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Identification and Quantitation of Source from Hemoglobin of Blood and Blood Mixtures by High Performance Liquid Chromatography

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ABSTRACT: The described technique offers a sensitive and reproducible method for inferring the source of over 50 different animal species from bloodstains and blood mixtures. Hemoglobins from each of the species were examined using reversed-phase high performance liquid chromatography (HPLC) in chromatographic times of less than 25 mins. The HPLC method complements and furthers current methodology for identification of species of origin. HPLC analysis is particularly well suited for the quantitative analysis of blood and blood mixtures and is applicable to species for which antisera are unavailable. The sensitivity of the method (hemoglobin amounts down to 1.2 μg) lends itself to the analysis of blood mixtures in which only a small percentage of the mixture represents blood from a given species. Such resolution and quantitation is applicable to wildlife forensic casework.

KEYWORDS: forensic science, species identification, hemoglobin, bloodstains, high performance liquid chromatography

Blood evidence is common in wildlife forensic science. The wildlife forensic community examines blood evidence similarly to the human forensic community. In many instances, whole blood offers the greatest amount of usable evidence in the form of protein and DNA genetic markers identifying family, genus, or species of animals. The greatest underlying difference between the human and wildlife forensic application is that blood evidence from humans arises from a single species, while blood evidence from animals may be one of many different species. Erythrocytes are the most abundant of the blood's formed elements. Hemoglobin represents about one-third the erythrocyte weight that corresponds to approximately 0.15 g/mL in adults (1). Analytical blood techniques range from presumptive testing (i.e., benzidine, luminol, etc.) to identify analysis (i.e., DNA techniques).

The National Fish and Wildlife Forensics Laboratory (NFWFL) uses immunodiffusion or immunoelectrophoresis to determine family of origin depending on the amount and quality of blood or tissue evidence. Once family has been established for an item. Species is determined by isoelectric focusing and staining for proteins that are known to differentiate one species within a family from another. The primary limitation of both immunological methods is that antisera are not available for many wildlife families. While antisera for such families as deer, bear, dog, and cat families are commercially available, antisera for bird, amphibian, and reptile families are difficult to obtain and sometimes must be developed. Isoelectric focusing represents a significant advance in electrophoretic methods, reducing the amount of evidence required and increasing the repeatability between analyses. The major limitation with isoelectric focusing as with other electrophoretic methods is finding species-specific protein markers that are stable and detectable in degraded evidence samples. Additionally, isoelectric focusing has difficulty differentiating between neutral amino acid substitutions that have occurred in two otherwise similar proteins and thus is limited in its ability to distinguish globin differences completely (2). Because blood is collected from so many crime scenes, the HPLC technique on hemoglobin provides a powerful species-specific marker that could identify evidence where other methods have been inconclusive.

Previous work has pointed out how discriminating, even with very minor differences, HPLC can be for distinguishing various hemoglobins (3–9). Kutlar and coworkers showed that variations in retention time signals were observed even for proteins with similar single amino acid residue substitutions (3). In addition, food sciences practitioners have applied HPLC techniques to the separation of hemoglobin, myoglobin, and other pigments from tissue samples (10–12).

Hemoglobin is most generally characterized as consisting of two α-β dimers that couple to form a tetramer in physiological conditions. Each globin chain is associated with a prosthetic heme unit. Analysis by HPLC of animal hemoglobin produces chromatograms with three or more significant peaks. Generally these peaks correspond to the α-globin chain, the β-globin chain, and the heme unit. We found some species possessed as many as four distinct chromatographic peaks. This is not unexpected as some animals are known to contain heterogenous globins (6,13). For the well-represented species studied by HPLC, the number of major globin chain signals and their positions (unique retention times, e.g., cow, human, etc.) were reproducible. In this report we present the results of the HPLC analysis of 275 animal samples. The advantages of the HPLC technique (*vide infra*) over existing methodology are simplicity, time efficiency, and resolution.

Material and Methods

Blood and Bloodstain Samples

Hemoglobin standards were purchased from Sigma (St. Louis, Missouri) for the following species: horse, dog, sheep, pig, turkey,

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TABLE 1—*Family, genus, and species identified plus their associated retention times (in minutes) and retention time variance (S^2).*

ORDER	FAMILY	GENUS	SPECIES	COMMON NAME	N	β CHAIN	β s^2	α CHAIN	α s^2	ω CHAIN	ω s^2
ANSERIFORMES	ANATIDAE	ANAS	PLATYRHYNCHOS	MALLARD	7	13.0	0.031	15.1	0.001	23.9	0.005
ARTIODACTYLA	ANILOCAPRIDAE	ANILOCAPRA	AMERICANA	PRONGHORN	6	12.0	0.111	16.3	0.016		
	BOVIDAE	ANTILOPINAE	AMERICANA	ANTELOPE	11	11.7	0.015	15.6	0.092	15.9	0.014
		BOS	TAURUS	COW	5	12.1	0.067	17.1	0.099		
		CAPRA	HIRCUS	GOAT	4	6.2	0.009	10.5	0.036	12.2	0.087
		ORYX	GAZELLA	ORYX	8	11.7	0.013	14.2	0.029		
		OVIS	ARIER	SHEEP DOMESTIC	6	5.5	0.0021	11.2	0.066	11.6	0.065
			CANADENSIS	BIGHORN SHEEP	11	6.8	0.076	12.0	0.014		
		PSEUDOIS	NAYAUR	BLUE SHEEP	1	7.1		12.0		16.2	
	CERVIDAE	ALCES	ALCES	MOOSE	11	11.6	0.050	12.0	0.006	13.3	0.014
		CERVUS	ELAPHUS	ELK	13	11.5	0.075	13.7	2.43		
			ELAPHUS	RED DEER	1	11.1		11.4			
			NIPPON	SIKA DEER	10	6.0	0.002	10.9	0.008	15.1	0.004
		ELAPHURUS	DAVIDIANUS	PERE DAVID DEER	3	6.7	0.0001	15.3	0.001		
		ODOCOILEUS	HEMIONUS	BLACK-TAILED DEER	11	11.0	0.036	15.9	0.0007	19.2	0.012
		ODOCOILEUS	HEMIONUS	MULE DEER	11	11.1	0.048	15.8	0.096		
		ODOCOILEUS	VIRGINIANUS	WHITE-TAILED DEER	13	11.4	0.101	19.2	1.437	19.1	
	SUIDAE	SUS	SCROFA	HOG	11	5.5	0.053	12.9	0.008		
	TAYASSUIDAE	TAYASSU	TAJACU	PECCARY	3	6.5	0.007	14.9	0.012	15.5	0.001
CARNIVORA	CANIDAE	CANIS	FAMILIARIS	DOG	27	9.8	0.003	10.3	0.837		
			LUPUS	WOLF	3	10.0	0.002	10.5	0.008		
			OCCIDENTALIS	WOLF	1	9.9		10.3			
		MEGALOTIS	OTOCYON	BAT EARED FOX	1	10.3		11.1			
		VULPES	VULPES	GREY FOX	1	10.1		10.8			
	FELIDAE	ACINONYX	JUBATUS	CHEETAH	1	10.3		13.3			
		FELIS	CATUS	CAT	11	9.4	0.019	12.8	0.015	13.9	0.086
			RUFUS	BOBCAT	1	10.3		13.0		14.2	

TABLE 1—*Continued.*

ORDER	FAMILY	GENUS	SPECIES	COMMON NAME	N	β CHAIN	β s^2	α CHAIN	α s^2	ω CHAIN	ω s^2
		PANTHERA	TIGRIS	TIGER	1	8.6		13.5		14.6	
	MUSTELIDAE	MARTES	AMERICANA	MARTEN	1	10.2		11.3			
CARNIVORA	MUSTELIDAE	MUSTELA	GRIPES	FERRET	5	9.0	0.003	10.9	0.002	12.3	0.001
	URSIDAE	URSUS	AMERICANUS	AMERICAN BLACK BEAR	9	7.5	0.013	10.5	0.005		
			MALAYANUS	SUN BEAR	2	7.5	0.003	10.5	0.002		
			MARITIMUS	POLAR BEAR	2	7.6	0.002	10.5	0.002		
			TIBETANUS	HIMILAYAN	1	7.4		10.4			
			URSINUS	SLOTH BEAR	2	7.6	0.034	10.6	0.02		
COLUMBIFORMES	COLUMBIDAE	STANDARD		PIGEON	1	15.7		16.0		18.9	
FALCONIFORMES	ACCIPITRIDAE	AQUILA	CHRYSAETOS	GOLDEN EAGLE	1	14.0		14.7		22.1	
		HALIAEETUS	LEUCOCEPHALUS	BALD EAGLE	1	13.7		14.4		20.7	
GALLIFORMES	PHASIANIDAE	MELEAGRIS	GALLOPAVO	TURKEY	1	14.3		15.5		21.6	
		PHASIANUS	COLCHICUS	RING-NECKED PHEASANT	7	13.8	0.012	15.2	0.006	22.5	0.014
LIGOMORPHA	LEPORIDAE	ORYCTOLAGUS	CUNICULUS	RABBIT	1	5.8		10.5			
PERISSODACTYLA	EQUIDAE	EQUUS	CABALLUS	HORSE	2	10.0	0.054	10.9	0.8		
			CABALLUS	PRZEWALSKI	2	9.8	0.002	10.3	0.001		
			GREVI	GREVYS ZEBRA	1	10.2		11.5			
			HEMIONES	KULAN	3	9.8	0.097	10.5	0.382	11.2	0.001
			ZEBRA	HARTMAN MOUNTAIN ZEBRA	10	10.2	0.003	11.2	0.191	12.6	2.358
PRIMATES	CERCOPITHECIDAE	PAPIO	BABOON	BABOON	1	10.0		12.3			
	HOMINIDAE	HOMO	SAPIENS	HUMAN	4	11.1	0.047	13.4	0.032		
PROBOSCIDAE	ELEPHANTIDAE	LOXODONTA	AFRICANA	AFRICAN ELEPHANT	3	9.0	0.009	13.0	0.352	13.6	0.001
RODENTIA	MURIDAE	RATTUS	RATTUS	RAT	1	6.7		9.1			
	SCIURIDAE	PSERMOPHILUS	BEECHEYI	GROUND SQUIRREL	1	10.6		12.5			
		BEECHEYI	PSERMOPHILUS	TREE SQUIRREL	2	11.1	0.001	12.5	0.001		
		SCIURUS	CAROLINENSIS	GREY SQUIRREL	1	13.5		15.4			
			NIGER	FOX SQUIRREL	1	10.7		12.9		15.4	
			TAMIASCUIRUS	RED SQUIRREL	1	11.3		12.5			
SIRENIA	TRICHECHIDAE	TRICHECHUS	MANATUS	MANATEE	1	9.9		10.6		11.2	

TABLE 2—*HPLC gradient time table for species identification.*

Time, min	Solvent, %		
0:00	A: 60	B: 40	
15:00	A: 50	B: 50	
25:00	A: 45	B: 55	
30:00	A: 0	B: 0	C: 100
35:00	A: 60	B: 40	

human (HbA), human sickle-cell (HbS), cat, goat, bovine, rabbit, pigeon, baboon, and rat. The domestic blood samples listed in Table 1 were obtained from local veterinary clinics. The other species listed in Table 1 were taken from the reference collection at the NFWFL.

Fresh Whole Blood—Five to twenty-five μL of fresh whole blood was added to 1 mL of pure distilled water, causing hypotonic lysis and then filtered through a syringe tip filter (25-mm, 0.45-μg Nylon filters from Lab Source Inc.). The EDTA or heparin or both present in the blood collection vacutaines used at veterinary clinics made no difference in the retention times of the globin signals.

Dried Blood—Dried blood samples collected on sterilized gauze were also analyzed. Many samples were obtained from the reference collection at the NFWFL which is maintained at −60°C. The dried blood samples were prepared by cutting a small blood-soaked piece from the gauze (25-mm^2 section). The blood was then removed from the gauze by wetting with 1 mL of distilled water and filtering.

High Performance Liquid Chromatography (HPLC)

The chromatograms were obtained using a Hewlett Packard 1090 Series II HPLC. The instrument's diode-array detector (DAD) allowed for the simultaneous spectrophotometric observation of the analytes. The detector's wavelength settings were established at 210 and 410 nm. These settings detected the proteins and the heme, respectively. A 125-μL injection loop was used. The average injection volume was 10 μL. A Merck, LiChroCART 125-4, LiChrospher 100 RP-8 (5-μm) column (125 by 4 mm) was used. A Merck, LiChroCART 4-4, LiChrospher 100 RP-8 (5-μm) guard column (4 by 4 mm) was placed in-line.

The mobile phase of the system consisted of the following solvents using the gradient displayed in Table 2.

Solvent A—HPLC grade water (Fisher Chemicals, Pittsburgh, Pennsylvania) with 0.1% trifluoroacetic acid (TFA) (Pierce Chemicals, Rockford, Illinois) and 0.1% triethylamine (TEA) (Fisher Chemicals) added.

Solvent B—HPLC grade acetonitrile (Fisher Chemicals) with 0.1% TFA and 0.1% TEA added.

Solvent C (wash)—1.36 g of sodium acetate (Fisher Chemicals), plus 0.02 g of Na_2EDTA (Fisher Biotech, electrophoretic grade) added to 100 mL of HPLC grade water, then pH balanced to 7.2. The pH was adjusted with dilute glacial acetic acid (Fisher Chemicals) and 6*M* sodium hydroxide (Fisher Chemicals). To the resulting solution, 400 mL of acetonitrile was added for a total volume of 0.5 L.

All the solvents were sparged for approximately 20 min with helium gas. The solvents were prepared freshly every 48 h, and the solvent flow rate was 1.0 mL/min at 36°C.

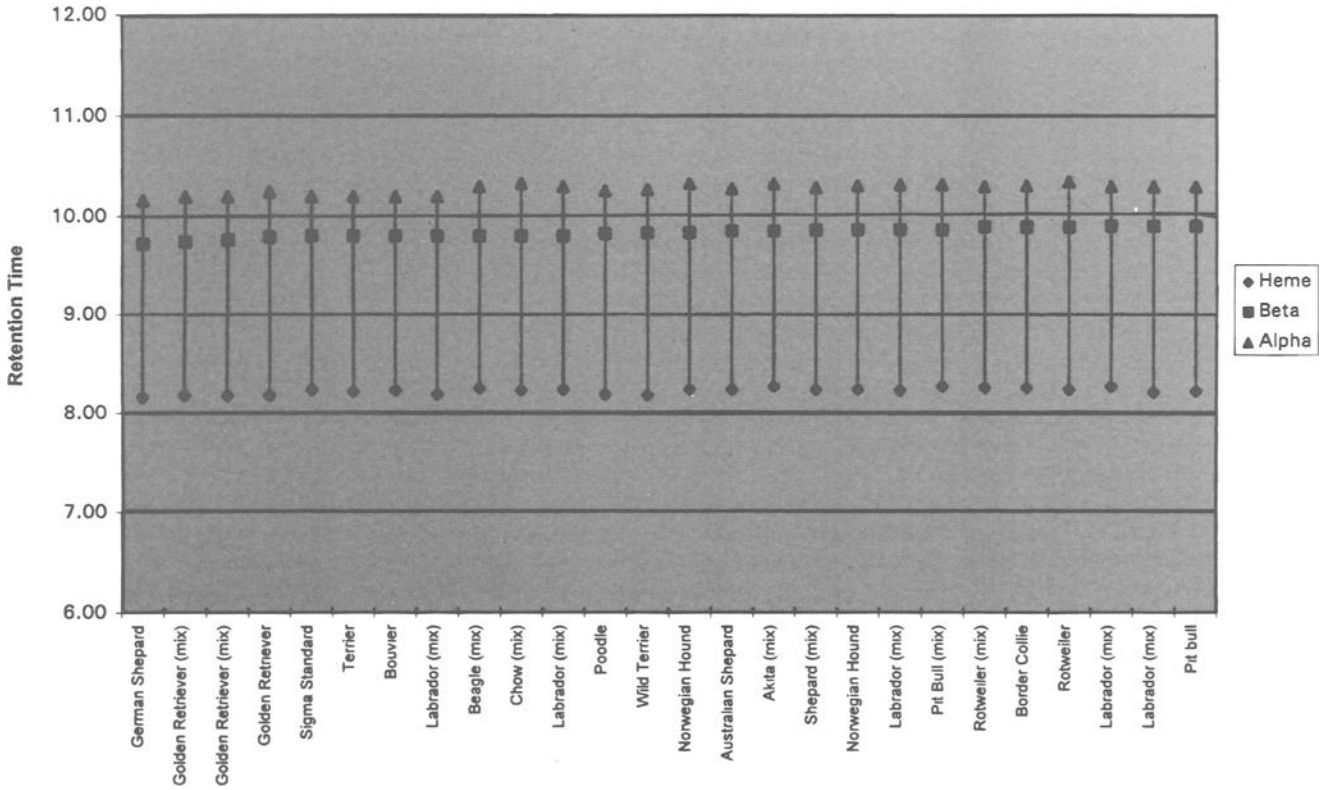

FIG. 1—*Graph of the reproducibility study for* C. familiaris.

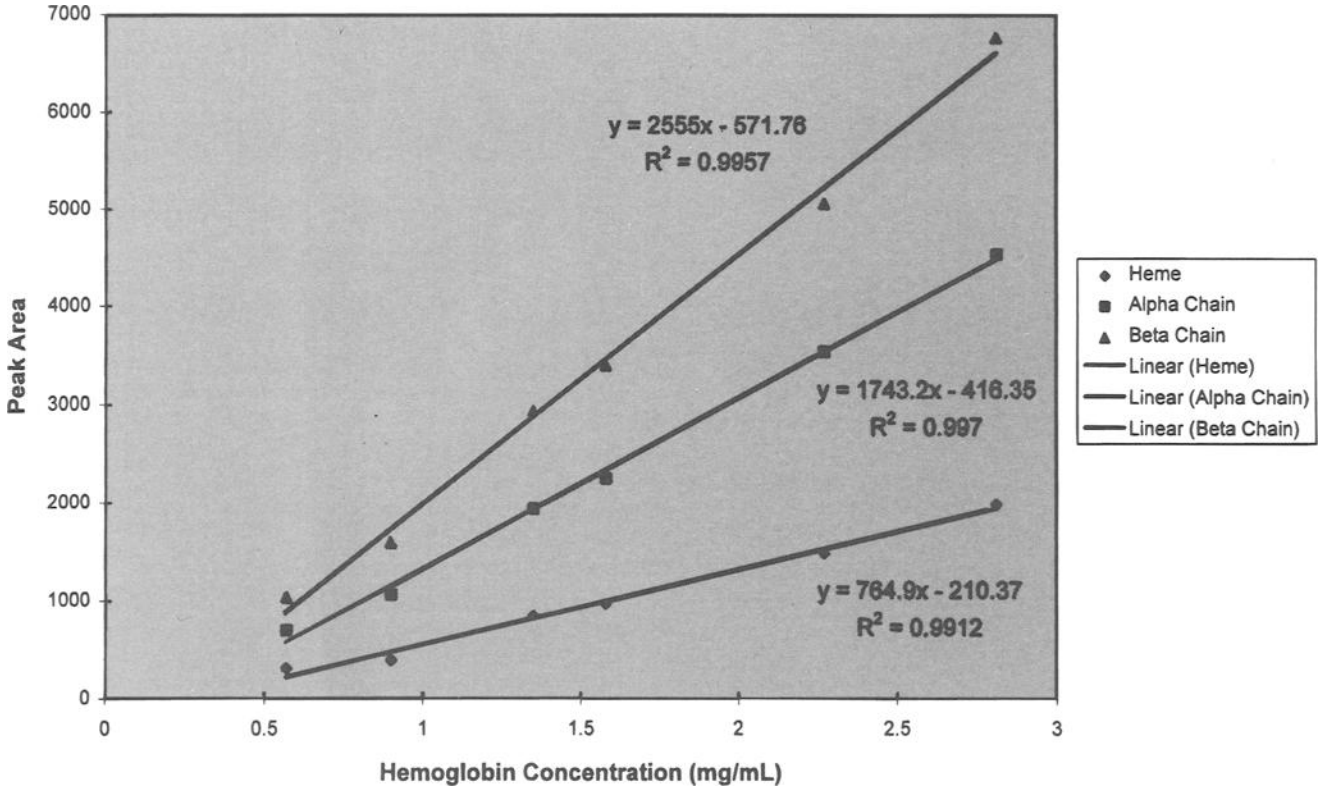

FIG. 2—*Regression of* C. familiaris *hemoglobin over peak area.*

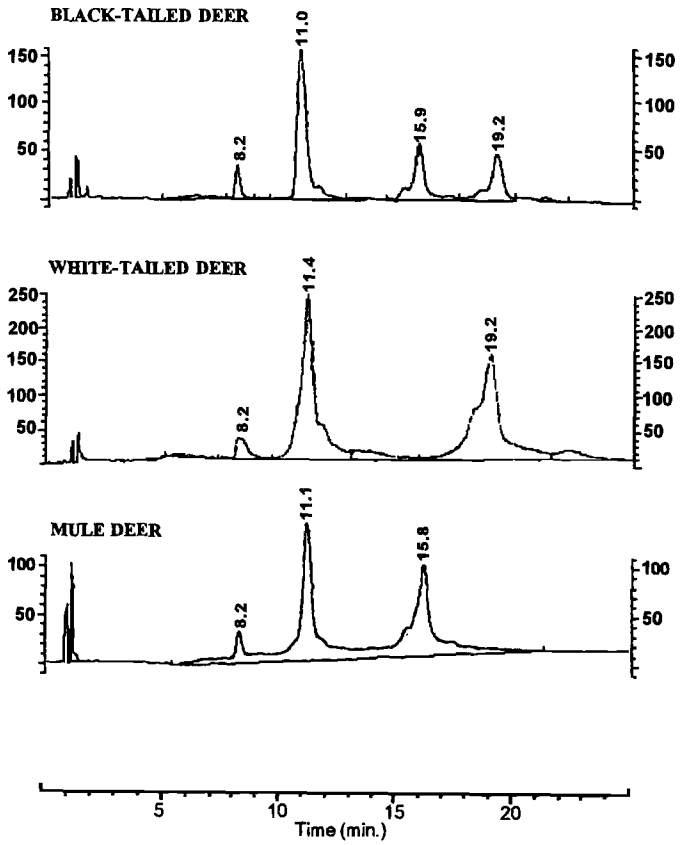

FIG. 3—*The comparison of chromatographic signatures between black-tailed deer, mule deer, and white-tailed deer.*

TABLE 3—*The comparison (calculated versus actual) of some blood mixtures.*

Animal Mixture	Actual		Calculated*		
	mg Hb	% Proportion	α Globin %	β Globin %	% Proportion
Cow	0.45	38.4	18.7	18.0	36.7
Horse	0.72	61.6	24.2	39.1	63.3
Cow	1.40	76.1	36.9	43.1	80.0
Horse	0.44	23.9	9.9	10.1	20.0
Goat	1.00	40.0	18.4	13.9	32.3
Pig	1.50	60.0	41.4	26.3	67.7
Human	3.10	70.5	30.7	45.5	75.5
Baboon	1.30	29.5	11.4	13.1	24.5
Human	0.70	60.9	28.8	29.9	58.7
Baboon	0.45	39.1	19.6	21.6	41.2
Cat	0.20	20.0	9.2	16.9	26.1
Pig	0.80	80.0	35.0	38.9	73.9

*Calculated by normalization of chromatographic peak areas for the major globin chains.

Results

Over 275 samples of blood (262 individual samples and 13 mixtures) have been analyzed to date. These samples were gathered from 22 discrete families comprising 38 genera and approximately 50 distinct species (see Table 1). Previously, both Andrasko et al. (12) (analyzing slightly over a dozen unique species) and Inoue et al. (6) (studying roughly 20 primates and a handful of domestic

TABLE 4—*Primary amino acid (β-globin chain) sequences for 19 distinct species.*

SeqEd NickName betas 7/12/95

			10	20	30	40	50	60	70	80	90	100
1	1...100	Golden Eagle	VHWTAEEKQL	ITGLWGKVNV	ADCGAEALAR	LLIVYPWTQR	FFASFGNLSS	PTAIIGNPMV	RAHGKKVLTS	FGEAVKNLDN	IKNTFAQLSE	LHCDKLHVDP
2	1...100	Cow	-MLTAEEKAA	VTAFWGKVKV	DEVGGEALGR	LLVVYPWTQR	FFESFGDLST	ADAVMNNPKV	KAHGKKVLDS	FSNGMKHLDD	LKGTFAALSE	LHCDKLHVDP
3	1...100	Dog	VHLTAEEKSL	VSGLWGKVNV	DEVGGEALGR	LLIVYPWTQR	FFDSFGDLST	PDAVMSNAKV	KAHGKKVLNS	FSDGLKNLDN	LKGTFAKLSE	LHCDKLHVDP
4	1...100	Kulan	VQLSGEEKAA	VLALWDKVNE	EEVGGEALGR	LLVVYPWTQR	FFDSFGDLSN	PAAVMGNPKV	KAHGKKVLHS	FGEGVHHLDN	LKGTFAQLSE	LHCDKLHVDP
5	1...100	Mtn. Zebra	VQLSGEEKAA	VLALWDKVNE	EEVGGEALGR	LLVVYPWTQR	FFDSFGDLSN	PAAVMGNPKV	KAHGKKVLHS	FGEGVHHLDN	LKGTFAQLSE	LHCDKLHVDP
6	1...100	Cat	GFLTAEEKGL	VNGLWGKVNV	DEVGGEALGR	LLVVYPWTQR	FFESFGDLSS	ADAIMSNAKV	KAHGKKVLNS	FSDGLKNIDD	LKGAFAKLSE	LHCDKLHVDP
7	1...100	Sun Bear	VHLTGEEKSL	VTGLWGKVNV	DEVGGEALGR	LLVVYPWTQR	FFDSFGDLSS	ADAIMNNPKV	KAHGKKVLNS	FSDGLKNLDN	LKGTFAKLSE	LHCDKLHVDP
8	1...100	Horse	VQLSGEEKAA	VLALWDKVNE	EEVGGEALGR	LLVVYPWTQR	FFDSFGDLSN	PGAVMGNPKV	KAHGKKVLHS	FGEGVHHLDN	LKGTFAALSE	LHCDKLHVDP
9	1...100	Human	VHLTPEEKSA	VTALWGKVNV	DEVGGEALGR	LLVVYPWTQR	FFESFGDLST	PDAVMGNPKV	KAHGKKVLGA	FSDGLAHLDN	LKGTFATLSE	LHCDKLHVDP
10	1...100	African Elephant	VNLTAAEKTQ	VTNLWGKVNV	KELGGEALSR	LLVVYPWTRR	FFEHFGDLST	AEAVLHNAKV	LAHGEKVLTS	FGEGLKHLDN	LKGTFADLSE	LHCDKLHVDP
11	1...100	Ferret	VHLTGEEKAA	VTALWGKVNV	DEVGGETLGR	LLVVYPWTQR	FFDSFGDLSS	PDAVMSNPKV	KAHGKKVLNS	FSEGLKNLDN	LKGTFAKLSE	LHCDKLHVDP
12	1...100	White Tail Deer	-MLTAEEKAA	VTGFWGKVNV	DVVGAEALGR	LLVVYPWTQR	FFEHFGDLSS	AGAVMGNPKV	KAHGKRVLDA	FSEGLKHLDD	LKGAFAELSE	LHCNKLHVDP
13	1...100	Rabbit	VHLSSEEKSA	VTALWGKVNV	EEVGGEALGR	LLVVYPWTQR	FFESFGDLSS	ANAVMNNPKV	KAHGKKVLAA	FSEGLSHLDN	LKGTFAKLSE	LHCDKLHVDP
14	1...100	Sheep	-MLTAEEKAA	VTGFWGKVKV	DEVGAEALGR	LLVVYPWTQR	FFEHFGDLSN	ADAVMNNPKV	KAHGKKVLDS	FSNGMKHLDD	LKGTFAQLSE	LHCDKLHVDP
15	1...100	Pheasant	VHWSAEEKQL	ITGLWGKVNV	ADCGAEALAR	LLIVYPWTQR	FFASFGNLSS	PTAILGNPMV	RAHGKKVLTS	FGDAVKNLDN	IKNTFSQLSE	LHCDKLHVDP
16	1...100	Rat	VHLTDAEKAA	VNGLWGKVNP	DDVGGEALGR	LLVVYPWTQR	YFDSFGDLSS	ASAIMGNPKV	KAHGKKVINA	FNDGLKHLDN	LKGTFAHLSE	LHCDKLHVDP
17	1...100	Pig	VHLSAEEKEA	VLGLWGKVNV	DEVGGEALGR	LLVVYPWTQR	FFESFGDLSN	ADAVMGNPKV	KAHGKKVLQS	FSDGLKHLDN	LKGTFAKLSE	LHCDQLHVDP
18	1...100	Polar Bear	VHLTGEEKSL	VTGLWGKVNV	DEVGGEALGR	LLVVYPWTQR	FFDSFGDLSS	ADAIMNNPKV	KAHGKKVLNS	FSDGLKNLDN	LKGTFAKLSE	LHCDKLHVDP
19	1...100	Asiatic Blk.Bear	VHLTGEEKSL	VTGLWGKVNV	DEVGGEALGR	LLVVYPWTQR	FFDSFGDLSS	ADAIMNNPKV	KAHGKKVLNS	FSDGLKNLDN	LKGTFAKLSE	LHCDKLHVDP
23	*1...100*		******EK**	****W*KVK*	***G*E*L*R	LL*VYPWT*R	*F**FGDLS*	**A***N**V	*AHG**V***	F*****H*DD	*KG*F**LSE	LHCD*LHVDP

			110	120	130	140	150	160	170	180	190	200
1	101...146	Golden Eagle	ENFRLLGDIL	IIVLAAHFTK	DFSPDCQAAW	QKLVRAVAHA	LARKYH					
2	101...146	Cow	ENFKLLGNVL	VVVLARNFGK	EFTPVLQADF	QKVVAGVANA	LAHRYH					
3	101...146	Dog	ENFKLLGNVL	VCVLAHHFGK	EFTPQVQAAY	QKVVAGVANA	LAHKYH					
4	101...146	Kulan	ENFRLLGNVL	VVVLARHFGK	DFTPELQASY	QKVVAGVANA	LAHKYH					
5	101...146	Mtn. Zebra	ENFRLLGNVL	VVVLARHFGK	DFTPELQASY	QKVVAGVANA	LAHKYH					
6	101...146	Cat	ENFRLLGNVL	VCVLAHHFGH	DFNPQVQAAF	QKVVAGVANA	LAHKYH					
7	101...146	Sun Bear	ENFKLLGNVL	VCVLAHHFGK	EFTPQVQAAY	QKVVAGVANA	LAHKYH					
8	101...146	Horse	ENFRLLGNVL	VVVLARHFGK	DFTPELQASY	QKVVAGVANA	LAHKYH					
9	101...146	Human	ENFRLLGNVL	VCVLAHHFGK	EFTPPVQAAY	QKVVAGVANA	LAHKYH					
10	101...146	African Elephant	ENFRLLGNVL	VIVLARHFGK	EFTPDVQAAY	EKVVAGVANA	LAHKYH					
11	101...146	Ferret	ENFKLLGNVL	VCVLAHHFGK	EFTPQVQAAY	QKVVAGVANA	LAHKYH					
12	101...146	White Tail Deer	ENFRLLGNVL	VVVLARNFGG	EFTPLVQADF	QKVVAGVANA	LAHRYH					
13	101...146	Rabbit	ENFRLLGNVL	VIVLSHHFGK	EFTPQVQAAY	QKVVAGVANA	LAHKYH					
14	101...146	Sheep	ENFRLLGNVL	VVVLARHHGN	EFTPVLQADF	QKVVAGVANA	LAHKYH					
15	101...146	Pheasant	ENFRLLGDIL	IIVLAAHFSK	DFTPECQAAW	QKLVRVVAHA	LARKYH					
16	101...146	Rat	ENFRLLGNMI	VIVLGHHLGK	EFTPCAQAAF	QKVVAGVASA	LAHKYH					
17	101...146	Pig	ENFRLLGNVI	VVVLARRLGH	DFNPDVQAAF	QKVVAGVANA	LAHKYH					
18	101...146	Polar Bear	ENFKLLGNVL	VCVLAHHFGK	EFTPQVQAAY	QKVVAGVANA	LAHKYH					
19	101...146	Asiatic Blk.Bear	ENFKLLGNVL	VCVLAHHFGK	EFTPQVQAAY	QKVVAGVANA	LAHKYH					
23	*101...146*		ENF*LLGD**	**VL******	*F*P**QA**	*K*V**VA*A	LA**YH					

animals) successfully characterized species using a C-4 column. However, no previous study has been as far reaching in the number of different species examined or in the number of individuals examined in a given family as the study described here.

The unique chromatographic retention times produced by the globin chain signals from species to species serve as the profiles to which an unknown species is compared and identified. Consequently, globin chain identity (i.e., whether a chromatographic signal represents the α- or the β-chain) is not needed to make a positive species identification. Earlier HPLC studies using similar columns and solvents as our method have shown the elution pattern of the globin signals to be the β-globin chain followed by the α-globin chain (3–7). As such, we have designated the first major globin chromatographic peak as the β-globin chain and the second as the α-globin chain.

To substantiate further this designation of the globin chain signals, chromatographic profiles of human hemoglobin (HbA) and human sickle-cell (HbS) hemoglobin were obtained. The first major globin peak obtained from the HbS was shifted 0.3 min longer than the first major globin peak from the HbA chromatogram. The second major globin peak had virtually the same retention time in both chromatograms. Since the sickle-cell mutation (glutamic acid → valine) is known to occur within the β-globin chain (a.a. Position 6) it was concluded that the first major peak represented the β-globin chain and the second the α-chain. There also exists a third major signal in the chromatograms of some species that is arbitrarily given the designation "ω-globin chain" (Table 1). The appearance of a third major globin chain signal suggests an abundant heterogeneous globin chain and its identity is currently under investigation. We recognize that the identity of the globins may be different than designated once further structural studies are undertaken. Several of the species α- and β-globin chain identities have been identified using electrospray ionization (ESI) mass spectrometry (Perkin Elmer PE SCIEX-API1 LC/MS). By comparing the experimentally determined molecular weights with known molecular weights, we were able to show in several species the chromatographic peaks followed the designation of the globin chains (14). We are currently studying the structural distinctions of more species' globin chains by ESI LC/MS.

Several of the species examined produced minor peaks in their corresponding chromatograms, and we have chosen to enumerate only the major chromatographic peaks (terming them β-globin chain, α-globin chain, and, in some cases, ω-globin chain). Prior work has postulated that the presence of minor peaks may be due to: heterogenous globins present in low concentration (i.e., other blood components) (13), globin-glutathione complexes (3), or denaturation products of the native globins (6–8).

The species specific hemoglobin chromatograms obtained with this method were reproducible. The statistical results of globin retention time reproducibility can be seen in Table 1. A key factor to the chromatographic retention time reproducibility within a given species was the development of the column reconditioning wash for the HPLC solvent system. The wash allows for large sequences of samples to be run without retention time drift or lengthy interruption for column purging. The resulting chromatograms have been free of retention time drift in over 275 such sequenced injections. The heme signal for all hemoglobin samples appeared at the same retention time (8.22 min ± 0.18 min, standard deviation = 0.006 17 for 275 samples). In essence, the heme signal for any sample serves as an internal reference for monitoring HPLC conditions. The sensitivity of this technique approached a lower limit of 1.2 μg of hemoglobin. The detection limit was obtained from a series of diluted hemoglobin standards from which the lowest discernible chromatogram was obtained from a 7-μL injection of a 0.17-mg/mL hemoglobin standard. Dried blood samples left at ambient temperatures showed consistent profiles for a period of at least three months. This is in agreement with previous work (7,8).

To evaluate species reproducibility, 27 breeds of domestic dogs (*Canis familiaris*) were analyzed. The *Canidae* family study also included several species of fox [*M. otocyon* (bat-eared fox), *V. vuples* (gray fox), and grey wolf (*C. lupus*). The large number of domestic dog samples provided a suitable population for statistical analysis within a single species (*C. familiaris*). A graph displaying the retention times versus dog breed demonstrates the reproducibility of this method (see Fig. 1). Although some of the species are represented by a small number of samples ($n \leq 5$), the reproducibility within the species ($n > 27$) allows one to expect similar reproducibility for all the species' chromatograms (independent of population variations).

Determining the amount of blood present from evidence can be crucial to forensic science casework. In addition to family or species identification or both of an unknown hemoglobin, hemoglobin quantity can also be determined using this method. Regression analysis for quantitative purposes was performed using chromatographic peak areas versus concentration from a series of standardized dog hemoglobin dilutions. The results (Fig. 2) produced good linearity and supports the ability of the method to quantitate an unknown concentration of blood.

Table 1 contains species that are forensically significant in North America. As can be seen the *Cervidae, Bovidae,* and *Canidae* were statistically well represented. These families were followed by the *Equidae, Ursidae, Felidae,* and *Suidae.* Four families *Cercopithecida, Columbidae, Leporidae,* and *Trichechidae* had only one representative. The remaining ten families studied ranged from two to eight species. As mentioned, the retention times of the globin chain signals change significantly enough from species to species within a given family (except with respect to the *Ursidae* family) to allow species signature profiles to be observed. Table 1 displays 20 families that show partial, if not complete, ability to be differentiated to the individual species level.

There are notable differences between species in the *Cervidae* family. These species were *A. alces* (moose), *E. davidianus* (Pere David deer), *C. elaphus* (elk), *C. elaphus-red deer* (red deer, a subspecies of elk), *C. nippon* (sika Deer), *Q. hemionus* (mule deer), *O. hemionus-black-tailed deer* (black-tailed deer, a subspecies of mule deer) and *O. Virginianus* (white-tailed deer). All of these species produced distinct chromatograms by this HPLC technique. Note that black-tailed deer, a subspecies of mule deer, has a unique HPLC signature, distinguishing it from both white-tailed or mule deer. The comparative chromatograms for these species can be seen in Fig. 3.

Of the numerous families studied, the *Ursidae* (bear) family was unique in its inability to be differentiated beyond the family level. The *Ursidae* family included; *Ursus americanas* (American black bear), *Helarctos malayanus* (sun bear), *U. maritimus* (polar bear), *Selanarctos thibetanus* (Himalayan bear), and *Melursus ursinus* (sloth bear). All bear samples produced chromatograms with nearly identical retention times regardless of the species (globin chain retention time variances, $\beta = 0.013$ and $\alpha = 0.049$). Such reproducibility suggests highly conserved globin chains among bears.

The detection of more than one animal's blood at a suspected crime scene may indicate a kill was due to natural predation rather

than from an illegal act. Likewise, the presence of human and animal blood may indicate more than just a wildlife crime. Therefore, the identification of blood mixtures is a forensically significant capability. The chromatographic nature of the HPLC technique lends itself nicely to the analysis of blood mixtures. The identification of the respective species' globin chains will be a result of their distinguishing HPLC signals. Several hemoglobin mixtures were studied (both from the standards and from fresh blood) and the quantitative results (actual versus calculated) can be seen in Table 3.

Table 3 indicates that quantitation was slightly less precise with respect to mixtures than it was for a single species hemoglobin (Fig. 2). Nonetheless, mixture analysis provides a good approximation of the relative abundance of each constituent. We have also noted slight retention time shifts associated with quantitating mixtures. The cause of this is under investigation. One possible explanation is the new globin—globin interaction that occurs between the increased number of unique globin chains in the mixture.

Discussion

The HPLC procedure developed for this study is rapid, sensitive, and reproducible. The technique allows for complete separation of the heme, α-, and β-globin chains in less than 25 min. This work has advanced earlier studies by exploring a large number of heretofore unanalyzed species. This analysis has also resolved a reproducibility problem associated with retention time drifting, which was inherent in previously published analyses (3–5). In the published cases of hemoglobin analysis by HPLC, the analyses were marked by chromatographies ranging from 40 to 60 min, and extensive purging of the HPLC column was required to obtain reproducibility of signal retention times. A problematic shift in retention times of a multiple run sequence occurred early in this study, as it did in the work of others (3–5).

The work of Andrasko et al. (4) deduced that the retention time shift in their studies was due to the samples being sequentially more dilute. We postulate the cause to be the repetition of sample injections that resulted in a buildup of small concentrations of protein, an accretion that over time caused a shift to slightly longer retention times (tenths of a minute).

The hemoglobin sequences of many of the species studied in this report have been obtained from the SWISS-PROT Protein Sequence Data Bank (14). These sequences indicate that approximately 50% of globin amino acid residues are conserved, and 50% exhibit neutral substitution in both the α- and the β-globin chains. Understanding the vast variability that can occur in the primary amino acid sequences of these globin chains offers, in part, a structural explanation for the resulting unique chromatographic globin chain signals. A representative comparison of the β-globin chain sequences of 19 different species can be seen in Table 4.

We have analyzed multiple blood samples from individuals of the same species that have been dried for one week, dried and stored in a freezer for as long as three years, dried and maintained at ambient conditions for up to three months, or sampled directly from fresh whole blood (diluted). There is no change in the chromatographic signature seen for these varied samples. HPLC analysis of hemoglobins provides sound inference for taxa identification. The HPLC method complements and furthers existing methodology. We feel HPLC analysis is also particularly well suited for the quantitative analysis of blood and blood mixtures and is directly applicable to species for which antisera is unavailable. This technique is of distinct interest to the forensic sciences, as it provides a rapid and reliable method for preliminary identification of species origin from blood or bloodstains. When a more thorough globin-chain molecular weight study of these selected species is complete (using ESI LC/MS), more robust statements of identification may be possible.

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