J Forensic Sci, Oct. 1978, Vol. 23, No. 4

R. C. Shaler,[1] *Ph.D.; F. P. Smith,*[1] *M.S.; and*
C. E. Mortimer,[1] *Ph.D.*

Detection of Drugs in a Bloodstain. I: Diphenylhydantoin

The blood of individuals differs in many ways. Forensic scientists have taken advantage of identifiable genetic differences that have permitted calculations to be made regarding the individuality of a particular blood specimen in the general population.

Although these genetic markers are of great utility in forensic serological analyses they have, with few exceptions, a limited persistence in the dried state. Thus, although very old stains can be typed for the ABO and Gm systems, other genetic markers such as red cell antigens and polymorphic protein and enzymatic systems cannot be detected sometimes even after only a few weeks.

Attempts have been made to utilize nongenetic factors of the blood to differentiate bloodstain samples. Sweet and Elvins [*1*] have attempted immunoelectrophoretic protein separations to arrive at an individualization index of the unknown stain. Werrett and Whitehead [*2*] have described a technique that permits the detection of allergen-associated antibodies in bloodstains. In both of these studies the blood factors being detected are proteins of endogenous origin and thus are subjected to the same degradative influences as the genetic markers.

It would be advantageous to identify substances in blood that would differentiate bloodstained evidence and not be subjected to the same degradative influences as the genetic markers. Shaler et al [*3*] and McWright and Brown [*4*] have used hormonal steroid concentrations to estimate the sexual origin of the bloodstain. This report is concerned with identification of the drug diphenylhydantoin in bloodstains, saliva, and semen with a radioimmunoassay (RIA) procedure. This communication illustrates a technique that has great potential utility in forensic science not only as a tool for differentiating and individualizing bloodstains but also as an investigative tool for police agencies.

Experimental Procedure

Standard Curve

A standard concentration curve was prepared by assaying known amounts of unlabeled diphenylhydantoin (DPH) equal to 0, 50, 100, 250, 500, and 1000 pg (10^{-12} g) in duplicate by the RIA method. This assay was accomplished by dispensing 0, 5, 10, 25, 50, and 100

Presented at the 29th Annual Meeting of the American Academy of Forensic Sciences, San Diego, Calif., February 1977. Received for publication 28 March 1977; revised manuscript received 11 Jan. 1978; accepted for publication 16 Jan. 1978.

[1]Research assistant professor, forensic chemist, and research associate, respectively, Department of Chemistry, University of Pittsburgh, Pittsburgh, Pa.

μl, respectively, of a 10 ng/ml DPH standard solution into 10- by 75-mm round-bottom glass culture tubes. Next, bovine serum albumin (BSA) buffer, pH 7.4 (0.06% BSA, 0.01*M* phosphate-saline) was added by pipetting to a final volume of 1.0 ml, followed by 50 μl ^{3}H-DPH equivalent to approximately 7000 counts per minute (cpm) per tube. The standards were then vortexed to disperse the ^{3}H-DPH in solution; then 50 μl of DPH-antibody solution, commercially prepared from rabbit serum, was transferred into each of the tubes, vortexed, and incubated at 4°C for 30 min. Dextran-coated charcoal suspension (500 μl, 0.625% charcoal, 0.0625% dextran-T 70, 0.01*M* phosphate-saline buffer, pH 7.4) was then added. The suspension was vortexed, incubated at 4°C for 10 min, and precipitated by centrifugation at 2000 *g* for 7 min. The supernatants were decanted into glass scintillation vials containing 10 ml of a suitable scintillator and counted in a Beckman LS-100C liquid scintillation counter for 5 min per sample. In addition to these standards, "total" samples (samples containing radioactivity but no antibody) containing only 1.0 ml BSA buffer and 50 μl ^{3}H-DPH were run as well as "blank" samples containing 1.0 ml buffer, 50 μl ^{3}H-DPH, and 500 μl charcoal suspension.

Data obtained were plotted on two different types of graphs. The first graph[2] is simply a ratio of the "zero" standard count per minute to each standard count per minute versus picograms DPH added per standard. The logit method [5] represents a mathematical manipulation of the same numbers where the logit of the efficiency-corrected count per minute is plotted versus the log of the dose. Results quoted in this report were extrapolated from the second (logit) standard curve. Experimental error of this RIA for DPH is ±9%.

Sample Preparation

Dried Bloodstains—Whole blood drawn by venipuncture into ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes was measured by using a 10-μl pipetting device (Sigma, St. Louis) and placed on a white, unbleached cotton sheet to dry. Storage was at room temperature in an open space for the designated aging times. At the time of assay the dried bloodstains were cut with scissors into approximately 1-mm squares, placed in 10- by 75-mm round-bottom glass culture tubes, and eluted with 2.0 ml of an appropriate solvent for the described time intervals. For DPH determination by RIA, 10-μl aliquots of the eluant were placed in BSA buffer to a final volume of 1.0 ml, as previously described. This dilution produced an equivalent of 0.05 μl of the original 10-μl bloodstain.

Saliva and Semen—Saliva and semen samples were collected and stored at 4°C in glass culture tubes. Dilution for RIA was as follows: 10 μl of the physiological sample was added to 100 μl of the physiological saline solution. Aliquots, 10 μl, of this dilution were tested by adding BSA buffer to a final volume of 1.0 ml, as previously described. This sample was the equivalent of 0.91 μl of the original sample.

Saliva Stains—Saliva stains on a cigarette filter were created by having an individual on DPH therapy hold a cigarette in his mouth as if he were smoking. The filter and filter paper were separated from the body of the cigarette. The paper was then cut into approximately 1-mm squares and eluted with 400-μl 0.1% sodium dodecyl sulfate-saline for 24 h at room temperature in a covered round-bottom disposable glass culture tube. This amount of solvent was sufficient to cover the sample. Similarly, 800 μl of the same solution was used to elute the filter under identical conditions, and 100-μl aliquots of the eluates were tested by pipetting BSA buffer to a volume of 1.0 ml and proceeding with the RIA method previously described.

Whole Blood—A 10-ml sample of whole blood obtained by venipuncture into EDTA-anticoagulated tubes was diluted with 5.0 ml saline. The 10-μl sample of the diluted blood was analyzed for the presence of DPH by adding 1.0 ml BSA buffer and performing the RIA.

[2]Wien Laboratories, commercially available RIA test kit.

Plasma—Whole blood was centrifuged for 3 min at 1000 *g* to separate plasma from the red blood cells. The plasma (10 μl) was dispensed into 5.0 ml of saline and 10 μl of this dilution was tested by adding 1.0 ml BSA buffer and continuing with RIA.

Red Blood Cells—Red blood cells, 10 μl, obtained by centrifugation of whole blood, were transferred to 1.0 ml of saline and resuspended by gently inverting the covered glass disposable culture tube. The cells were then recollected by centrifugation at 1000 *g* for 3 min. The supernatant was discarded, and the cells were resuspended in 1.0 ml of saline. Five such washes were done before the red blood cell precipitate was taken up in 1.0 ml of BSA buffer and tested by the RIA procedure.

Elution by Detergents

Ten microlitres of DPH and control dried bloodstains were prepared as described above. A nonionic detergent, Triton X-100, $CH_3C(CH_3)_2CH_2C(CH_3)_2C_6H_4\text{-}O\text{-}[CH_2\text{-}CH_2\text{-}O]_XH$, and an ionic detergent, sodium dodecyl sulfate, $CH_3(CH_2)_{11}O\text{-}SO_3^-Na^+$ (SDS), were examined for their efficiency at extracting DPH from dried bloodstains. Detergent solutions (0.1 and 1.0%) of each were prepared in physiological saline. Each 10-μl bloodstain was cut into approximately 1-mm squares and eluted with 2.0 ml of these solvents in glass round-bottom disposable culture tubes for 60 min at 23°C. Each sample was vortexed for 5 s at the start and finish of incubation, and 10-μl aliquots of eluant were transferred for RIA.

Materials

Blood, saliva, and semen containing DPH were provided by an individual taking Dilantin®. Negative control samples for comparison were obtained from various sources including the Central Blood Bank of Pittsburgh. A "DPH Test Set" was purchased from Wien Laboratories, Inc., Succasunna, N.J. Triton X-100 was purchased from Rohm and Haas, Philadelphia, Pa. and SDS from Fisher Scientific, Pittsburgh, Pa.

Results

The standard curves (Figs. 1 and 2) illustrate the extreme sensitivity of the RIA procedure for DPH determinations. It is possible to detect DPH to the 50-pg level. Figure 3

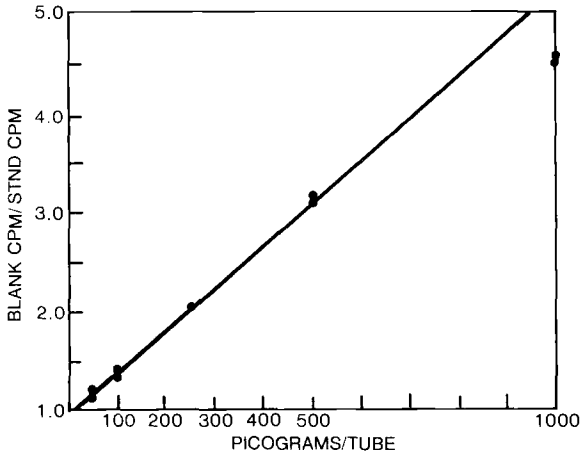

FIG. 1—*Standard curve for DPH.*

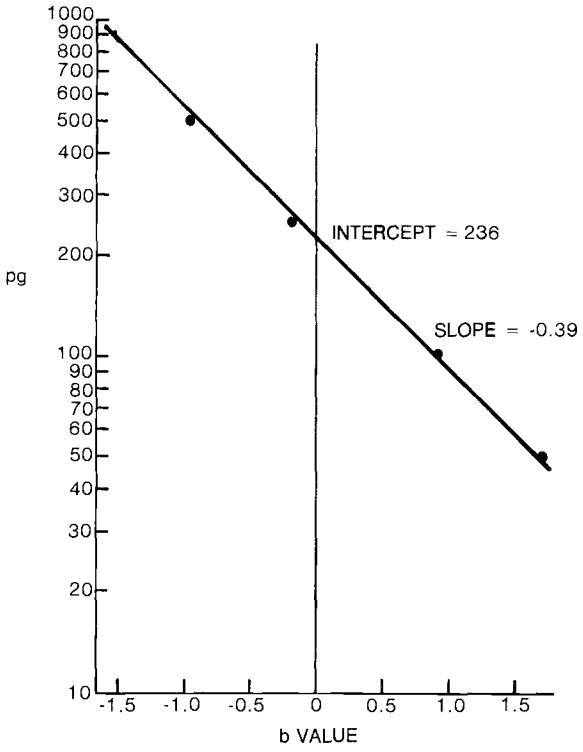

FIG. 2—*Logit plot for DPH.*

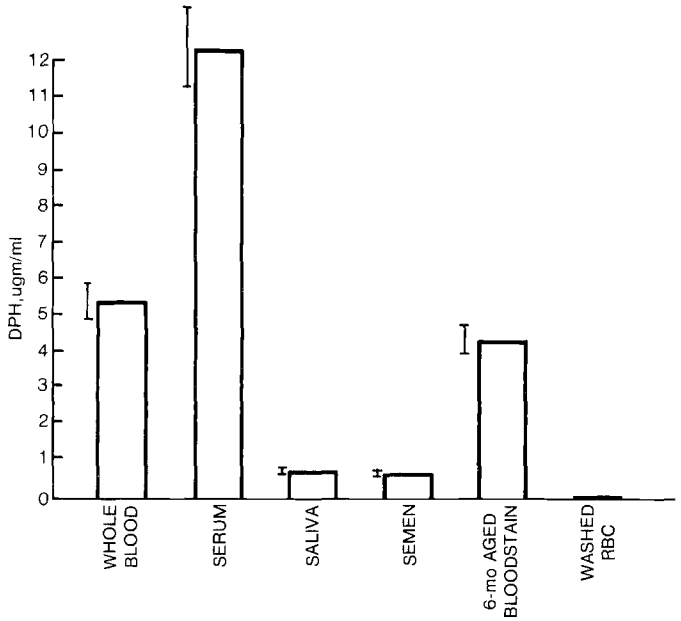

FIG. 3—*Levels of DPH in physiological samples.*

illustrates the concentrations of DPH found in whole blood, blood plasma, and washed red blood cells. The data shown, as expected, indicate that blood plasma has larger concentrations of DPH than whole blood, 12.5 ± 1.1 μg/ml versus 5.5 ± 0.5 μg/ml, respectively. The whole blood samples were analyzed to give concentrations that would be expected from bloodstains made from that same volume of blood. Figure 3 also shows that red blood cells contain no readily detectable levels of DPH, and thus the difference in concentration between whole blood and blood plasma represents the dilution factor of the red blood cells (blood particulates) in the particular blood sample.

Figure 3 also shows the amount of DPH found in saliva and seminal fluid. The amounts present in each, although lower than blood plasma (0.7 ± 0.1 μg/ml versus 12.5 ± 1.1 μg/ml, respectively), are not significantly different from each other. Although there is significantly less DPH in the saliva and seminal fluid, the concentration is sufficient to reliably detect DPH in 0.9 μl of undiluted sample. Thus it is possible to detect the drug in physiological stains other than blood.

One theoretical concern with aged stains might be associated with the extractability of the drug. This problem was investigated and the results are shown in Fig. 4. Bloodstains were aged for six months and the ability to extract DPH was compared to a fresh (less than one week old) stain prepared from the same volume of blood. The length of time required to extract the stain is important. For example, the data show that extraction of the drug from a fresh stain for 1 h in physiological saline yields 2.2 ± 0.2 μg DPH/ml, or 40% of the total, while extraction for 24 h yields 3.8 ± 0.3 μg DPH/ml, or 70% of the total, which is a 75% increase in the extractability of the drug. Similarly, extraction of the six-month-old stain for 24 h in physiological saline gave 4.4 ± 0.4 μg DPH/ml, which is not significantly different than that obtained from the fresh stain incubated for the same length of time. In addition, these results indicate an upper limit (70%) for the extractability of the drug from stains incubated in physiological saline whether the stain is relatively fresh or not.

Methods were investigated which would permit the extraction of the drug from stains with greater efficiency and shorter incubation times. Thus different detergents at various concentrations were studied. Figure 4 illustrates these results. The stains were extracted with the nonionic detergent Triton X-100 and the anionic SDS detergent at concentrations of 0.1 and 1.0% in physiological saline for 1 h. The Triton X-100 extracted 4.6 ± 0.4

SAMPLE	AMT. DET.	CALCULATED SAMPLE
SERUM	250 pg/0.02 ul	12.5 ± 1.1
WHOLE BLOOD	110 pg/0.02 ul	5.5 ± 0.5
FR. STN 1-hr SALINE	110 pg/0.05 ul	2.2 ± 0.2
24-hr SALINE	190 pg/0.05 ul	3.8 ± 0.3
1-hr 1.0% TRITON X-100	200 pg/0.05 ul	4.0 ± 0.4
1-hr 0.1% TRITON X-100	230 pg/0.05 ul	4.6 ± 0.4
1-hr 1.0% SDS	260 pg/0.05 ul	5.2 ± 0.5
1-hr 0.1% SDS	290 pg/0.05 ul	5.8 ± 0.5
AGED STN. - 6 mo 24-hr SALINE	220 pg/0.05 ul	4.4 ± 0.4

FIG. 4—*Extraction of DPH.*

and 4.0 ± 0.4 μg DPH/ml at concentrations of 0.1 and 1.0%, respectively. This corresponds to approximately 80% of the total drug available. The anionic detergent SDS extracted 5.8 ± 0.5 and 5.2 ± 0.5 μg DPH/ml, or 100% of the total for the 0.1 and 1.0% solutions respectively. Thus the detergents, especially the anionic detergent SDS, quantitatively extract the drug in very short periods of time.

Saliva stains prepared on a cigarette were extracted in SDS detergent solution and analyzed by the RIA procedure for the presence of DPH. The cigarette was separated into its filter paper and filter components. The results are interesting since the amount of DPH on the outside filter paper was less than that in the filter, 320 pg versus 2600 pg, respectively.

Discussion

The RIA technique works on the principle that nonradioactive endogenous antigens (DPH) compete with exogenous radioactive ^{3}H-DPH for antibody combining sites in a manner proportional to their concentrations [6]. Radioimmunoassay, because of its extreme sensitivity, is rapidly becoming a technique of great utility in forensic science.

This technique for identifying DPH in bloodstains is not necessarily an end in itself but is a model system for the potential of generating nongenetic information available in bloodstain evidence. Similar techniques for other drugs could be worked out so that a very small stain could describe the state of health or the habits of an individual. A more pragmatic aspect of this model system may be its value to police officers. If the victim of a violent crime was taking drugs, in this case an epileptic on Dilantin therapy, the presence of DPH found in the bloodstain on the clothing of the suspected perpetrator of the crime would go a long way toward establishing that that stain may have been derived from the victim. Thus, although these tests were conducted with DPH because of the availability of a known DPH donor, the potential exists for the detection of many other drugs. This general method would then be of interest to those involved in the investigation of burglaries, homicides, motor vehicle violations, assaults, and any crime where blood and physiological fluid evidence exists.

Acknowledgments

This work is supported by the Law Enforcement Assistance Administration Grants in Aid 75I-99-0011 and 76I-99-0099.

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Address requests for reprints or additional information to
Robert C. Shaler, Ph.D.
Director, Forensic Sciences
The Aerospace Corporation
Suite 4040, 955 L'Enfant Plaza, S.W.
Washington, D.C. 20024