Detection of Hemophilia A Carriers by Use of Frozen Plasma Samples

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The efficacy of using promptly frozen plasma samples in the diagnosis of the carrier state for hemophilia A was evaluated by simultaneous measurement of factor VIII activity and antigen in 20 normal women and 20 obligate carriers. Factor VIII antigen was measured by two methods, electroimmunoassay and immunoradiometric assay. When the factor VIII activity and antigen data were evaluated by regression analysis, 94% of the carriers were correctly identified at the 95% confidence level.

Since the development of a monospecific heterologous antiserum to factor VIII antigen, some investigators have been able to detect the carrier state for hemophilia A with 90–95% accuracy by simultaneous measurement of factor VIII activity and antigen (1–3). However, other investigators have not had similar success (4–6). The reasons for the discrepancy among investigators are not known but could be due to differences in patient population, statistical methods, or laboratory techniques, or some combination of these.

Detection of carriers would be greatly facilitated at regional hemophilia care centers if the plasma samples from at-risk individuals in satellite areas could be frozen and forwarded to the centers for study. However, data have been presented suggesting that only fresh plasma samples used in carrier studies (7), which has led to the exclusive use of fresh plasma samples at some centers, thus requiring individuals to travel—sometimes great distances—and laboratories to study individuals singly rather than in batches. Here, we re-examine whether fresh-frozen plasma samples stored at −70 °C are satisfactory for carrier detection.

Materials and Methods

Subjects

We studied 20 obligate carriers of hemophilia A. Factor VIII antigen was measured in the same plasma sample by electroimmunoassay and immunoradiometric assay in 14 of the carriers and in different samples in one of the carriers. Four carriers were studied by only immunoradiometric assay; one carrier was studied only by electroimmunoassay. Two obligate carriers were from pedigrees with moderate hemophilia (factor VIII activity 0.01–0.05 unit/ml) and five obligate carriers were from pedigrees with mild hemophilia (factor VIII activity >0.05 unit/ml); the other obligate carriers were from pedigrees with severe hemophilia (factor VIII activity <0.01 unit/ml). An obligate carrier was defined as either the daughter of a hemophilic father, the mother of more than one hemophilic son, or the mother of a hemophilic son with a positive family history of hemophilia in other male members. The use of contraceptive pills was not ascertained in the obligate carriers.

The normal female group consisted of 20 healthy volunteers between the ages of 19 and 47 (mean age, 30) who had no personal or family history of bleeding disorders. The factor VIII antigen was measured by both electroimmunoassay and immunoradiometric assay in the same plasma sample in four of the normal women. The other 16 were studied by the two methods on plasma samples drawn on different days. Of the 20 normal women, six were taking contraceptive pills. No subject was known to be pregnant at the time of study.

Plasma Collection

Plasma was obtained from the subjects by centrifugation of anticoagulated blood (one volume of sodium citrate solution, 38 g/liter, to nine volumes of blood) at 2400 X g and 4 °C for 15 min. Aliquots of 1 ml were immediately frozen by placing them in a −70 °C freezer, where they were stored for subsequent factor VIII activity and antigen assays. Normal reference plasma pools were similarly obtained and stored by combining plasma specimens from 15–20 normal individuals, about half of whom were men and the other half women. Several pools were used in the course of this study, but factor VIII activity and antigen were always measured against the same pool for a given sample. Normal pooled plasma is arbitrarily defined to contain 1 unit of factor VIII activity and antigen per milliliter.

Factor VIII Activity Assay

Factor VIII activity was measured by a one-stage method, with use of a deficient substrate with less than 0.01 unit of factor VIII activity per milliliter (8). Test plasma was assayed in duplicate at two dilutions and, with a few exceptions, on two different days. The average of the values from the two days was used; the discrepancy between the two values was usually less than 10%. Assays were completed within six weeks of the sample date.

Factor VIII Antigen Assays

The monospecific factor VIII antisera used were obtained commercially (Behring Diagnostics, Somerville, N.J. 08876) or were produced in our laboratory (9). The commercial antiserum was produced by the method of Zimmerman et al. (10).

Electroimmunoassay of factor VIII antigen was performed as previously described (9). Test plasma was studied at two dilutions in two different experiments. The average of the values from the two experiments was used; the discrepancy between the two values was usually less than 20%.

Immunoradiometric assay of factor VIII antigen was performed by the method of Hoyer (11). Radiodination of IgG was performed by a modified Chloramine T method (12). Test plasma was studied in duplicate in two dilutions, usually 1:40
Fig. 1. Linear regression line, with 95% confidence limits, for factor VIII activity and antigen by electroimmunoassay
20 normal women (A); $y = 0.65x + 0.38$ ($r = 0.81$, Student’s t-test, $P < 0.005$). Obligate carriers studied by both electroimmunoassay and immunoradiometric assay on the same sample (O). Obligate carrier samples studied by electroimmunoassay only (O).

Fig. 2. Linear regression line, with 95% confidence limits, for factor VIII activity and antigen by immunoradiometric assay
20 normal females (A); $y = 0.81x + 0.33$ ($r = 0.81$, Student’s t-test, $P < 0.005$). Obligate carriers studied by both immunoradiometric assay and electroimmunoassay on the same sample (O). Obligate carrier samples studied by immunoradiometric assay only (O).

and 1:80, on two days. The average of the values from the two days was used; the discrepancy between the values was usually less than 20%. All antigen assays were completed within 4 months of the sample date.

Statistical Methods

Simple linear regression was used for the analysis of data in the 20 normal women, factor VIII antigen being the independent variable and factor VIII activity the dependent variable (13). A 95% confidence limit was established. A subject was classified as a carrier of hemophilia A when the activity value was below the 95% confidence limit for the observed antigen value.

Results

Figures 1 and 2 show the relationship between factor VIII activity and antigen, with the 95% confidence limits, in the 20 normal women.

By electroimmunoassay, 14 of 16 obligate carriers (12 of 14 common samples) were correctly identified as carriers of hemophilia A (Figure 1). By immunoradiometric assay, 19 of 19 obligate carriers (14 of 14 common samples) were correctly identified as carriers (Figure 2). The comparative rates of correct identification of obligate carriers were not statistically different between the two methods (chi-square test, $P > 0.30$). Although there was good correlation ($r = 0.81$, Student’s t-test, $P < 0.001$) between the antigen values measured by both electroimmunoassay and immunoradiometric assay in the 14 common carrier samples, there were two notable discrepancies: two subjects had respective factor VIII antigen values of 0.94 and 2.12 unit/ml by electroimmunoassay and 1.74 and 1.03 unit/ml by immunoradiometric assay.

Discussion

Our data demonstrate that freshly frozen plasma samples, stored at $-70 \, ^\circ C$, can be used validly to identify carriers of hemophilia A. The combined rate of correct identification of obligate carriers by electroimmunoassay and immunoradiometric assay was 33 of 35, or 94%. Our success with frozen plasma samples contrasts with the failure encountered by Hoyer and Rick (7), who could correctly identify only eight of 24 obligate carrier samples that had been frozen and sent from another institution. Several investigators have reported a 10–20% loss of factor VIII activity with freezing and storage of plasma at $-70 \, ^\circ C$ (10, 14), although others have found negligible loss (6). However, since our reference normal pooled plasma is processed in the same manner as individual samples, one would expect the losses to be proportional and not to alter significantly the relative factor VIII activity values. Difficulties encountered by Hoyer and Rick (7) may not be related to the freezing process but to other technical difficulties such as transportation of the plasma samples. It also appears inconsistent to compare a fresh test plasma sample with a frozen reference plasma pool as in some previous studies (1, 3, 15).

In a cooperative study for the detection of hemophilia A carriers, two laboratories had success rates of 66% and 86% in identifying obligate carriers with use of immunoelectrophoretic techniques for factor VIII antigen; a third laboratory, using an immunoradiometric method, had a success rate of 96% with the same subjects. It was not possible to distinguish the quality of the laboratory method from the ability of the investigator to detect carriers in the previous study. Our data suggest that, within the same laboratory, the electroimmunoassay and immunoradiometric assay are equally efficacious in carrier detection.

The success rates for carrier detection do not appear to be affected by the choice of statistical methods such as ratio, linear regression, and discriminant function (16), in spite of a previous study suggesting the superiority of the discriminant function method (17). We used the linear regression method because of its simplicity. A 95% confidence limit was arbitrarily chosen as in previous studies (5–7). If a 99% confidence limit had been used, only 10 of 16 obligate carriers with the electroimmunoassay and 14 of 19 obligate carriers with the immunoradiometric assay would have been correctly identified.

There are several logistic advantages in the use of promptly frozen plasma samples for carrier studies. Samples can be tested for factor VIII activity and antigen in batches, and repeated on another day. A large number of individuals would not be needed to form a fresh normal pool on each assay day. At-risk females would not have to travel long distances for a carrier test. Thus identification of carriers would be greatly facilitated.

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Emergency Gas-Chromatographic Assay of Phenobarbital and Phenytion and Liquid-Chromatographic Assay of Theophylline

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Phenobarbital and phenytion are extracted and concentrated in a single step, without solvent evaporation, followed by on-column methylation and gas-chromatographic quantitation. A similar extraction step for the assay of theophylline by high-pressure liquid chromatography is described. The extraction step can be completed in less than 2 min, the chromatographic step in less than 8 min. This extraction method yields clean chromatograms, avoids evaporating health-hazardous solvents, and is applicable to other drugs.

Additional Keyphrases: drug assay • high-performance liquid chromatography •

Two main approaches are commonly used to extract anticonvulsant drugs from serum for assay by gas chromatography. The first is to extract the drug from serum with large volumes of organic solvents, about 10-fold the serum volume, followed by solvent evaporation to concentrate the drugs. The evaporation is a time-consuming step, involving health-hazardous vapors with the possibility of partial or total loss of the drug, as in the case of ethosuximide. The second approach is to extract the drug initially into an organic solvent followed by a second extraction into a concentrated solution of methylvating agent such as trimethylphenylammonium hydroxide (TMAH). This double-extraction approach not only involves extra steps but requires a high concentration of TMAH, which often causes breakdown of phenobarbital (1–3).

Certain drugs such as phenytion and phenobarbital are poorly soluble in chloroform, so large proportions of the solvent are necessary to effectively extract these drugs from serum. However, if ethyl acetate and methanol are added to the chloroform the solubility of these drugs is increased by 10- to 20-fold. Thus, the drug can be extracted in small amounts of the solvent and at the same time concentrated several fold in a single step.

This approach can be extended for the high-performance liquid chromatographic assay of drugs. The advantage of this approach over the more common method of acetonitrile deproteinization is that the chromatograms are cleaner. With the proper choice of pH and extracting solvent, many interferences can be avoided. Drugs present in serum in small concentrations that cannot be adequately assayed after deproteinization with acetonitrile can be concentrated with this simple extraction method.