Int J Leg Med (1990) 104:9–12

International Journal of
Legal Medicine

Species identification of blood and bloodstains by high-performance liquid chromatography

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Received February 1, 1990

Summary. A reverse-phase high-performance liquid chromatographic method for species identification of blood and bloodstains is described. The method employs a 300 Å pore SynChropak RP-4 column and ternary solvents (acetonitrile-trifluoroacetic acid-water) and can not only identify a species by its characteristic chromatogram, but also simultaneously demonstrates that it is of blood origin by the existence of the heme peak. Deformations in chromatographic profiles obtained with older bloodstains were observed, but the retention times of heme and the major peaks showed only minor changes. The species could be identified from bloodstains at least 3 months old and the present method has the advantage of simplicity, speed and sensitivity in the practice of forensic science.

Key words: Blood stains – Species identification – Hemoglobin – HPLC

Zusammenfassung. Beschrieben wird eine „reversed-phase" HPLC-Methode zur Identifizierung und Spezifizierung von Blut und Blutspuren. Die Analysenmethode besteht aus einer SynChropak RP-4-Säule und einem ternären Lösungsmittelgradientensystem (Acetonitril/Trifluoressigsäure/Wasser). Mit dieser Methode wird durch die Detektion des Häm-Peaks die Probe als Blut identifiziert und die Herkunft (human/tierisch) gleichzeitig durch charakteristische Chromatogramme UV-spektrometrisch spezifiziert. Deformationen der chromatographischen Profile wurden bei älteren Blutspuren beobachtet, wobei sich aber die Retentionzeiten der Hauptkomponenten nur geringfügig änderten. Der Speziesnachweis gelang bei bis zu drei Monate alten Blutspuren. Die vorgestellte Methode hat den Vorteil der Einfachheit, der Schnelligkeit und der Empfindlichkeit für die forensische Praxis.

Schlüsselwörter: Blutspuren – Spezies-Identifizierung – Hämoglobin – HPLC

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Introduction

Differentiation between human and animal bloodstains is of great importance in the identification of an individual specimen. The first step in bloodstain examination is to determine whether a stain is actually of blood origin [1]. Preliminary and confirmatory tests for this purpose generally depend on the demonstration of heme or its peroxidase activity. Following identification of a stain as blood, suitable tests may be conducted to determine the species. Numerous immunological methods, such as the precipitin test [2], immunodiffusion tests and immunoelectrophoresis, as summarized by Lee et al. [3], have been developed using antisera specific for proteins contained in human blood. In recent years enzyme-linked immunosorbent assay and radioimmunoassay have also been developed [4, 5]. However, for the determination of other species, it is necessary to use antisera specific for those species.

In the field of forensic science, high-performance liquid chromatography (HPLC) has often been used for qualitative and quantitative determination of various kinds of drugs, narcotics and stimulants [6], as its resolution, sensitivity and speed compare favorably with the more traditional chromatographic methods. The usefulness of HPLC for the identification of human fetal hemoglobin in bloodstains has recently been described [7] and in this paper, species identification of blood and bloodstains using reverse-phase HPLC is described.

Materials and methods

Preparation of samples and heme standard solution. Human blood and blood of other primates and various other species (see Table 1) were collected in EDTA or heparin, with the exception of rainbow trout and sardine blood, which was used neat. The methods used for preparing hemolysates, bloodstains on filter paper and the extracts from these bloodstains have been described in a previous paper [7]. Mixed bloodstain samples were made by dropping equal volumes of human blood and blood of other species (pig, cow, dog, cat and chicken) onto filter paper.

Table 1. Species of blood samples investigated using HPLC

Name	No.	Name	No.
Humans		*New World monkeys*	
Adult	10	Tufted capuchin	3
Neonate	11	Night monkey	4
Apes		*Prosimians*	
Chimpanzee	3	Black lemur	1
Orang-utan	1	*Non-primate vertebrates*	
Agile gibbon	2	Pig	8
White-handed gibbon	2	Cow	8
Old World monkeys		Dog	3
François' lutong	1	Cat	3
Mandrill	1	Rabbit	3
Hamadryas baboon	2	Rat	3
Japanese monkey	3	Mouse	4
Pig-tailed monkey	2	Chicken	1
Assamese monkey	2	Quail	1
Rhesus monkey	2	Rainbow trout	4
Crab-eating monkey	2	Sardine	4
Patas monkey	2		
Savannah monkey	3		

Hemin (bovine, type I) was purchased from Sigma Chemical Co., and was dissolved in dimethylsulfoxide (amino acid sequencing grade, Wako Pure Chemical Industries, Osaka, Japan) to give a concentration of 5 m*M* and diluted with 0.1% trifluoroacetic acid in 40% acetonitrile to give a final concentration of 0.1 m*M*.

Apparatus. The liquid chromatographic system used in this study was a Shimadzu LC-6A liquid chromatograph consisting of two LC-6A pumps, an SCL-6A system controller, an SIL-6A auto injector, a CTO-6A column oven, an SPD-7AV UV-VIS spectrophotometric detector and a C-R6A computing integrator.

Chromatographic conditions. The method is a minor modification of the procedure described previously [7]. The column used was a 300 Å pore SynChropak RP-4 (250 × 4.6 mm I.D., SynChrom, Lafayette, Ind.). The solvents consisted of acetonitrile, trifluoroacetic acid and water, as detailed by Shelton et al. [8]. The first gradient (40%–50% B in 30 min, 50%–55% B in 30 min, and 55%–70% B in 30 min) was used to separate globin chains from all species studied. The second gradient (44%–50% B in 30 min, 50%–52% B in 20 min, and 52%–56% B in 20 min) was used to separate globin chains in humans and other primates. After each gradient, the solvents were maintained at final percentages of solvent B for the appropriate time and then returned to starting conditions in 2 min. Re-equilibration was carried out for at least 20 min. The flow rate was 1.0 ml/min at a column temperature of 35°C. The column effluent was monitored at 220 nm.

The heme standard solution and hemolysates (approximately 3 mg/ml hemoglobin) were applied to the column in 20-µl volumes while those of the extracts varied from 10 to 100 µl.

Results

Figure 1 illustrates the typical chromatograms of bloodstain extracts (1 week old) from several species using the first gradient. Chromatographic profiles of hemolysates and relatively fresh stain extracts obtained from the same species were almost identical. A peak at a retention time of about 11 min was identified as heme by comparison with that of the standard solution. The retention times of two or more major peaks, which were considered the α and β globin chains, varied according to the species, while those of heme were identical regardless of species. It appears that some minor peaks that eluted before and after heme are various plasma components. The major peaks on chromatograms from two canine bloods were incompletely separated by this HPLC procedure, but were similar to those described by Schroeder et al. [9].

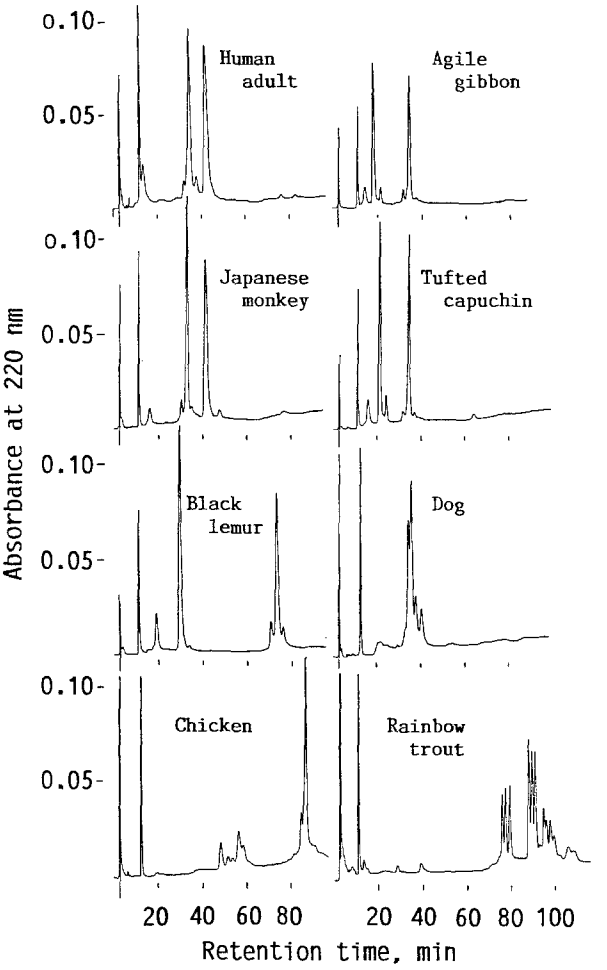

Fig. 1. Chromatograms from extracts of 1 week-old bloodstains of several species, obtained using reverse-phase HPLC. The first gradient (40%–50% B in 30 min, 50%–55% B in 30 min, and 55%–70% B in 30 min) was used

Figure 2 is a schematic comparing the major peak positions on chromatograms from hemolysates of the species studied using the first gradient. The data on the two fish samples were from the bloodstain extracts. As for chicken and quail, the chromatographic profiles obtained from the two species were similar, although only one individual of each species was examined. Rat hemoglobin has long been known to be highly heterogeneous [10, 11]. The chromatograms obtained from rats (Wistar) also exhibited a major peak and some minor ones, which were thought to be the α and some heterogeneous β globin chains, respectively, on comparison with the report of Schroeder et al. [9]. The chromatographic profiles of

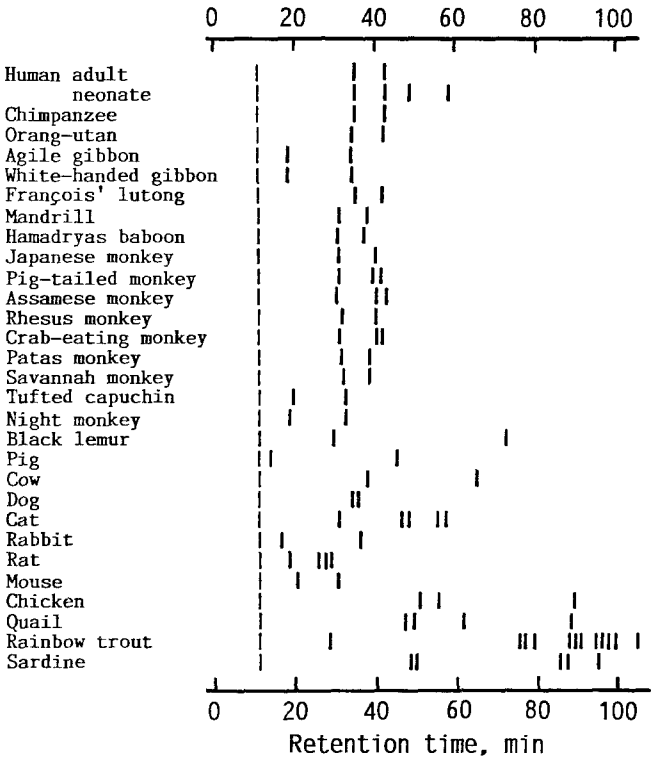

Fig. 2. Comparison of peak positions on chromatograms of hemolysates from species studied. The first gradient was used

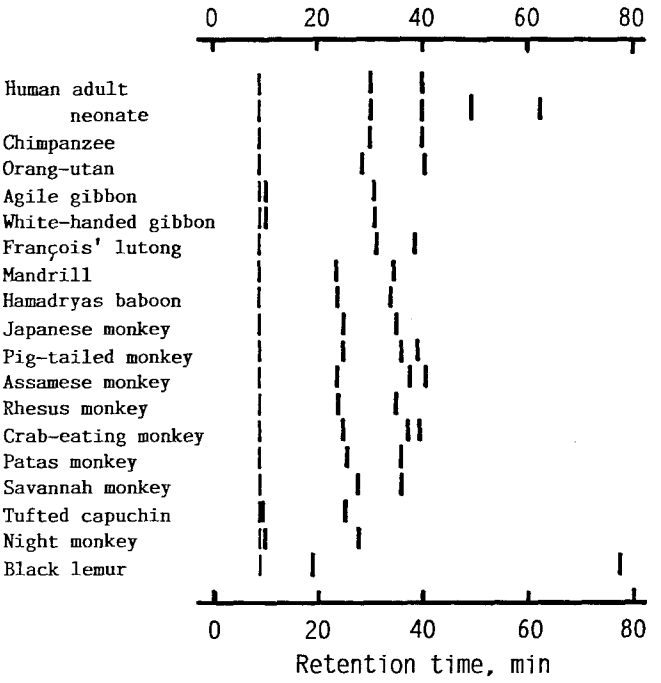

Fig. 3. Comparison of peak positions on chromatograms of hemolysates from the primates. The second gradient (44%–50% B in 30 min, 50%–52% B in 20 min, and 52%–56% B in 20 min) was used

the two gibbons were more similar to those of New World monkeys than to those of other apes, so far as the retention times were concerned. Human blood and bloodstains could quite easily be differentiated from those of gibbons, New World monkeys and other species ranking below New World monkeys within 90 min using the triphasic linear gradient of 40% to 70% solvent B.

The chromatographic profiles of primates ranking above Old World monkeys were closely similar to the human profiles, with the exception of the two gibbons. HPLC analysis was subsequently performed on samples of primate origin using the second gradient, which was suitable for separating the human globin chains. Figure 3 shows a schematic diagram comparing the major peak positions of hemolysates from primates using the second gradient. The retention times of the two main peaks of the black lemur, a prosimian, were considerably different from those of the other primates. One of the main peaks obtained from New World monkeys eluted together with heme or in close proximity to it, so that there was a clear difference in the elution profiles of the New and Old World monkeys. Many investigators have reported that some Old World monkeys such as, the pig-tailed monkeys, the Assamese monkeys and the crab-eating monkeys [12], have phenotypic variability of hemoglobin. This variability also appeared in the chromatograms. There was no difference between human and chimpanzee profiles, because both have identical amino acid sequences in the hemoglobin. It is clear that the second gradient is more effective for the differentiation of human blood and bloodstains from those of the higher primates.

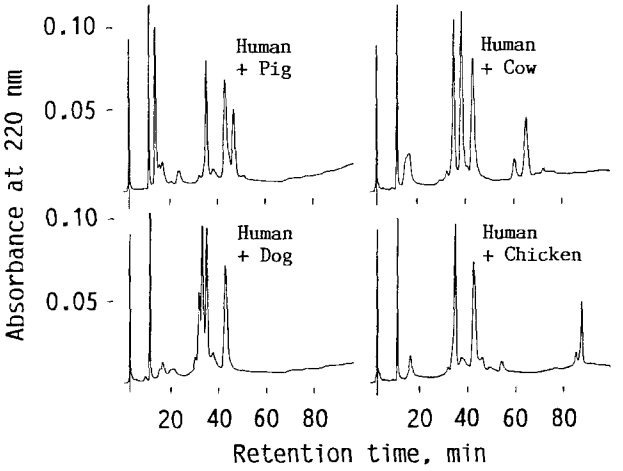

Fig. 4. Chromatograms from extracts of mixed bloodstains aged 4 weeks by reverse-phase HPLC. The first gradient was used

Figure 4 illustrates the first-gradient chromatograms from the extracts of mixed bloodstains which were made from a mixture of blood from human and other species and stored for 4 weeks. The globin chains in the mixed samples were separated on a column and eluted with their characteristic retention times. The results demonstrate that the present method is effective for identifying the species, even if a bloodstain sample is a mixture of blood from various species.

Discussion

The identification of blood and determination of human origin must be carried out on a doubtful stain prior to

blood group typing for personal identification, because human blood group antigens, such as the ABO system, are widely distributed in nature. Springer et al. [13] demonstrated that some bacteria have substances resembling human blood group antigens. Cowan [14] also reported that substances similar to blood factors have been found in some animals. Therefore, it is preferable to develop a rapid and reliable method for the identification of blood and species of origin. Ito et al. [15] described HPLC analysis for the identification of species, based on the amino acid composition of hemoglobin α-T4 tryptic peptide, which was characteristic for each species studied. Oshima et al. [16] reported the application of isoelectric focusing in polyacrylamide gel plates, with particular use of *p*-chloromercuribenzoate in treating hemoglobin. These methods could not only determine whether a stained material was of human origin but, in the case of a non-human stain, could also identify the species. The HPLC method described in the present study demonstrated that a stain was actually blood on the basis of the existence of a heme peak at a retention time of about 11 min on the first-gradient chromatograms, and the same chromatograms also revealed the species of origin. In this study, the minimal volume of hemoglobin applied to the column was 40 μg, which corresponded to approximately 0.3 μl fresh whole blood. This method is very simple, rapid and sensitive.

Some extra peaks appeared before and after the major ones with older bloodstain samples, and were often incompletely separated from the major ones. These were thought to be denaturation products of the native globin chains, because these increased with decreasing area of the native major peaks. However, the retention times of heme and the major peaks were little changed. No problems were encountered in identifying the species from bloodstains up to 3 months old.

It is generally considered that the mechanism of separation using reverse-phase HPLC functions mainly through hydrophobic interactions [17, 18] and amino acid substitutions of globin chains may alter their retention times. As numerous variants of globin chains in human and other primates have been reported [12, 18], this must be borne in mind in attempts to determine the origin of bloodstains.

Studies are in progress which will enable the identification of various species from blood and bloodstains by comparison of the α and β globin chain retention times, as well as chromatographic profiles. The present method can simultaneously identify blood and determine the species of doubtful stains and has the advantage of simplicity, speed and sensitivity.

Acknowledgements. We wish to thank Dr. Haruhisa Inagaki and Dr. Shigetaka Kotera, Japan Monkey Centre, for supplying us with some of the primate blood samples. This study was supported in part by a Grant for Cooperative Research from the Primate Research Institute, Kyoto University.

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