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Sensitive Identification of Hemoglobin in Bloodstains from Different Species by High Performance Liquid Chromatography with Combined UV and Fluorescence Detection

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ABSTRACT: HPLC with reversed phase large pore columns (5 μm particle size, C_4-phase, 300Å pore size) has been used to analyze extracts from bloodstains. Bloodstains from humans as well as various animals often encountered in forensic casework have been investigated. Bloodstains from common wild animals as well as domestic animals could be distinguished by this technique with one exception—dog and wolf. The use of fluorescence detection increased the sensitivity of the method. For human bloodstains, the detection limit was about 1 to 2 nanoliter blood.

The method has the advantage of simplicity, speed and sensitivity for use in forensic practice.

KEYWORDS: pathology and biology, bloodstains, species identification, HPLC, fluorescence detection, forensic science, *hemoglobin*

Differentiation between human and animal bloodstains, as well as identification of the animal species, is of considerable importance in forensic science. Hemoglobin is a useful marker for this identification.

High performance liquid chromatography (HPLC) is one of the analytical techniques that has been used successfully to separate the globin chains in hemoglobin. During the 1980s, large pore reverse phase HPLC columns with shorter hydrocarbon chains (particularly C_4-chains) were developed. These columns are very useful for making rapid and reproducible separations of globin chains [*1,2*]. Inoue et al. [*3–6*] applied HPLC with a C_4-reversed phase column to bloodstain examinations. This technique was used to identify human fetal hemoglobin in bloodstains [*3*] as well as species identification of bloodstains from primates and some common animals [*4*]. Inoue et al. also reported two different methods for estimation of bloodstain age by HPLC [*5,6*].

In this study HPLC with large pore C_4-reversed phase columns was used for species identification of bloodstains from some common household and wild animals from Sweden.

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By a combination of ultraviolet (UV) and fluorescence detection, the sensitivity of the detection was improved and a better differentiation between human and animal bloodstains could be made. The method can identify human bloodstains in amounts down to 1 to 2 nanoliters of blood.

Materials and Methods

Blood and Bloodstain Samples

Venous blood samples were collected from human adults just before analysis. Animal blood samples were obtained from the reference collection at our laboratory. These samples had been frozen and held at −67°C for several years. Bloodstain samples were obtained by dropping whole blood (fresh or melted) on filter paper (Munktell No. 3) and dried overnight at room temperature.

A piece of bloodstain corresponding to about 4 μL blood was cut out from the filter paper and immersed in 200 μL distilled water for 15 minutes at 37°C. Aliquots of 10 μL (equivalent to about 0.2 μL fresh blood) were taken for analysis if not otherwise stated.

High Performance Liquid Chromatography

HPLC chromatograms were run on a Varian Model 5000 Liquid Chromatograph equipped with a variable wavelength detector (model 100). The detector wavelength was set to 220 nm. In series with this 220 nm detector, two other detectors were connected. A fixed wavelength detector (Waters Model 440) operated at 254 nm and a Perkin Elmer fluorescence detector (LC 240) worked with the excitation wavelength of 275 nm and the emission wavelength of 345 nm. The fluorescence detector was connected to a Hewlett Packard HP 3396 A integrator. The signals from the UV-VIS detectors were monitored and recorded with an Omega Data system from Perkin Elmer.

Two different columns were used for the analysis of hemoglobin. The first column was a Supelcosil LC-304 (250 × 4.6 mm I.D., 5 μm particle size, 300 Å pore size, Labkemi AB). The second column was a Vydac 214TP54 (250 × 4.6 mm I.D., 5 μm particle size, 300 Å pore size, Scandinaviska GeneTec AB). Both columns were large pore C_4-columns. A 2 cm Supelguard guard column with 5 μm Supelcosil LC-304 packing was used for both separation columns. The samples were injected into a 10 μL loop (Valco Valves).

The chromatographic conditions were similar to those described previously [*2,3*]. Each mobile phase consisted of two solvents, A and B. Solvent A was a 80/20 mixture of 0.1% aqueous trifluoroacetic acid (TFA) and 0.1% TFA in acetonitrile. Solvent B was a mixture of these solutions in a 40/60 ratio. The gradient for the Supelcosil column was: 40% B at time 0, 50% B at 30 minutes, 55% B at 60 minutes and 70% B at 90 minutes. For the Vydac column: 43% B at time 0, 53% B at 30 minutes, 63% B at 60 minutes and 70% at 70 minutes. After each gradient, the system was returned to starting conditions in 5 minutes. Re-equilibration of the column was carried out by maintaining the initial composition for 10 minutes. The temperature was ambient and the flow rate was 1.0 mL/min.

Results

The chromatographic procedure described above resulted in separating hemoglobin into several peaks. Hemoglobin is the major protein in blood (for example, 15 percent of human blood) and the main peaks detected at short wavelengths in extracts from fresh bloodstains are those for the heme group and for the globin chains. The interpretation of the origin of different peaks has already been reported in the literature [*1–6*]. The heme peak has the same retention time for all hemoglobin species and its presence indicates that the stain is

actually blood. The globin peaks show a variety of retention times and are used for family or species identification. This agrees with the results reported in the literature [*1–4*].

Most of the species investigated showed two major peaks in addition to the heme peak. Both heme and globin peaks are detected with a good sensitivity at 220 nm. At 254 nm, the heme peak is strong, but the globin peaks are rather weak. Only the globin peaks exhibit fluorescence under the experimental conditions. Figure 1 illustrates the different chromatograms obtained by the three detectors for extracts from roe deer bloodstains. The native fluorescence of proteins is fairly weak [*7*] and only from some amino acids - tyrosine, tryptophan and phenylalanine. Yet the sensitivity of fluorescence detection is higher than that of UV detection. The injected amount, corresponding to 0.2 μL blood, resulted in globin peaks which almost saturated the fluorescence detector.

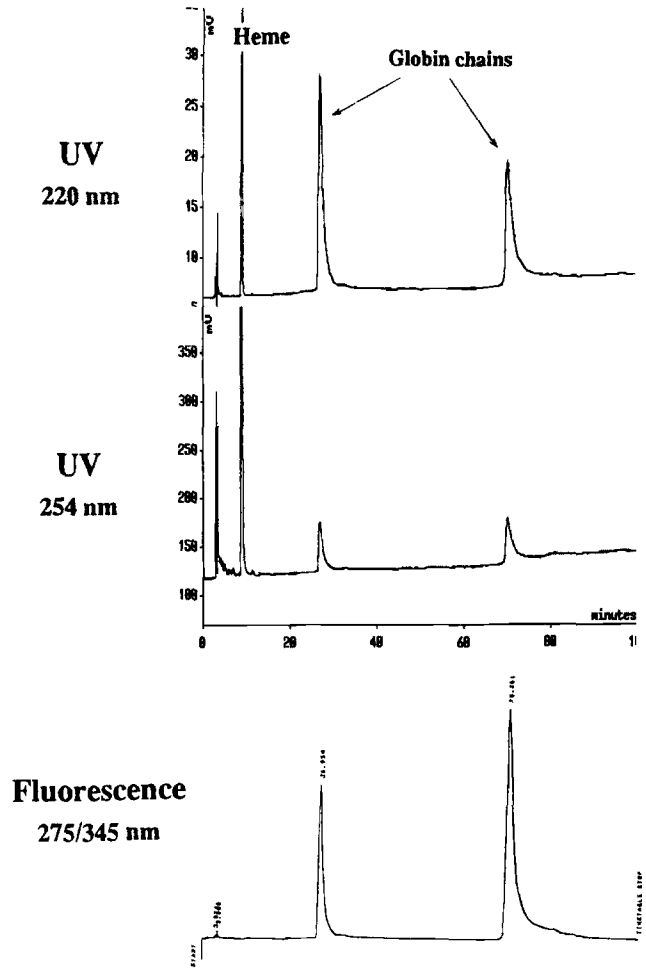

FIG. 1—*Separation of globin chains of a roe-deer. The sample was extracted from bloodstains on filter paper. The Supelcosil C_4 column and corresponding gradient was used. The sample size was about 0.2 μL blood. The three chromatograms are the results of detection at 220 nm, 254 nm and of fluorescence detection (the excitation wavelength 275 nm, the emission wavelength 345 nm). The heme peak is not detected by fluorescence detection.*

Figure 2 is a schematic diagram of the major peak positions in chromatograms of the species studied. These species are representative for the species often encountered in forensic examination, mostly common household animals and the animals living in Swedish forest. Between 2–6 individuals of each species were analyzed and the mean retention times are shown in this figure. The peak intensities are based on the detection at 220 nm. The column used was Supelcosil. Figure 2 shows that the polypeptide peaks are most often eluted after the heme peak. Some of the globin chains of pig and hare have, however, eluted before the heme peak. All the species presented in Fig. 2 could be distinguished with one exception—dog and wolf, which are genetically very similar. When several representants of the same species were compared, the retention times within each group varied by only a few tenths of a minute. These variations were comparable with the reproducibility achieved for the same sample.

The large pore columns used in this study (Supelcosil and Vydac) separated the globin chains in the same order but with different retention times. With the same chromatographic conditions the retention times on the Vydac column were much longer compared with the Supelco column. Therefore, different conditions were employed for the two columns used in this work. We have no data about the separation characteristic of columns from the same manufacturer produced at different times. It is probable, that the positions of the major globin peaks must be determined for each new column otherwise extrapolation of the retention times may be necessary.

Many species in Fig. 2 show two major polypeptide peaks in addition to the heme peak. There should not be any problem in distinguishing between these species on the basis of retention times alone. The combination of UV and fluorescence detection makes the differentiation between human hemoglobin and that of other species even better. We have calculated two different ratios between the two globin peaks for human hemoglobin and for some other species. One of these ratios is based on the ratio between peak absorbance at 220 and 254 nm, respectively. The second ratio expresses the ratio between peak absorbances at 220 nm and peak fluorescence. These ratios are shown in Table 1. The uncertainties in the values presented in Table 1 are about 5%. The results show clear

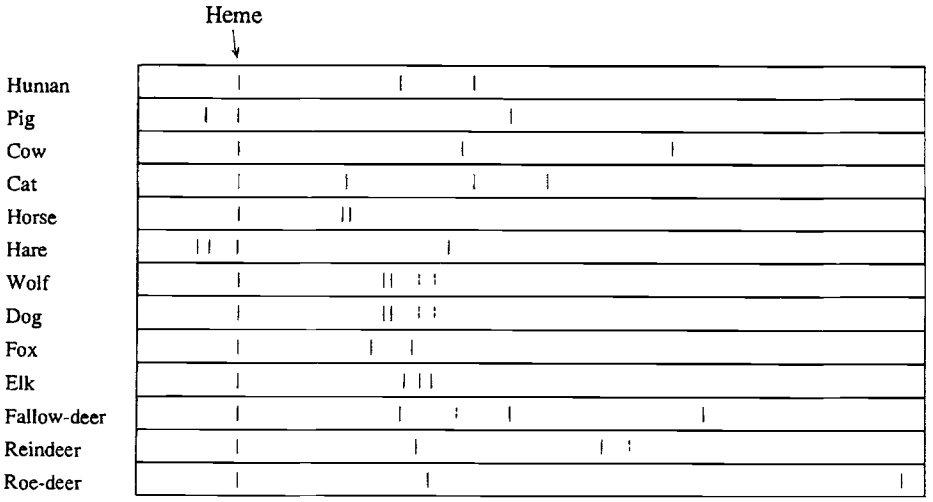

FIG. 2—*Comparison of main peak positions on chromatograms of bloodstain extracts from various species. The data are based on the detection at 220 nm and the use of the Supelcosil column. The retention time of the heme group is about 9 minutes. The dashed lines represent weaker peaks.*

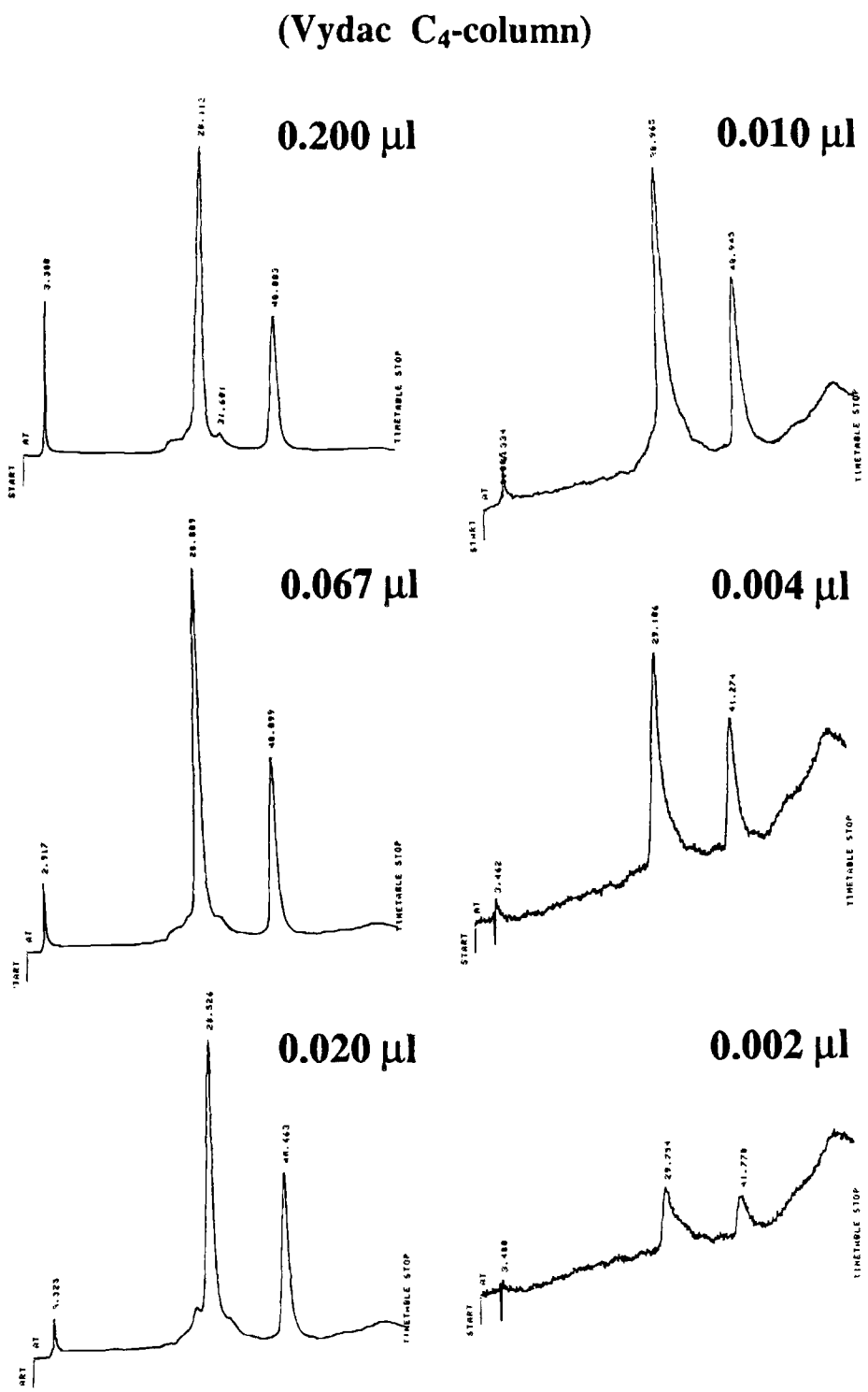

FIG. 3—*Fluorescence detection of various amounts of human blood. The amount of blood ranged from 0.2 µL to 0.002 µL and the Vydac C_4 column was used. Note a slight increase in retention times of the globin peaks with dilution of the sample.*

TABLE 1—*The differentiation between human hemoglobin and that of some other species based on the combination of UV and fluorescence detection. The ratios between the globin peak heights of various species detected at 220 nm, 254 nm and by fluorescence.*

	(Vydac C_4 - column)	
Species	Ratio 220 nm/254 nm[b]	Ratio 220 nm/fluorescence[c]
Pig	1.46	2.3
Cow	1.47	3.0
Reindeer	1.47	2.3
Roe-deer	1.38	2.7
Cat[a]	1.38	3.1
Fallow-deer[a]	1.40	2.3
Human	0.70	0.50

[a] The ratios were calculated for the first two of three globin peaks.

[b] This ratio is equal to R_1/R_2, where R_1 = Peak 1 (220 nm)/Peak 1(254 nm) and R_2 = Peak 2(220 nm)/Peak 2(254 nm).

[c] This ratio is equal to R_1/R_2, where R_1 = Peak 1(220 nm)/Peak 1(fluorescence) and R_2 = Peak 2(220 nm)/Peak 2(fluorescence).

differences between human hemoglobin and the other species investigated. The ratios varied slightly between the Supelcosil and the Vydac columns, because the composition of the mobile phase for peaks eluted from these two columns was not exactly the same.

The sensitivity of fluorescence detection has the advantage that minute amounts of blood can be detected. Figure 3 shows analyses of various amounts of human blood, ranging from 0.2 μL to 0.002 μL, with fluorescence detection. The first peak eluated was assigned β-globin and the second peak α-globin, according to corresponding peaks detected by UV and the literature data on the UV-detection [*1–3,5*]. The calibration curve for the β-and α-globulin peaks was linear down to 0.004 μL. The detection limit is estimated as 0.001–0.002 μL blood, lower amounts will be lost on the column. The chromatograms in Fig. 3. were obtained with the Vydac column, which showed better properties than the Supelcosil column

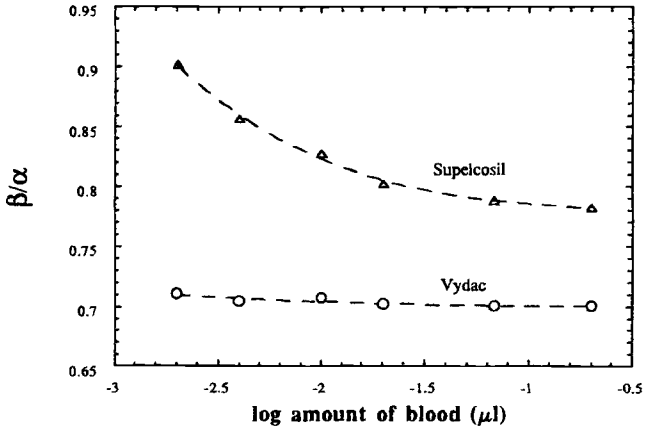

FIG. 4—*Comparison between the Supelcosil and the Vydac large pore columns. Extracts from human bloodstains corresponding to the amount of blood in the range of 0.002 to 0.2 μL were analyzed. The figure shows the dependence of the ratio between the retention times of the globin chains (β/α) on the amount of blood (logarithmic scale). This dependence is negligible for the Vydac column in the concentration range studied.*

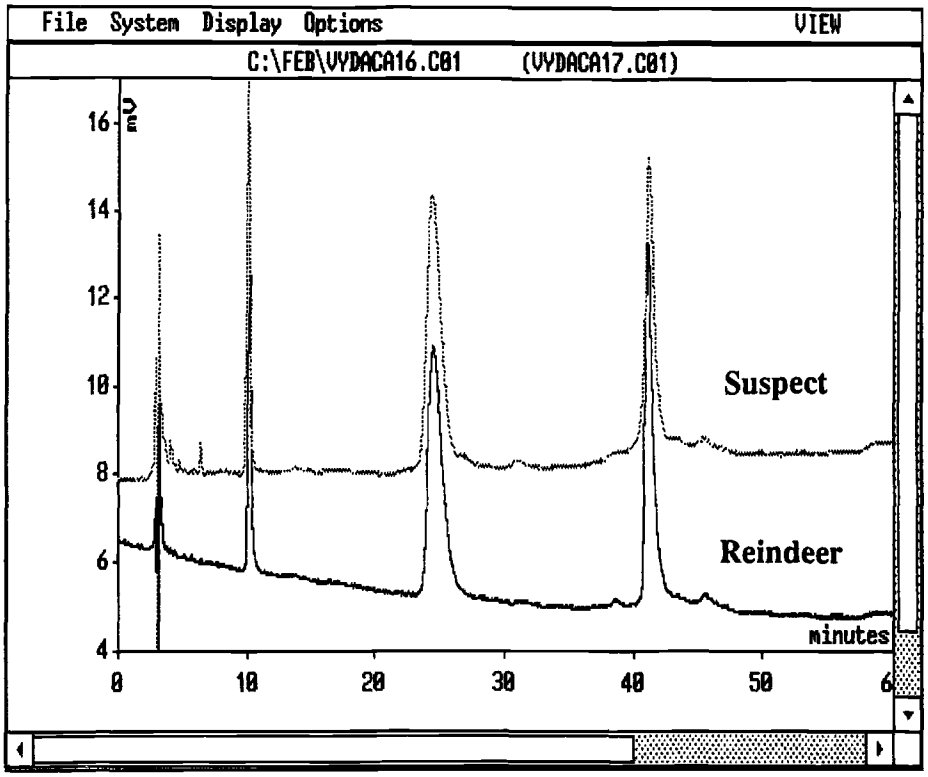

FIG. 5—*Comparison of the HPLC analysis of output water, suspected of containing blood, and authentic bloodstains from reindeer. The chromatograms were detected at 220 nm using the Vydac column. The reference chromatogram corresponds to about 0.07 μL of reindeer blood. The concentration of blood in the output water was about 1% and the species was reindeer. The small peak with a retention time of about 6 minutes is an impurity or a decomposition product.*

when very small amounts of blood were injected. With the Supelcosil column, the retention times of the globin peaks (particularly that of the β-globin chain) increased considerably with decreasing amount of blood injected. The β-globin peak was also broadened and the separation between the globin peaks decreased with decreasing concentration. These effects were much less serious when the Vydac column was used. Figure 4 shows a comparison between these two column when expressed as the variation of the ratio between the retention times of the globin peaks with the amount of human blood analyzed. A similar dependence of retention times on the concentration of hemoglobin was observed for other species. Clearly a calibration of retention times against the concentration of hemoglobin is recommended when using a new column, if low concentrations of hemoglobin are to be determined.

The UV detection of the globin peaks at 220 nm has a detection limit of about 0.01 μL. The sensitivity of detection at 254 nm is lower for the globin peaks, but the heme peak may be detected for amounts below 0.001 μL blood. Thus the combination of fluorescence detection (of the globin peaks) and UV detection at 254 nm (of the heme peak) can identify human bloodstains in amounts down to a few nanoliters of blood. Very small bloodstains can be used for identification of different species and human bloodstains can be identified prior to DNA analysis with little loss of the forensic evidence—the blood sample.

Figure 5 shows an application of the HPLC method in casework. Water used for sewage treatment was suspected of containing blood. Was this really blood, what kind of blood,

and what was its concentration? A sample of the output water was injected (10 μL) directly onto the column (Vydac). The chromatogram (λ = 220 nm) is presented in Fig. 5. The positions of two major globin peaks corresponded to those determined for blood from a reindeer. An extract from bloodstains of reindeer in the amount corresponding to 0.07 μL blood was also analyzed (Fig. 5). The globin peaks in these two chromatograms matched exactly. The output water contained blood from reindeer and the concentration was estimated to be about 1% from the height of the heme peaks in Fig. 5. The blood came from a slaughter-house for reindeers.

The HPLC method reported here is recommended for screening work and the results should be confirmed by another independent, (for example, immunological) method. This is particularly recommended for bloodstains from young species. It is known, that additional globin chains are detected for fetal human hemoglobin compared with hemoglobin from normal adults [*1–3*]. Similar situation can be expected when analyzing other very young species. Also abnormal hemoglobins give another pattern [*2,3*]. The globin peaks in Fig. 2 were obtained for normal hemoglobin from adults.

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