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Preparation, Purification and Characterization of Chlorohaemin

H. Uwe Wolf[a], Hans Link[b] and Werner Lang[c]

[a] Abteilung Pharmakologie und Toxikologie der Universität Ulm, Ulm, Germany
[b] Sektion Schwingungsspektroskopie der Universität Ulm, Ulm, Germany
[c] Institut für Physiologie und Pathophysiologie der Universität Mainz, Mainz, Germany

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Summary: Preparation of chlorohaemin (CAS 16009-13-5) is performed on the basis of the method of Labbe and Nishida (acetone-acetic acid-$SrCl_2$ method) with some significant modifications. Instead of blood as starting material, a fresh preparation of purified human erythrocytes is used. This avoids any contamination with serum and erythrocyte proteins and lipids of the end product. Special care is taken to remove contaminating globin by introducing some specific purification steps during isolation and recrystallisation.

The yield is in the range 65–75% of theory, and the purity of the product is better than 99.9% as shown by elemental analysis and specific tests on various compounds as possible contaminants which originate from the starting material such as lipids and proteins and/or from the different steps of preparation and purification during the procedure.

The pure chlorohaemin which is the compound of choice as reference substance for the AHD method in haemoglobinometry is characterized by LSI mass spectrometry (m/z = 616, haemin ion), field desorption-mass spectrometry (m/z = 652), by IR-spectroscopy, and by UV/VIS absorption spectroscopy (pyridine haemochrome spectrum, AHD spectrum).

Darstellung, Reinigung und Charakterisierung von Chlorhaemin

Zusammenfassung: Die Darstellung von Chlorhaemin (CAS 16009-13-5) erfolgt mit einigen bedeutsamen Modifikationen nach der Methode von Labbe und Nishida (Aceton-Essigsäure-$SrCl_2$-Methode). Anstelle von Blut als Ausgangsmaterial wird ein frisch hergestelltes Präparat von gereinigten Human-Erythrozyten eingesetzt. Hierdurch kann eine Kontamination des Endprodukts mit Serum- und Erythrozyten-Proteinen und -Lipiden vermieden werden. Besondere Beachtung findet die Abtrennung des Globins, die durch einige zusätzliche Reinigungsschritte während der Präparation und der Rekristallisation erreicht wird.

Die Ausbeute liegt bei 65–75% der Theorie und die Reinheit des Produkts bei mehr als 99.9%. Dies wird durch die Elementaranalyse der Substanz und durch spezifische Tests auf mögliche Verunreinigungen, die aus dem Ausgangsmaterial (wie Lipide und Proteine) und/oder aus den verschiedenen Präparations- und Reinigungsschritten stammen, nachgewiesen.

Das reine Chlorhämin, das die Verbindung der Wahl als Referenzsubstanz für die Standardisierung der AHD-Methode in der Haemoglobinometrie darstellt, wird durch LSI-Massenspektrometrie (m/z = 616, Haemin-Ion), Felddesorptions-Massenspektrometrie (m/z = 652), IR-Spektroskopie und durch UV/VIS-Absorptionsspektroskopie (Pyridinhaemochromspektrum, AHD-Spektrum) charakterisiert.

Abbreviations:
ADH, alkaline haematin detergent; DSC, differential scanning calorimetry; SD, standard deviation; LSI, liquid secondary ionization.

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Key terms: Chlorohaemin, pyridine haemochrome, alkaline haematin detergent complex (AHD), reference substance for haemoglobinometry.

Chlorohaemin (CAS 16009-13-5),* haemin, protohaemin, haemin chloride, haematin hydrochloride, protohaemin-IX, protochlorohaemin-IX, ferriprotoporphyrin-IX chloride, chloroprotoporphyrin, chloroferriprotoporphyrin, or chloro(protoporphyrinato)iron(III), is a pigment which contains the same chromophoric group as haemoglobin which is present in the blood of humans and of higher animals. Its structure and relative molecular mass (651.96) is well-known, and the first total synthesis of chlorohaemin was published in the famous work of Fischer and his school[1] in 1929. On the other hand, following the method of Schalfejeff[2] described in 1885, it can be prepared easily by treatment of haemoglobin or whole blood with glacial acetic acid in the presence of NaCl, the precipitate formed by this reaction being known since a long time as "Teichmann's crystals".

Because of its close relationship to haemoglobin, chlorohaemin was thought even in the beginnings of haemoglobinometry to be the ideal candidate for a reference substance. Pioneers like Clegg and King[3], Drabkin[4] and Horecker[5] tried to establish crystalline chlorohaemin as a reference substance, however, without having great success. Based on iron analysis, equal concentrations of chlorohaemin and of haemoglobin, dissolved in aqueous alkaline solution (0.1 mol/*l* NaOH) showed differences in the light absorbance when measured under the same conditions. As another disadvantage, solutions of chlorohaemin in aqueous alkali were not stable.

Recently in 1984, Zander, Lang and Wolf[6–10] reexamined the classical alkaline haematin method. The reagent, 0.1 mol/*l* NaOH, simply modified by the addition of 2.5% (w/v) Triton X-100, the so-called "AHD reagent", revealed unexpected results when applied to blood or chlorohaemin. The green solution obtained after mixing the AHD reagent with either blood or chlorohaemin had the same spectroscopic properties, and the discrepancy between haemoglobin and chlorohaemin known so far disappeared completely when purified chlorohaemin was used.

Products of chlorohaemin commercially available which had been used, did not meet the requirements for a reference substance in the AHD method, since varying amounts of impurities which are colloidal to some extent, lead to irreproducible values of absorbance. Therefore, a procedure for preparation, purification and characterization had to be worked out. This was done based on the method of Labbe and Nishida[11] and on experimental contributions from Fuhrhop and Smith[12].

The present paper describes the isolation and purification of chlorohaemin obtained from human erythrocyte concentrate, its identification, its properties, and the criteria applied for the proof of its purity.

Materials and Methods

MATERIALS

Starting material for the isolation of chlorohaemin was fresh human erythrocyte concentrate obtained from the Blood Bank of the Deutsches Rotes Kreuz (German Red Cross) at Ulm. (Alternatively, fresh bovine erythrocytes may be used.)

Acetone, ammonium chloride, acetic acid (96%), chloroform, ethanol, hydrochloric acid, methanol, pyridine, sodium chloride, sodium hydroxide, and strontium chloride ($SrCl_2 \times 6\ H_2O$) were obtained from Merck, Darmstadt, Germany, in the highest grade of purity available. Triton X-100 (for scintillation techniques, analytical grade) was from Serva, Heidelberg, Germany.

The water used throughout all steps of the procedure was twice distilled over quartz.

DETERMINATION METHODS

The tests of the end product for impurities of lipids, especially of erythrocyte membrane lipids, proteins (e.g. globin), acetone, acetic acid, pyridine, and water were carried out as follows:

Lipids

Ca. 100 mg of the end product were extracted by 1 m*l* of chloroform. This solution was evaporated to 100 μl and analysed by thin-layer chromatography according to the method of Peter and Wolf[13]. The detection limit for the complete mixture of erythrocyte lipids is in the order of 100 ng of lipids which is equivalent to 1 μg/g lipid contamination.

Protein

Since it was not possible to determine the amount of contaminating protein in chlorohaemin by using a conventional method for protein determination, an amino-acid analysis as a measure for protein content was done after boiling a sample of ground chlorohaemin (10–50 mg) with 6 mol/*l* HCl for 24 h at 120 °C. The determination of the amino acids was performed in a Biotronic AS-Analyzator device.

Acetone, acetic acid, and pyridine

Ca. 100 mg of the end product was dissolved completely in aqueous 0.1 mol/*l* NaOH, the solution acidified to pH 5–6 by 1 mol/*l* HCl and the precipitate was removed by centrifugation (15 min at 4000 $\times$ *g*). Aliquots of the supernatant were analysed for acetone, acetic acid, and pyridine by gas chromatography. The limits of detection were: acetone: 10 μg, acetic acid: 20 μg, and pyridine: 10 μg. The pure compounds were used for spiking the samples in order to detect possible adsorption on the chlorohaemin precipitate.

Sodium and strontium ions

Sodium and strontium ions were determined by atomic absorption flame photometry (Na) and atomic emission flame spectroscopy (Sr, excited plasma) in a commercial analytical laboratory (Fresenius, W-6200 Wiesbaden, Germany). The limits of detection were in the order of 1 μg/g in both cases.

Water and other volatile compounds

The content of water and other volatile compounds were determined by differential thermo analysis, i.e. thermogravimetric measurements and differential scanning calorimetry (DSC). The sample size was 1.0–1.2 mg of chlorohaemin.

Mass spectrometry

LSI mass spectrum: mass spectrometer MAT 900, Finnigan MAT, Bremen; a solution of chlorohaemin in dimethylsulfoxide was mixed with *m*-nitrobenzyl alcohol in the ratio 1:50. FD mass spectrum: mass spectrometer Varian MAT 711; chlorohaemin was dissolved in dimethylsulfoxide.

ISOLATION PROCEDURES

The procedure for the preparation of purified chlorohaemin consists of three different steps: 1. Preparation of haemoglobin from human erythrocytes; 2. Isolation of chlorohaemin by dissociation of haemoglobin in acetic acid solution; and 3. Purification of chlorohaemin by recrystallization.

1) Preparation of haemoglobin

One liter of human erythrocyte concentrate is suspended in physiological saline (0.155 mol/*l* NaCl) to a final volume of 3 *l*, and the erythrocytes are sedimented at 3–5000 × *g*. All centrifugation steps are carried out at 0–4 °C. The supernatant and the buffy coat of leucocytes is thoroughly removed and the procedure is repeated once using 0.155 mol/*l* NaCl and afterwards twice using 0.155 mol/*l* NH_4Cl.

The washed erythrocytes are frozen at a temperature of – 80 °C in order to haemolyse the cells. After thawing, the cell membranes are removed by centrifugation for 60 min at 40000 × *g*. Then the supernatant is collected and frozen in portions of 200 m*l* at – 80 °C and stored for the preparation of chlorohaemin at – 20 °C to – 25 °C.

2) Isolation of chlorohaemin

A mixture of 10 g of $SrCl_2 \times 6\ H_2O$ dissolved in 10 m*l* of water and 450 m*l* of 96% acetic acid is added quickly to 1500 m*l* of acetone at room temperature under vigorous stirring. The appearence of a precipitate of strontium chloride is without significance, as long as it can be kept in suspension. In all following steps of the procedure direct light, especially sunlight, has to be avoided.

Then a 200-m*l* aliquot of the haemoglobin solution described in step 1 is thawed and added slowly and continuously into the vigorously stirred acetone solution at room temperature. After finishing the addition, the stirring of the dark brown solution with the white fluffy precipitate of globin is continued for at least 10 min.

The precipitated globin is removed from the chlorohaemin solution by filtration. Then the clear, dark brown solution is heated to the boiling point (ca. 65–70 °C) for 10 min to precipitate traces of globin, and allowed to cool to room temperature. The globin precipitate is removed by filtration through sintered glass (Schott D-4 (G-4), nominal maximum pore size: 10–16 μm).

The chlorohaemin solution is then transferred into a round flask and the acetone is removed using a rotation evaporator and a water bath starting with a temperature of 70 °C. During the removal of acetone, the temperature is elevated slowly and continuously to 90 °C within 90–120 minutes. The last 100–200 m*l* of acetone are removed at 90 °C by use of slight vacuum.

After complete removal of acetone, the mixture is allowed to cool to room temperature during the next 2–3 h under continuous rotation of the evaporator. During this time, the chlorohaemin appears in form of the typical crystals. After termination of the crystallization process, the chlorohaemin is separated from the solution, and washed with 50% acetic acid/water, 50% ethanol/water, 96% ethanol, and acetone. Finally, it is dried under vacuum over solid NaOH.

The yield is generally 1600–1800 mg of chlorohaemin, equivalent to 65–75% of the theoretical yield of 2400 mg contained in 200 m*l* of the starting haemoglobin solution.

3) Purification of chlorohaemin

For further separation of possible contaminants the chlorohaemin obtained in step 2 is recrystallized.

Appr. 6000 mg of chlorohaemin (pooled preparations from step 2) are dissolved in a mixture of 15 m*l* pyridine and 60 m*l* methanol at room temperature. Immediately after dissolution (crystallization of the chlorohaemin-pyridine complex formed must be avoided!), the solution is poured under stirring into 1000 m*l* of acetone heated nearly to the boiling point. The mixture is kept for 10 min at the boiling point and then allowed to cool to room temperature. Then the solution is filtered through sintered glass (Schott G-4) and poured into a solution of 1.25 g NH_4Cl dissolved in 100 m*l* H_2O and 300 m*l* 96% acetic acid.

The subsequent removal of the acetone from the mixture in the rotation evaporator as well as other steps for isolation and washing of the recrystallized material is carried out as described in step 2.

The procedure of recrystallization has to be repeated until the specific absorbance of the sample at 575 nm, measured under defined conditions in "AHD-solution"[6,7], has become a constant and characteristic value. This is generally the case after only one single recrystallization.

Results and Discussion

1) Optical appearance

Chlorohaemin prepared by the method described above appears as blue shining, rhombic crystals of ca. 1–2 mm in size with dark brown colour in the passing light. A microphotograph of the product is presented in Fig. 1a and b.

Fig. 1b shows for comparison an own preparation (large crystals) and a commercial product (small crystals).

2) Elemental analysis

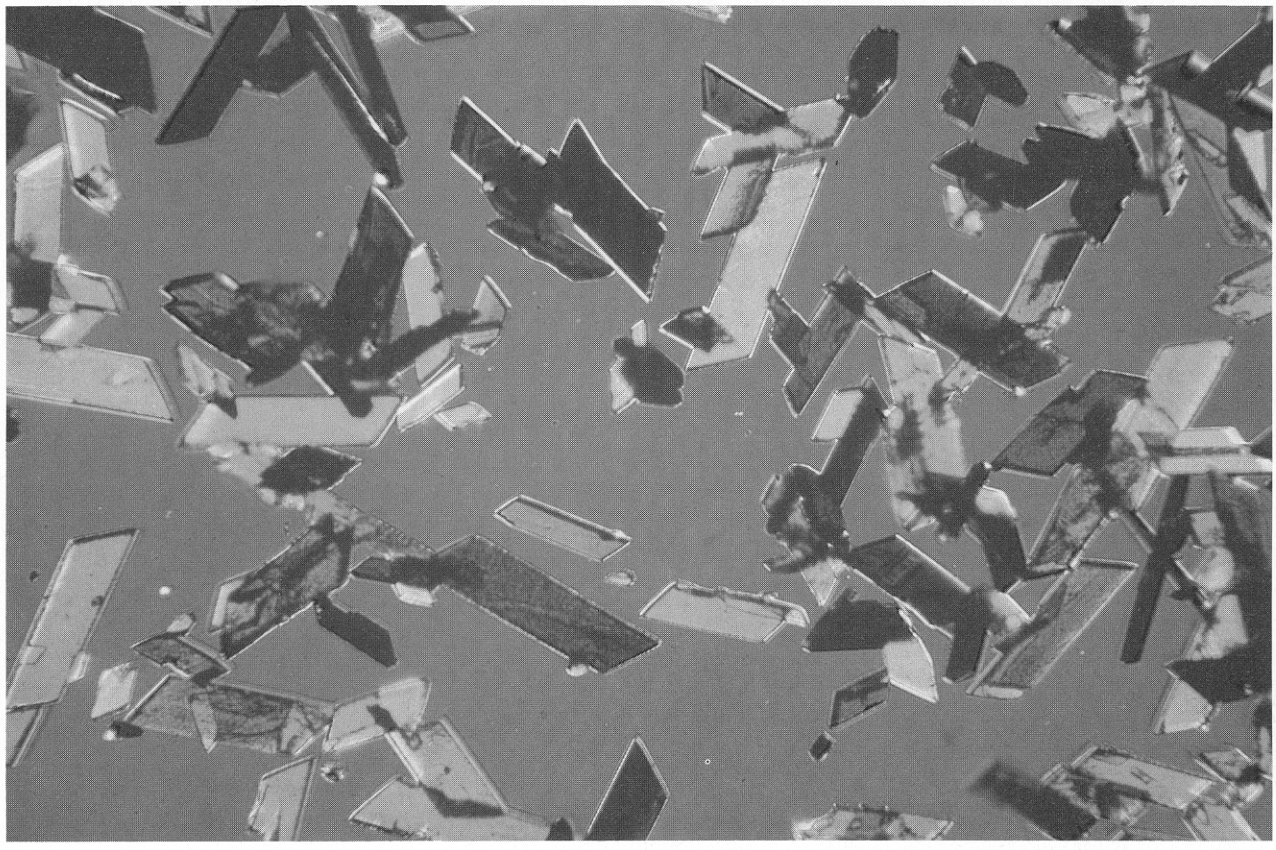
The elemental analysis of chlorohaemin was carried out with a total number of 15 samples obtained from different preparations, with the exception of Fe which was determined in 9 preparations. The results including the standard deviation of the mean values are given in Table 1.

3) Determination of impurities in the end product

In principle, the end product can be contaminated by various compounds resulting from different sources as described in material and methods.

Concerning analogous compounds of chlorohaemin contaminating the end product, the only compound which could be formed under the conditions of the preparation procedure is acetato-haemin (protohaemin-acetate, acetylhaemin) in which the chloride anion is replaced by the acetate anion. However, this

a)



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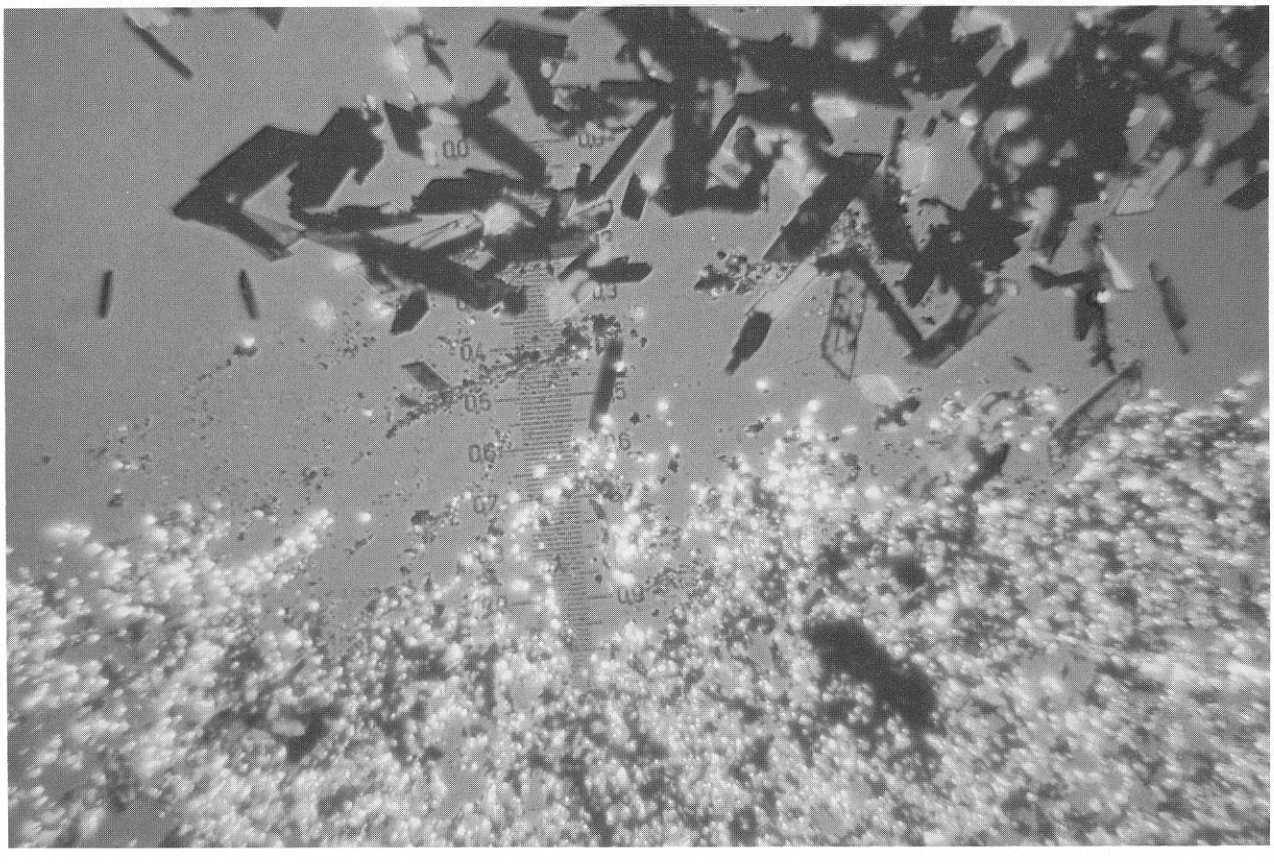

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Fig. 1. Micrographs of the isolated and purified chlorohaemin.

a) Total magnification ca. 1:15; b) Comparison of the purified material (large crystals) with a commercial product (small crystals), total magnification ca. 1:7.

Table 1. Elemental analysis of purified chlorohaemin.

The values represent the mean of determinations from 15 different preparations with the exception of Fe, which was determined in 9 different preparations.

Element	Theor. content [%]	Experimental data [%] (mean ± SD)	% of theory
C	62.64	62.42 ± 0.20	99.65
H	4.95	4.98 ± 0.06	102.68
Cl	5.44	5.37 ± 0.21	98.71
Fe	8.57	8.55 ± 0.16	99.77
N	8.59	8.56 ± 0.11	99.65
O	9.82		
Sum	100.01		

compound has been reported to be formed with acetic acid only in the absence of chloride ions[15].

Therefore, the end product was tested for impurities such as lipids, protein, pyridine, acetone, acetic acid, sodium ion, strontium ion, and water.

Lipids

Lipids could not be detected in the chlorohaemin sample by thin-layer chromatography. Therefore, the residual lipid content in the chlorohaemin must be below 1 μg/g.

Protein

The protein content of samples of 4 different preparations, estimated by means of an amino-acid analysis as a measure for protein, and based on the relative content of the most abundant amino acids alanine, leucine, and valine, was < 0.03% in all cases.

Acetic acid, acetone and pyridine

The content of pyridine in samples of 2 different preparations was < 0.02% for each compound in both cases.

Water and other volatile compounds

In the thermogravimetric measurements, heating of samples of 4 different chlorohaemin preparations to 130 °C yielded a loss of 0.1% of the total weight. This indicates that the content of volatile compounds such as water, acetic acid (boiling point 116 °C), acetone (56 °C), and pyridine (115 °C) cannot exceed a total amount of 0.1% in the chlorohaemin. Thus, the results obtained by the method of the thermogravimetry confirm the results obtained by gas chromatography.

Sodium ion, strontium ion

The content of 2 different chlorohaemin preparations was < 2 μg/g for both ions.

4) Stability

The DSC diagram, presented in Fig. 2, does not show any phase transition of chlorohaemin in the temperature range between 25 °C and 275 °C. This indicates that there are no changes in the crystal structure of this compound, no processes of phase separation and no melting point. This thermic stability indicates that there is no decomposition in the temperature range tested.

5) Spectral data

Spectra in the visible range

The typical spectrum of chlorohaemin in AHD solution (2.5% Triton X-100 in 0.1 mol/l NaOH) in the range between 500 and 750 nm is shown in Fig. 3. Characteristic features of this spectrum are 1. the minimum at 533 nm, 2. the first maximum at 575 nm, and 3. the second maximum at 600 nm. For comparison, this figure also contains the spectrum of alkaline haematin generated in 0.1 mol/l NaOH.

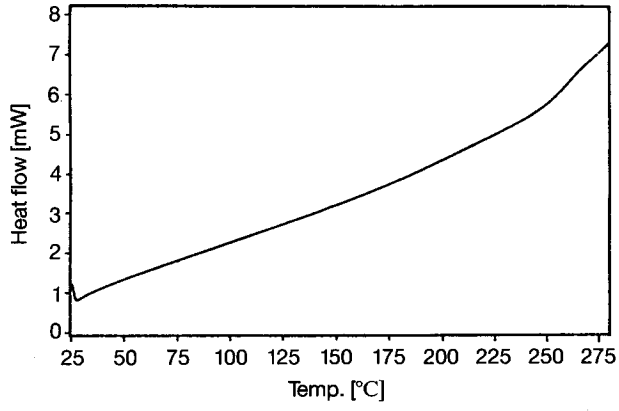

Fig. 2. Typical recording of the differential scanning calorimetry of chlorohaemin in the temperature range of 25–275 °C.

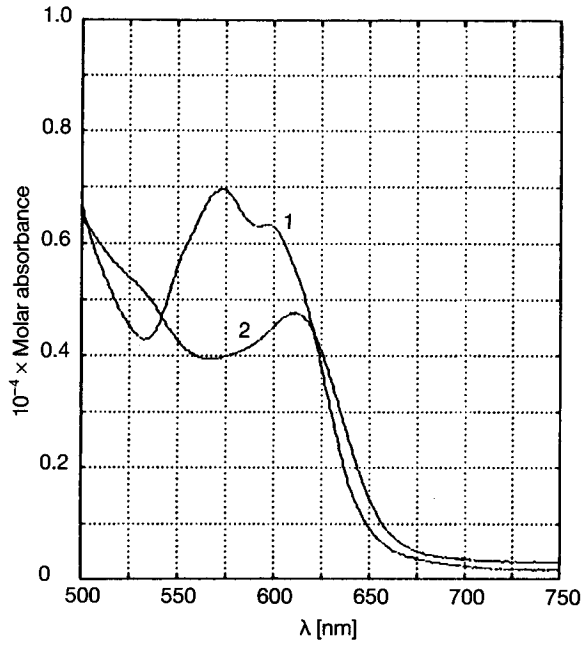

Fig. 3. Spectra of 0.1 mmol/*l* chlorohaemin in AHD solution (curve 1) and of 0.1 mmol/*l* chlorohaemin in 0.1 mol/*l* NaOH (curve 2).

Mass spectrometry

In the LSI mass spectrum a signal is observed with m/z = 616 which can be attributed to the haemin ion $[C_{34}H_{32}N_4O_4Fe]^{\oplus}$; the intensities of the isotope cluster are consistent with the calculated values. In the FD mass spectrum a signal with m/z = 652 (addition of a proton to the chlorohaemin) is observed.

IR spectra

Fig. 4 shows the IR spectrum of chlorohaemin with CsI as embedding material at ambient temperature. The strong bands around 1600 cm^{-1} (c = 0) valence and the fingerprint area between 1500 cm^{-1} and 1000 cm^{-1} are highly characteristic. (A complete set of IR spectra at ambient temperature and at low temperature (liquid N_2) with different embedding materials (CsI, Nujol, and Hostaflon) and peak lists of the spectra can be provided on request.)

Characterization as pyridine haemochrome

The pyridine haemochrome spectrum of chlorohaemin was measured as recommended by Paul et al.[16] in an aqueous solution containing 2.1 mol/*l* pyridine and 0.075 mol/*l* NaOH immediately after reduction with solid sodium dithionite in the cuvette (Procedure A). A typical spectrum of chlorohaemin as pyridine haematin before reduction (green solution) and as pyridine haemochrome after reduction (red solution) with dithionite in the visible range of 450–700 nm is shown in Fig. 5.

For characterization of chlorohaemin, the following wavelength of its pyridine haemochrome spectrum have been chosen at which the specific absorptivities were determined:

Maxima: 556.5 nm (α-band), 525 nm (β-band), and 480 nm.
Minima: 539 nm, 502 nm, and 464 nm.

However, because of stability problems with the reduced form of the pyridine complex in the cuvette under aerobic conditions, the reduction was also performed according to a modified procedure under pure nitrogen (Procedure B). Under these conditions, all absorbancies measured at the selected wavelengths were stable during an interval of 15–30 min. Therefore, these values are considered to be the more reliable ones.

(A detailed description of the two Procedures A and B will be included into a subsequent paper dealing with standardization methods in haemoglobinometry.)

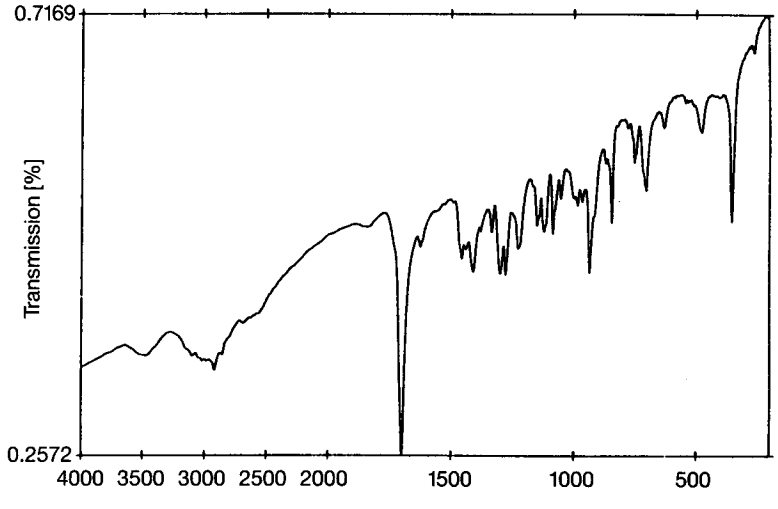

Fig. 4. IR-spectrum of purified chlorohaemin. Conditions: embedding material: CsI, ambient temperature.

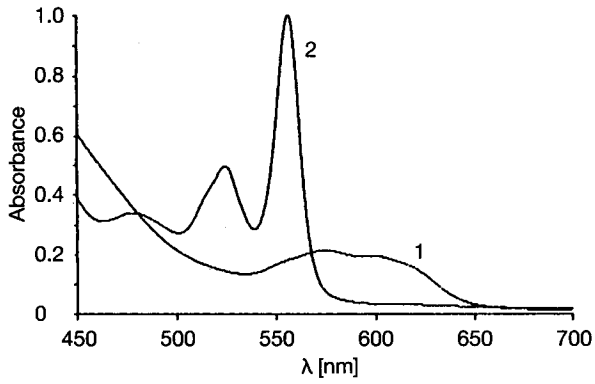

Fig. 5. Spectrum of purified chlorohaemin (0.031 mmol/*l*) dissolved in aqueous alkaline pyridine (2.1 mol/*l* in 0.075 mol/*l* NaOH) before reduction (curve 1) and after reduction (curve 2) with sodium dithionite: "pyridine haemochrome" spectrum. Spectra recorded on PERKIN-ELMER LAMBDA 17 with 2 nm band-width.

The results of these experiments are given in Table 2 as the mean of the millimolar absorptivity (2 stock solutions, 5 measurements) at the selected wavelengths under anaerobic conditions (pure nitrogen, Procedure B) and under aerobic conditions (Procedure A), and compared with the literature values.

The millimolar absorptivity values determined under pure nitrogen are considerably lower in nearly all cases than those measured under aerobic conditions. (The only exception is the α-band, at which the values are identical.)

Table 2. Millimolar absorptivity values (in $l \times mmol^{-1} \times cm^{-1}$) at the maxima (480 nm, 525 nm, and 556.5 nm, resp.) and at the minima (464 nm, 502 nm, and 539 nm, resp.) of the pyridine haemochrome spectrum of chlorohaemin in the visible range, and comparison with literature values.

All photometric measurements were performed on a ZEISS PM6 photometer (bandwidth 2 nm, wavelength accuracy ± 0.5 nm)

	Reducing conditions		Literature values	
Wavelength	Anaerobic	Aerobic	(16)	(17)
464 nm	8.65 ± 0.09	8.97 ± 0.04	11.29 (460 nm)	
480 nm	10.24 ± 0.07	10.45 ± 0.10	12.29	10.60
502 nm	8.14 ± 0.03	8.31 ± 0.07	9.94 (503 nm)	
525 nm (β-band)	15.89 ± 0.06	16.00 ± 0.13	17.50 (526 nm)	16.00
539 nm	8.82 ± 0.04	9.03 ± 0.07	9.90 (540 nm)	
556.5 nm (α-band)	32.74 ± 0.11	32.75 ± 0.22	34.22 (557 nm)	32.50 (557 nm)

The values obtained under pure nitrogen agree moderately with the values published by Johnson[17], but not at all with those by Paul et al.[16] which apparently are too high. A good reason to presume this is the appearance of an extra band or shoulder at about 625 nm in their published spectra of the pyridine haemochrome indicating a possible contamination which cannot be detected in the corresponding spectrum of chlorohaemin prepared and used within this work (Fig. 5).

Therefore, whenever the pyridine haemochrome method is used in characterizing chlorohaemin, the conditions for the reduction have to be controlled very carefully in order to obtain reliable values. The circumstantial procedure to exclude atmospheric oxygen from the reduced pyridine complex, however, is a significant disadvantage of this method preventing it from being used routinely. Therefore, the following procedure may be proposed as an alternative:

Characterization on the basis of the spectral behaviour in "AHD solution"

Chlorohaemin dissolved in aqueous alkali alone yields a brown solution which is unstable and thus does not show constant spectroscopic properties. Therefore this solution cannot be used to characterize chlorohaemin. However, in the presence of 2.5% (w/v) Triton X-100 (in 0.1 mol/*l* NaOH), the resulting green solution is stable, and the "alkaline haematin detergent" complex formed ("AHD" complex, see refs.[6,7]) of chlorohaemin can be well characterized by spectroscopy, even though its exact structure is not known as yet. In the absorption spectrum of the AHD complex, there are two bands in the visible range: one at 575 nm (1. maximum), the other at 600 nm (2. maximum) with a minimum at 533 nm.

Based on the properties of the spectrum of chlorohaemin in AHD solution in the visible range, the following criteria are defined for characterization of this compound with respect to quality control:

Characterization by Lambert-Beer's law at 575 nm

The first maximum of the AHD complex at 575 nm is the wavelength which is used in the AHD method for the determination of haemoglobin in blood diluted 1:151[6,7]. Under these conditions, a 0–25 g/d*l* range of haemoglobin concentration corresponds to 0–0.10 mmol/*l* of chlorohaemin in AHD reagent, for which the validity of the Lambert-Beer's law was tested. It was found that the measured absorbance at 575 nm (A_{575}) was strictly linear to the concentration of chlorohaemin (c, in mmol/*l*) in the range of 0.01–0.10 mmol/*l* chlorohaemin, however, with a very slight de-

viation from linearity in the range of 0–0.01 mmol/*l*, a range with no physiological significance. By a linear regression analysis the best fit of the experimental data of 7 different chlorohaemin preparations yielded a straight line with a slope of 6.914 and, due to the slight deviation from linearity below 0.01 mmol/*l*, a small positive intercept of 0.0027, which may be caused methodologically. Using statistical criteria described by v. Klein-Wisenberg[18] one can assume direct proportionality between concentration and absorbance, and the best straight line including all experimental data and the point of origin yields the millimolar absorptivity of the AHD complex of chlorohaemin at 575 nm:

$$A_{575}^{\text{mmol}} = 6.945 \pm 0.019\, l \times \text{mmol}^{-1} \times \text{cm}^{-1}.$$

This value is in good agreement with that (6.960) published in the past[7], and can be used for spectral characterization of chlorohaemin. Only those samples of chlorohaemin should be used as a standard in haemoglobinometry (AHD method) of which the millimolar absorptivity is in the recommended range of ± 3 SD: 6.888–7.002.

Characterization by absorption ratios and by A_{750}

As another criterium for quality control of chlorohaemin in AHD solution, the following ratios of absorbances (A_λ) at the minimum (533 nm) and the two maxima (575 nm, 600 nm) were defined:

Ratio $Q_1 = A_{533}/A_{575}$
Ratio $Q_2 = A_{600}/A_{575}$

In addition, as a measure of possible contaminants causing some turbidity in the solution, the absolute absorbance at 750 nm (A_{750}) in the non-absorbing range of the spectrum was routinely checked.

Characteristic values for Q_1, Q_2, and A_{750}, based on a series of careful absorbance measurements of 7 different preparations of purified chlorohaemin in AHD solution (0.1 mmol/*l*, absorbance measurements not before one day after preparation of the solution), and the corresponding ranges are the following:

Q_1 = 0.603 (± 1.0%)	Range Q_1: 0.597–0.609
Q_2 = 0.906 (± 1.0%)	Range Q_2: 0.897–0.915
A_{750} = 0.014	Range A_{750}: 0.012–0.016

These values are recommended in a concentration range of 0.075–0.150 mmol/*l* of chlorohaemin and can be used for reference, if samples of chlorohaemin with unknown degree of purity are to be checked. In the presence of impurities, these three characteristic values are increased, Q_1 and A_{750} showing the most pronounced effect. E.g., the values of the commercial product shown in Fig. 1 were $Q_1 = 0.625$ and $A_{750} = 0.022$.

For determination of Q_1 and Q_2, however, the necessary absorbance measurements at 533, 575 and 600 nm should not be performed in a too low concentration range of chlorohaemin (< 0.075 mmol/*l*), because the relative photometric error increases strongly within the corresponding low absorbance range. Also, the effect on Q_1 and Q_2, caused by the experimental relationships between absorbance and concentration (see above) at the selected wave-lengths which all show by linear regression analysis a slightly positive intercept near the origin (intercept at 533 and 600 nm: 0.007–0.010, at 575 nm: 0.002–0.005), is the higher, the lower the concentration and absorbance, respectively. Therefore, for comparison the concentration of chlorohaemin should be in the recommended range of 0.075–0.150 mmol/*l*.

Conclusions

On the basis of the experimental data presented in this work it can be concluded that the chlorohaemin obtained by the method described here

- contains an amount of impurities not exceeding 0.1% in total, i.e. the purity grade of this chlorohaemin is ≥ 99.9%
- can be well characterized in the range of 0.01–0.10 mmol/*l* chlorohaemin by spectroscopy, preferably by the specific optical properties of its spectrum in the visible range in AHD solution based on Triton X-100
- can be used as a reference compound for quality control in the AHD method for determination of haemoglobin[6–8,10].

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Prof. Dr. H. Uwe Wolf*, Abteilung Pharmakologie und Toxikologie der Universität Ulm, Albert-Einstein-Allee 11, N26-430, W-7900 Ulm/Donau;

Hans Link, Sektion Schwingungsspektroskopie der Universität Ulm, Albert-Einstein-Allee 11, N25-360, W-7900 Ulm/Donau;

Dr. Werner Lang, Institut für Physiologie und Pathophysiologie der Universität Mainz, Saarstraße 21, W-6500 Mainz/Rhein.

* To whom correspondence should be addressed.