using water as a standard.

We traced the spectral absorption curve over the interval from 390 to 500 nm with a Cary recording spectrophotometer, Model 14, Serial 225, using a cell of 1.0-cm path length and a slit width of 0.03 cm. Figure 4 is a typical absorption spectrum of canine CSF gathered six days after hemorrhage.

The absorbance at wavelengths of 412 and 480 nm, corresponding to oxyhemoglobin and bilirubin, respectively, was read from the curve, and, using the nomogram presented by Kronholm and Lintrup [5] and reproduced in Fig. 5, we were able to calculate the concentrations of oxyhemoglobin and bilirubin, expressed in micromoles per liter.

To determine the time of injury, an expression relating the concentrations of pigments in the CSF was formed. This ratio, called the hemoglobin coefficient *HC*, is defined as

$$HC = \frac{Hb}{Hb + bil}$$

where *Hb* is the oxyhemoglobin concentration and bil is the bilirubin concentration. To adapt the *HC* to the demands of parametric statistical methods, the coefficient was transformed to an inverse trigonometric function by using the following formula:

$$\phi = \text{arc sine} \sqrt{\frac{Hb}{Hb + bil + 1}} + \text{arc sine} \sqrt{\frac{Hb + 1}{Hb + bil + 1}}$$

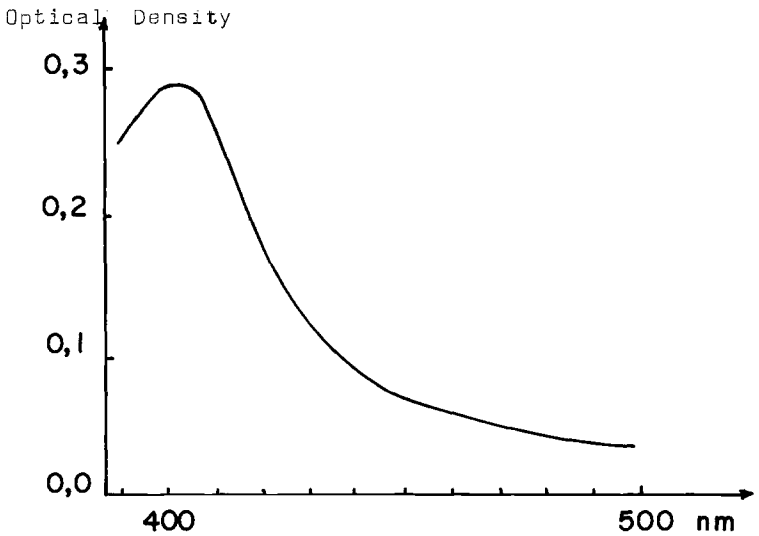

FIG. 4—*Spectral absorption curve of the cerebrospinal fluid of a dog, six days after hemorrhage.*

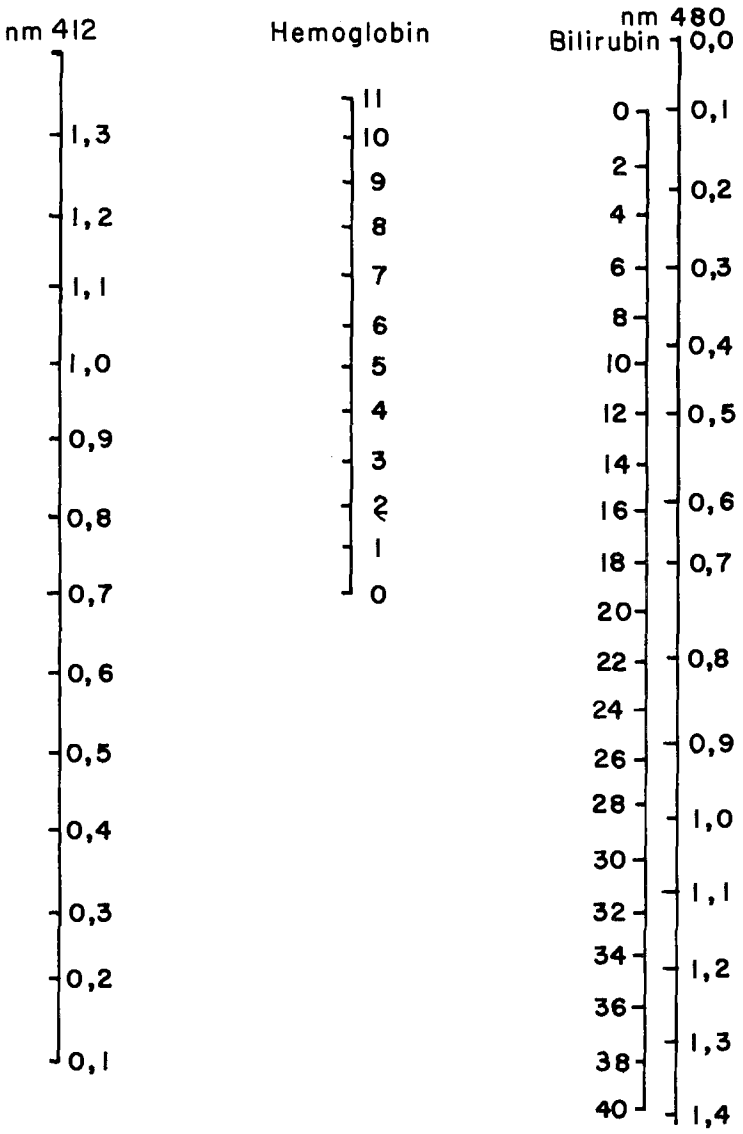

FIG. 5—*Nomogram for calculating hemoglobin and bilirubin concentrations, in micromoles per litre, from extinction at 412 and 480 nm (taken from Kronholm and Lintrup* [5] *).*

Results

The average concentrations of hemoglobin and bilirubin, expressed in micromoles per litre of cerebrospinal fluid on the day of the hemorrhage, are given in Table 1. Table 2 presents values for the arc-sine transformation of the hemoglobin coefficients. In that table are given, for each time of injury, the arithmetic mean, the standard deviation, and the 90% confidence limits of the transformed variable.

Discussion

Because of the difficulty in performing the arc-sine transformation of *HC*, particularly for those not familiar with mathematical manipulations, the following index was proposed:

$$HBI = HB/bil$$

Although this ratio, called the hemoglobin-bilirubin index, lacked the theoretical foundation of the *HC*, its use proved satisfactory. The differences in the results obtained with the *HC* and the *HBI* were not statistically significant (about 10%).

Table 3 lists the average values of *HBI* corresponding to various times since injury. Figure 6 shows the change in the arithmetic mean of *HBI* as a function of the time since injury.

To illustrate the use of the two indicators, assume that the concentrations (expressed in micromoles per litre) of oxyhemoglobin and bilirubin are 3.1 and 1.6, respectively. The transformation formula yields an arc-sine value of

$$\phi = \text{arc sine}\sqrt{\frac{3.1}{3.1 + 1.6 + 1.0}} + \text{arc sine}\sqrt{\frac{3.1 + 1.0}{3.1 + 1.6 + 1.00}} = 104.85$$

TABLE 1—*Arithmetic means of the concentrations of hemoglobin and bilirubin, in micromoles per litre, in the xanthochromic CSF of dogs from the day of the subarachnoid hemorrhage.*

Day	Hemoglobin	Bilirubin
0	0.09	0.23
1	4.36	0.46
2	14.78	0.71
3	7.72	1.03
4	5.17	1.37
5	4.30	1.78
6	3.52	2.23
7	2.79	3.27
8	2.14	5.61
9	1.58	4.54
10	1.11	3.30
11	0.80	2.16
12	0.50	1.41
13	0.20	0.60
14	0.04	0.16

TABLE 2—*Statistical values (arithmetic means* $\bar{X}$*, standard deviation* s*, and 90% confidence limits) of the transformed variable* ϕ.

Day	$\bar{X}$	s	Confidence Limits	
			Upper	Lower
0	89.94	19.06	129.29	50.69
1	134.43	15.22	159.46	109.40
2	148.97	5.14	157.43	140.51
3	134.66	8.92	149.33	120.01
4	121.69	7.34	133.76	109.62
5	111.98	9.43	127.49	96.47
6	101.81	7.41	114.00	89.62
7	86.10	7.50	98.44	73.76
8	65.74	5.38	74.59	56.89
9	64.80	6.10	74.83	54.77
10	65.20	7.30	77.20	53.20
11	67.64	8.50	81.62	53.66
12	69.26	10.37	86.32	52.20
13	69.83	13.12	91.45	48.25

TABLE 3—*Average values of* HBI *corresponding to days since hemorrhage occurred.*

Day	*HBI*
0	0.22
1	14.07
2	24.04
3	8.63
4	4.46
5	2.82
6	1.62
7	0.91
8	0.39
9	0.37
10	0.37
11	0.40
12	0.37
13	0.32
14	0.26

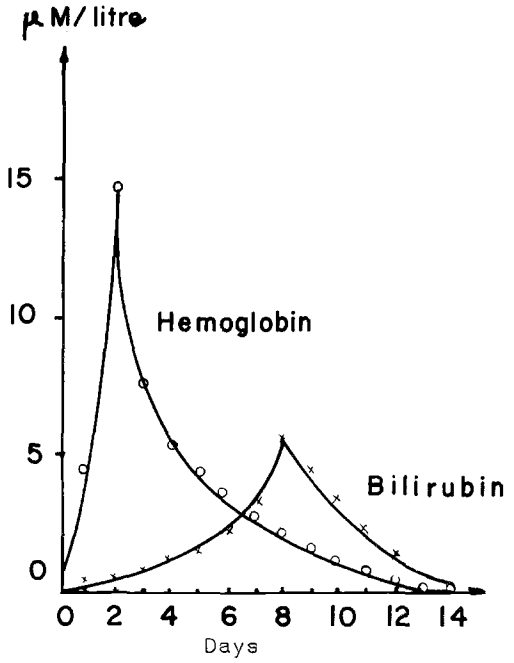

FIG. 6—*Arithmetic average of hemoglobin-bilirubin indexes for cerebrospinal fluid of dogs, plotted against time elapsed since hemorrhage.*

This value, according to the graph of Fig. 7, affirms that hemorrhage occurred less than 10 days before, within the period of 4.6 to 6.5 days, 5.3 days being most probable. With the pigment concentrations, we can calculate the *HBI*:

$$HBI = 3.1/1.6 = 1.8$$

Using the graph of Fig. 6 and the *HBI*, we can estimate that the time of hemorrhage was probably 6.0 days before. This example, chosen at random, corresponds to actual pigment

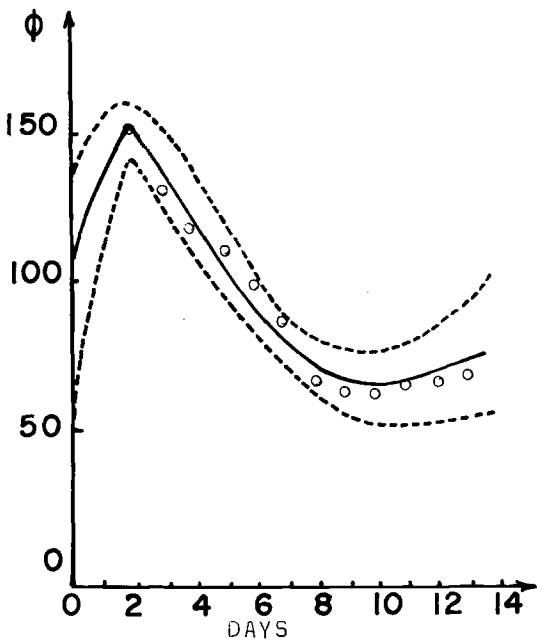

FIG. 7—*Arithmetic mean and 90% confidence limits of the arc-sine transformation of the hemoglobin coefficient for the cerebrospinal fluid of dogs, plotted against days elapsed since hemorrhage.*

concentrations measured on the fifth day after hemorrhage in one of the experimental animals.

It should be noted that ϕ sometimes yields two probable dates for the hemorrhage. This problem is easily overcome by puncturing the animal again one day later. If the hemorrhage occurred less than two days before, the value of ϕ of the second sample of the cerebrospinal fluid will be greater than that of the first; if the hemorrhage occurred more than two days before, ϕ should be smaller. Both indicators have the inconvenience of not being sensitive to hemorrhages that occurred more than eight days before.

Table 4 presents a quick method for establishing the time of the hemorrhage once ϕ is known. The method is not completely accurate owing to the interpolation inherent to its use.

TABLE 4—*Probable time since subarachnoid hemorrhage in dogs, according to the value of transformed hemoglobin coefficient.*

ϕ	Probable Time Since Hemorrhage
60	more than 8 days
70	more than 7 days (probably 8 days)
80	recently (less than 12 h) or 6.5 to 8.5 days (probably 7 days)
90	recently (less than 24 h) or 5.5 to 7.5 days (probably 6.5 days)
100	recently (less than 24 h) or 5.0 to 7.0 days (probably 6 days)
110	recently (less than 24 h; probably 12 h) or 4.5 to 6.0 days (probably 5 days)
120	recently (less than 36 h; probably 12 h) or 3.5 to 5.5 days (probably 4.5 days)
140	12 h to 4 days (probably 1.5 or 3.0 days)
150	1.0 to 3.0 days (probably 2 days)

It should be noted that the low concentrations of protein (less than 2 mg/mL) as well as the constant negativity of Pandy reaction made the corrections recommended by Meulen [*10*] unnecessary.

Conclusions

Both the transformed *HC* (ϕ) and *HBI* are useful in establishing the time from the onset of intrameningeal hemorrhage to the moment of the cerebrospinal fluid examination, when this time span does not exceed eight days and the patient remains alive. Also, the method presented appears to have great practical value owing to its technical simplicity.

References

[*1*] Scully, F. J., "Yellow Spinal Fluid. Its Origin and Significance," *Archives of Neurology and Psychiatry*, Vol. 10, No. 1, July 1923, pp. 23-99.

[*2*] Robinson, F. H. and Miller, B. N., "On the Differentiation of Colored Cerebrospinal Fluid," *The American Journal of the Medical Sciences*, Vol. 191, No. 4, April 1936, pp. 538-542.

[*3*] Merritt, H. H. and Fremont-Smith, in *The Cerebrospinal Fluid*, W. B. Saunders, Philadelphia, 1938.

[*4*] Barrows, L. J., Hunter, F. T., and Banker, B. Q., "The Nature and Clinical Significance of Pigments in the Cerebrospinal Fluid," *Brain*, Vol. 78, No. 1, March 1955, pp. 59-80.

[*5*] Kronholm, V. and Lintrup, J., "Spectrophotometric Investigation of the Cerebrospinal Fluid in the Near Ultraviolet Region. A Possible Diagnostic Aid in Diseases of the Central Nervous System," *Acta Psychiatrica et Neurologica Scandinavica*, Vol. 35, No. 3, 1960, pp. 314-329.

[*6*] Kronholm, V. and Lintrup, J., "Pigment Changes in the Lumbar Fluid as a Diagnostic Aid in Cranial Haemorrhagic Lesions," *Danish Medical Bulletin*, Vol. 8, No. 2, May 1961, pp. 47-49.

[*7*] Kronholm, V., "A Method of Diagnosis by Means of Spectrophotometric Measurements of the Cerebrospinal Fluid of the Central Nervous System," *Nordiske Neurologkongres*, Copenhagen, 1960.

[*8*] Kronholm, V., "Spectrophotometric Investigation of Spinal Fluid," *World Neurology*, Vol. 2, No. 5, May 1961, pp. 435-441.

[*9*] Levere, R. D., Swerdlow, F., and Garavey, M. R., "Measurements of Human Plasma Hemoglobin by Difference Spectrophotometry," *The Journal of Laboratory and Clinical Medicine*, Vol. 77, No. 1, Jan. 1971, pp. 168-173.

[*10*] van der Meulen, J. P., "Cerebrospinal Fluid Xanthochromia: An Objective Index," *Neurology*, Vol. 16, No. 2, Feb. 1966, pp. 170-178.

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