TECHNICAL NOTE

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Isotachophoretic Analysis of Bloodstains: Differentiation of Human, Menstrual, Bovine, and Ovine Bloods

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ABSTRACT: Isotachophoresis, a technique to separate components by constant current electrophoresis, was used to differentiate between bloodstains of male, female, menstrual, bovine, and ovine bloods on cotton cloth and filter paper. Bloodstain analysis by isotachophoresis of stains from male and female subjects showed identical cationic patterns, but gave different profiles in the anionic system. Plasma had one extra peak in the anionic system when compared to the profile of serum. This extra peak is due to the presence of fibrinogen in plasma. Some hemoglobin peaks overlapped with serum protein peaks, but these could be identified by comparisons at lower concentrations. Menstrual blood had a much different pattern than normal human blood as was expected since many more compounds are found in menstrual blood than in normally circulating blood. Human, bovine, and ovine bloodstains showed different profiles both in the cationic and anionic systems. These results indicate that isotachophoresis can be used for the rapid and simple analysis of bloodstains to differentiate reliably human male, female, and menstrual blood and also to distinguish human bloodstains from those of cattle or sheep.

KEYWORDS: pathology and biology, blood, isotachophoresis

As bloodstains are frequently present in the physical evidence of most crimes of violence, information obtained from them can be used to reconstruct the sequence of events in a crime and to link a suspect to the crime. Therefore, blood identification in the forensic sciences is the first most important step in the examination of a suspected stain. Because of the importance of bloodstain analysis in the forensic sciences, a number of analytical procedures for blood identification have been established. These, however, only differentiate blood type into a few basic groups. Microscopic [*1,2*], immunologic [*3-6*], and biochemical analysis of bloodstain components [*7-12*] are frequently used. It is frequently important to determine the sex of the person from whom a bloodstain originated. For such a purpose, the fluorochrome stain, quinacrine mustard, or related stain, quinacrine dihydrochloride, have been

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used to stain the Y chromosome characteristic of the human male specifically with variable results. Also, for determining the sex of an individual, radioimmunoassay of testosterone, progesterone, and 17β-estradiol has been used [*13-16*].[3,4] Each of these many different methods has its own characteristic advantages and uses, depending on the particular circumstances surrounding the case. However, many more different methods are needed still for the investigation of bloodstain evidence. Moreover, as new biochemical markers are discovered, new methods must be developed. We can be optimistic in expecting continued improvement in the forensic science of bloodstain analysis.

Because many of the possible amino acid substitutions involve changes in net electrophoretic charge, electrophoretic methods offer considerable potential for the recognition of protein sequence variations. Isotachophoresis, a technique to separate components by constant current electrophoresis, can be valuably applied to the field of bloodstain analysis. Much of the credit for introducing electrophoretic markers to the forensic science community goes to the Metropolitan Police Forensic Science Laboratory in London [*17*].

Capillary isotachophoresis was first used in forensic science for the analysis of snake venoms in a suicide case [*18*]. However, this highly sensitive and reliable analytical tool has not yet been used for routine bloodstain analysis. In this paper, we present our results from the bloodstain analysis of blood from humans (male, female, and menstrual), cattle, and sheep by isotachoelectrophoresis. To simulate normal crime conditions, analyses were made from the aqueous extracts of routine bloodstains on cotton cloth or filter paper.

Materials and Methods

Human Blood

Fresh blood from 20 healthy donors was obtained by pricking the fingertip with a blood lancet. The blood was immediately smeared on cotton cloth or filter paper.

Menstrual blood from the first three days of the period was collected from seven healthy women donors.

Animal Blood

Fresh blood was collected from sheep and cattle at a slaughter house. This was smeared on cotton cloth or filter paper, and the number of bloodstains for each blood type is shown in Table 1.

Extraction of Bloodstains

A 0.5-cm^2 piece of cotton cloth with a dried bloodstain (24 h to one week old) was soaked in 3 to 4 mL of glass distilled water for 30 to 60 min at room temperature. The clear extract was pipetted out and centrifuged at $\times 8000\ g$ for 10 min. The supernatant liquid was used for isotachophoretic analysis.

Isotachophoresis

Capillary isotachophoresis was performed on an LKB tachophore, model 2127, Uppsala, Sweden, at 75 μA and at variable voltages from 5 000 to 12 000 V (Fig. 1). Both anionic and

[3]P. H. Whitehead, Research work at the H.O.C.R.E., Aldermaston, U.K., Note 34359B, personal communication, 1983.

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TABLE 1—*Number of bloodstains for each blood type.*

	N.H.[a] Male	N.H. Female	Menst.[a]	Bovine	Ovine	Total
No. of bloodstains examined	10	10	7	15	15	57

[a]N.H. = normal human and Menst. = menstrual.

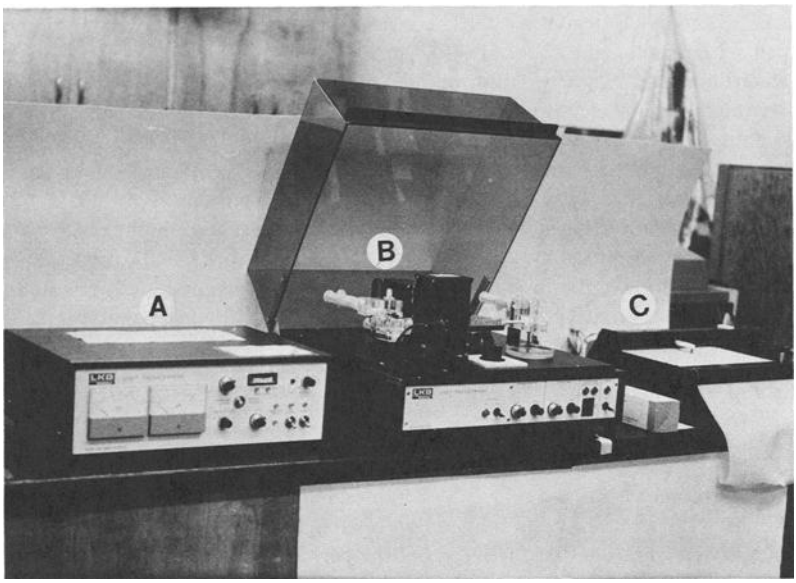

FIG. 1—*Overall view of capillary isotachophoresis, LKB 2127 Tachophor where* (a) *is power supply,* (b) *is isotachophoretic system, and* (c) *is recorder.*

cationic systems were used. For the anionic system, 2-amino-2-methyl-1,3 propanediol was used as the leading anion (pH 9.0) and ε-amino-*N*-caproic acid was used as the terminating electrolyte (pH 10.5). For the cationic system, cacodylic acid at pH 7.0 was used as the leading ion and creatinine at pH 4.5 was used as the terminating ion. Ampholines (1%) from Pharmacia with the pH range of 3 to 10 were used as spacers. For each analysis the same concentration of blood protein 0.5 μg/μL was used. About 5 to 10 μL of blood extract was used.

The Blind Studies

Analysis was made for bloodstains on cotton cloth and filter paper. Therefore, three blind studies were conducted for each type of blood examined: normal human, menstrual, bovine, and ovine bloodstains, as follows:

(1) dried bloodstains on cotton cloth, 0.5-cm^2 pieces;
(2) dried bloodstains on cotton cloth, 0.5-cm-long threads; and
(3) dried bloodstains on filter paper, 0.5-cm^2 pieces.

In all of the blind studies, the blood specimens were assigned a code number and submitted to the analyst (K. A.) who was told nothing concerning the nature of the samples to be analyzed.

Results

Human Plasma, Serum, and Hemoglobin

For controls, five plasma and five serum samples were analyzed. Isotachophoretic patterns are shown in Fig. 2. Examination of plasma and serum revealed that plasma showed one extra band (f in Fig. 2*a*), which is obviously fibrinogen. The rest of the patterns are identical between plasma and serum.

Hemolysis may take place on some occasions and hemoglobin within the red blood cells can be released into the bloodstream. It is known that hemoglobin in blood exists as a tetramer containing 2 α and 2 β chains. Normal hemoglobin may also consist of δ chain. This probably is the reason for giving more than one band. Heterogeneity of normal hemoglobin in isotachophoresis was also shown by other investigators [*19*] and our patterns are almost identical to earlier patterns reported. To identify free hemoglobin in the blood, two control experiments were performed. One used hemoglobin alone (Fig. 2*c*) and the other hemoglobin mixed with serum (Fig. 3). Hemoglobin and serum protein peaks overlapped slightly. Two mixtures of hemoglobin and serum were made. To identify the hemoglobin peaks from serum protein peaks, different amounts of hemoglobin were mixed with serum (Fig. 3*a* and *b*).

Human Bloodstains

Human bloodstains on cotton cloth and filter paper dried for 2 to 6 h were extracted with distilled water and isotachophoretic (ITP) patterns were obtained (Figs. 4 and 5). In the anionic system the ITP patterns of both male and female bloodstains (Fig. 4*a* and *b*) showed some differences between the two sexes. However, in both results the main prominent peak from serum protein (S) was evident. Moreover, this main serum protein peak is symmetric in

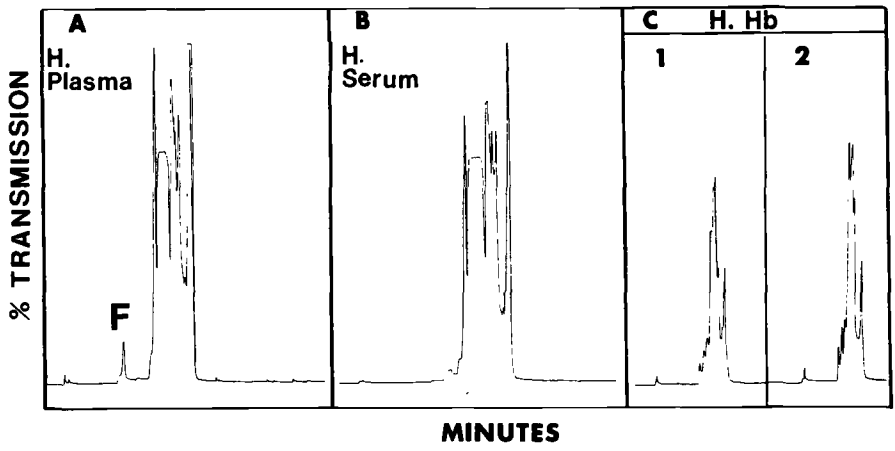

FIG. 2—*Positive standard runs for human plasma* (a), *serum* (b), *and hemoglobin* (c) *in anionic system.* 1, 2 *in* c *were obtained by adding different amounts of spacer (ampholine).*

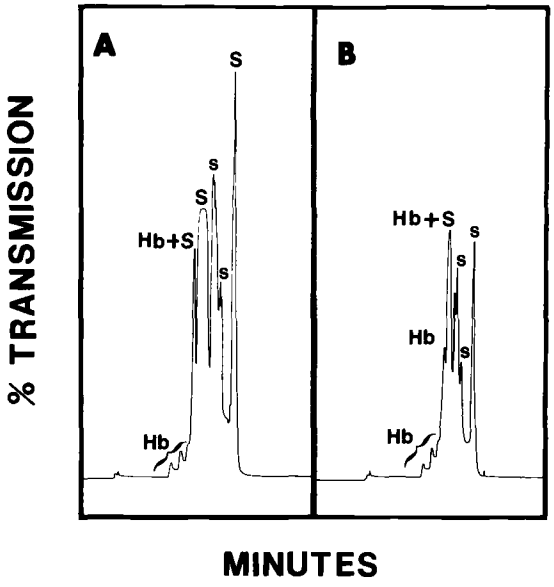

FIG. 3—*Identification of hemoglobin* (Hb) *peaks in serum* (S) *in anionic system. More hemoglobin was added in* b *than in* a.

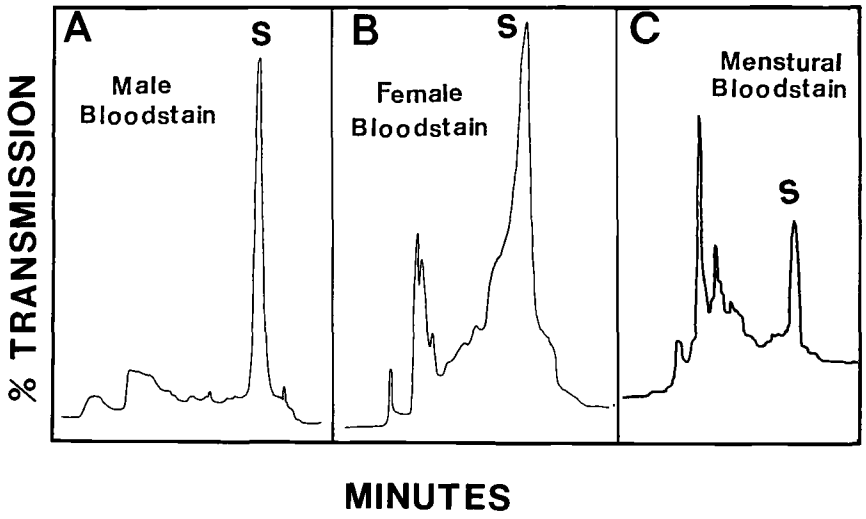

FIG. 4—*Isotachophoretic analysis of human bloodstain extracts in anionic system:* (a) *male,* (b) *female, and* (c) *menstrual blood.*

males (Fig. 4*a*, S peak) while it is asymmetric in females (Fig. 4*b*, S peak) as a result of the overlapping of some other components which are not identified in this investigation. On the other hand, menstrual bloodstain ITP patterns (Fig. 4*c*), when compared to male bloodstain patterns, showed some good differences. Moreover, when they are compared to those of normal circulatory female blood, they still show some differences. The serum protein peak in menstrual bloodstains was evident also (Fig. 4*c*, S peak). Several other peaks were shown

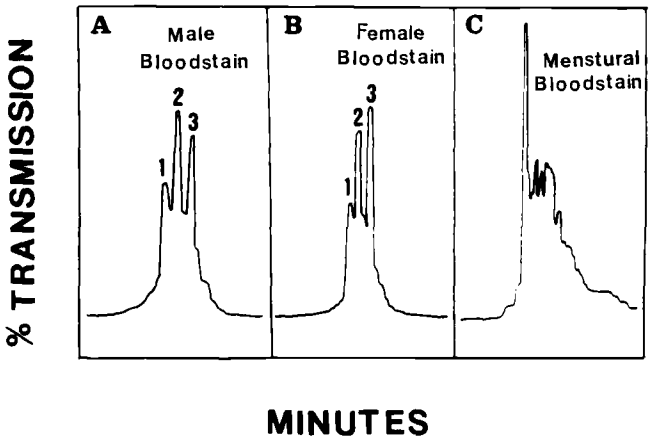

FIG. 5—*Isotachophoretic analysis of human bloodstain extracts in cationic system:* (a) *male,* (b) *female, and* (c) *menstrual blood.*

and they were probably caused by many other miscellaneous biological components present in menstrual blood.

The differentiation between normal male and female bloodstains and menstrual bloodstains is much clearer in the cationic system. Consistently, male bloodstains had a higher intensity of Peak 2 relative to Peak 3, while for normal female bloodstains Peak 3 was higher than Peak 2 (Fig. 5*a* and *b*). Altogether ten male and ten female blood samples were obtained for bloodstain analysis. The results for each sex were identical, so there is a difference in male versus female bloodstains. For menstrual bloodstains, the isotachophoretic patterns were totally different from normal male and female bloodstains. This is reasonable because menstrual blood contains hormones and tissue debris of the uterus lining in addition to normal blood constituents.

Bovine and Ovine Bloodstains

When bovine and ovine bloodstains were examined, they were found to be completely different from those of humans in the anionic system (Fig. 6). Using the cationic system, there were similarities in their isotachophoretic patterns; however, there were still differences, and the three types of bloodstains could be differentiated by close inspection (Fig. 7).

The Blind Studies

A total of 37 different bloodstains were examined in these blind trials. The results of these blind studies were compared to the known bloodstains analysis results and then each bloodstain found to be identical to one of the known results was assigned the same origin as the known one.

Table 2 shows the different types of blood and the size of each bloodstain that was used in this blind trial after they were identified.

Reproducibility of the Technique

Each bloodstain was analyzed isotachophoretically several times and the results observed for each bloodstain were completely reproducible under the same experimental conditions.

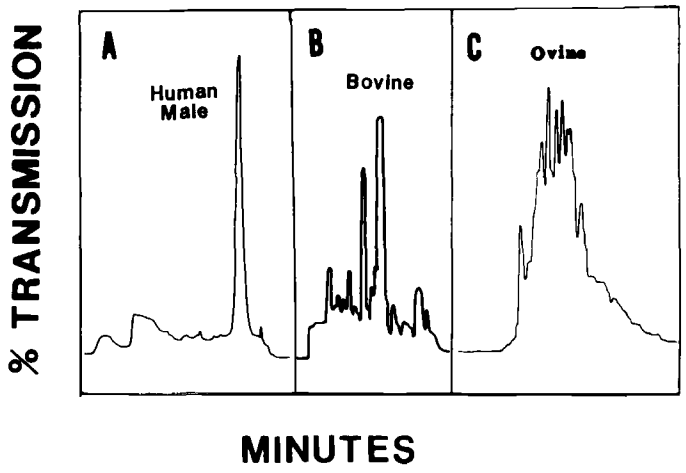

FIG. 6—*Isotachophoretic analysis of bloodstain extracts in anionic system:* (a) *male (human),* (b) *bovine, and* (c) *ovine.*

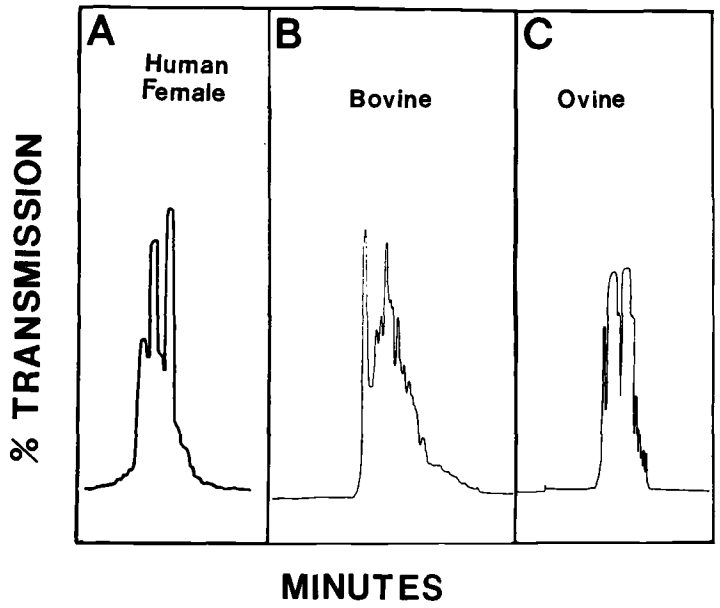

FIG. 7—*Isotachophoretic analysis of bloodstains in cationic system:* (a) *male (human),* (b) *bovine, and* (c) *ovine.*

Discussion

Isotachophoresis is known to biochemists as a simple, rapid, and precise analytical tool, but it has seldom been used for bloodstain analysis. Based on the present investigation, the following conclusions can be made:

1. Serum and plasma can be differentiated.
2. Human male and female bloodstains can be differentiated using the cationic system.

TABLE 2—*The numbers and the sizes of each type of bloodstain examined in the blind trials or studies.*

The Nature of the Bloodstain	No. of B.S.[a] on Cotton Cloth, 0.5-cm^2 Pieces	No. of B.S. on Cotton Cloth, 0.5-cm Threads	No. of B.S. on Filter Paper, 0.5-cm^2 Pieces	Total No. of B.S. Analyzed
N.H. male[b]	3	2	3	8
N.H. female	3	2	3	8
Menstrual	2	1	2	5
Bovine	3	2	3	8
Ovine	3	2	3	8
Total No. of B.S. examined in the blind trials				37

[a]B.S. = bloodstains.
[b]N.H. = normal human.

3. Bloodstains from menstrual blood can be differentiated from those of normally circulating human male and female blood.
4. Bovine and ovine bloodstains can be readily distinguished from those of humans because of distinct differences in their fingerprint patterns by isotachophoresis.

The significance of these findings to forensic science is obvious. Bloodstains are quite common evidence in the resolution of numerous crimes. The crime suspect may insist that bloodstains in his clothes are not human. Isotachophoretic analysis should clarify human blood from bovine and ovine by analyzing the bloodstains of a crime suspect. On the occasion of rape, bloodstains in the suspect's clothing may show whether the blood is of menstrual origin. Clearly this rapid and simple technique should be used more extensively in forensic science analysis.

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