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An Evaluation of Gamma-Glutamyl Transpeptidase (GGT) and p30 Determinations for the Identification of Semen on Postcoital Vaginal Swabs

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ABSTRACT: The following study entails the investigation of gamma-glutamyl transpeptidase (GGT) and p30 for the identification of seminal stains in sexual assault cases. A commercial kit was used to test for GGT activity, while p30 was demonstrated with a crossed electrophoresis technique. Specificity, sensitivity, and stability of both markers were studied. Postcoital swabs from lab staff were tested for GGT and p30. In addition, 144 postcoital swabs from case material were tested for p30, spermatozoa, and acid phosphatase. Results show p30 to be a useful semen marker particularly in cases of azoospermia. However, GGT was found to be unsuitable for forensic science casework.

KEYWORDS: criminalistics, criminal sex offenses, semen, postcoital vaginal swabs

The scientific corroboration of alleged sexual assault requires the conclusive identification of semen. Over the years, various constituents of semen such as acid phosphatase, choline, spermine, lactate dehydrogenase, and spermatozoa have been used for this purpose but there are inherent problems associated with the determination of each. Consequently one infallible identifying feature of semen has yet to be described.

Prostatic acid phosphatase was originally believed to be produced only in the prostate [*1*] and although high levels are found solely in semen, significant levels can be demonstrated in vaginal secretion [*2*].

Choline was accepted for many years as being unique to semen [*3*] but false negative reactions were a problem. Recently the specificity has been questioned, as crystals very similar to choline crystals have been found in noncoital postmortem vaginal swabs [*4*].

Spermine, like acid phosphatase, is quantitatively unique to semen [*5*], but the identification test of crystallization is difficult to perform. Leucine amino peptidase also occurs in greater quantities in semen than in other body fluids [*6*]; however, the technique requires reagents that are carcinogenic.

Since lactate dehydrogenase-C4 (LDH-C4) is specific to spermatozoa, its identification is

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valid evidence of the presence of semen. As it is a relatively unstable marker determined by an insensitive technique and would be absent in semen lacking spermatozoa, its identification is of limited practical application [7].

Spermatozoa are unique to semen with the exception of post-ejaculate urine, and their identification in vaginal samples is considered to be proof of ejaculation [8]. However, the number of individuals with semen containing low concentrations, or no spermatozoa, whether for clinical or surgical reasons, continues to increase [9].

Gamma-glutamyl transpeptidase (GGT) was proposed by Rosalki and Rowe in 1973 as a specific semen marker [10], but there is recent controversy regarding its value to forensic science. Gohara [11] recommends GGT not only as a semen specific marker for postcoital vaginal swabs but as a mathematically determinable indicator of the elapsed time since the coitus occurred. Nakanishi [12] found high GGT activity in semen that was independent of the spermatozoa density classification and more stable than acid phosphatase. However, Suzuki et al [13] suggested that the technique could only be used as a confirmatory test for semen, and Pragay et al [14] found both the sensitivity and specificity of GGT to be unacceptable for use either as a confirmatory test for semen or as a substitute for the acid phosphatase test. Rutter et al [15] identified GGT in vaginal secretion, as did Suzuki [13], and therefore disagreed with Gohara's claims of semen specificity and predictable GGT decay in the vagina.

In 1971 Li and Shulman [16] described a protein, El, which appeared to be semen-specific according to its mobility in conventional electrophoresis and prepared an antiserum against it. Sensabaugh [17] used this anti-El to confirm the identity of the semen-specific protein he had isolated and named p30. Independently, Wang et al [18] described a prostate-specific antigen useful in the early serological diagnosis of cancer of the prostate and since found to be identical to El and p30 [19].

Although population [20] and stability [21] studies confirmed the range of p30 concentration in liquid semen and its stability in uncontaminated dried seminal stains, only one group has studied the stability of p30 on postcoital vaginal swabs. Poyntz and Martin [22] obtained less than 20% positive p30 results with swabs from cases of alleged sexual assault and observed no correlation with either acid phosphatase or total protein levels.

The present study was designed to confirm and expand the published data in hopes of verifying specificity and predictable stability of both GGT and p30 for the conclusive identification of semen in vaginal samples.

Materials and Methods

Semen

Ninety-four men contributed liquid semen samples. Of these samples, 54 contained normal numbers of spermatozoa, 20 were oligospermic, 8 were azoospermic, and 12 were from vasectomized individuals. One donor submitted six successive ejaculates. Liquid semen from one donor was mixed with stains of saliva, urine, feces, and perspiration from another donor and incubated for 48 h in a moisture chamber at 37°C. Seminal stains from five donors were aged for ten weeks at 4, 22, and 37°C.

Vaginal Swabs

Semen-free vaginal swabs which were taken at least three days postcoitus were submitted by thirty donors. Four donors submitted forty-one postcoital swabs from nine acts of intercourse. Immediately after collection, semen-free vaginal swabs from four donors were immersed in liquid semen from one donor and incubated at 37°C for eight days. Vaginal swabs from 144 cases of alleged sexual assault were examined.

Other Body Fluids

Urine samples from ten donors, and post-ejaculate urine samples from four donors were tested. Saliva samples from ten donors, breast milk from seven mothers, and venous blood from five donors were tested. One donor submitted samples of menstrual blood, perspiration, and feces.

Samples were tested as dried stains. Those that could not be tested immediately were stored at −60°C.

Gamma-Glutamyl Transpeptidase

GGT amounts were determined using a commercial kit (Smith-Kline, Sunnyvale, CA) with which the absorbance of free paranitroaniline, a product of the transfer of a glutamyl group from gamma-glutamyl-paranitranilide to glycylglycine was measured at 405 nm (Bausch and Lomb Spec 20). The rate of absorbance increase is stated to be directly proportional to the enzyme activity present. Specifically, 1 IU of GGT activity is the amount of enzyme which transfers 1 μmole of glutamate per minute per liter of sample with concurrent release of 1 μmole of *p*-nitroaniline. When measurements are made in a cuvette with a 1.0-cm light path:

$$\text{IU} = \frac{\Delta A}{T} \times \frac{TV}{SV} \times \frac{1}{10^{-6}\epsilon} \times 1000$$

where

ΔA = absorbance change,
T = time interval in minutes over which ΔA is measured,
TV = total reaction volume in millilitres,
SV = sample volume in millilitres, and
ϵ = molar absorptivity of *p*-nitroaniline 9.9×10^6 cm^2/mol at 405 nm.

Approximately 0.5-cm^2 stain was soaked in 0.25 mL of distilled water for ½ h then actively extracted using a pasteur pipette. Fifty microlitres of stain extract and three millilitres of substrate were placed in a cuvette and incubated at 37°C in a water bath. Absorbance readings were taken at 5, 10, and 30 min. The 10-min absorbance reading is reported in this study, as the mathematical conversion to international units was not considered to be meaningful at this stage.

The reproducibility of results was tested by repeating a single dried semen sample five times. Seven samples were duplicated and all others were tested once.

p30

Cross-over electrophoresis with anti-p30 (Serological Research Institute (SERI), Emeryville, CA) was performed according to Wraxall [*23*] with some modifications. Fifteen millilitres of a 1% agarose (EEO 0.25, SERI) was layered onto the hydrophilic side of a 12.5 by 9-cm sheet of Gelbond (Mandell Scientific) to produce a layer approximately 1 mm thick using fiber tape (Tuck Tape, Canadian Technical Tape Ltd.) to create a chamber. The same buffer was used for the gel and the tank (0.208-mmol/L Trizma base, 8.55-mmol/L ethylenediaminetetraacetate (EDTA), 30.7-mmol/L boric acid, pH 9.1). Pairs of wells approximately 2 mm in diameter were punched 0.5 cm apart at right angles to the direction of the current. The sample extracts prepared for the GGT assay were used for p30 determination so that the relative sensi-

tivity of two different assays could be compared. The case work swabs were extracted in the same way in 0.45 mL of distilled water. Three microlitres of extract and of neat-anti-p30 were placed in the cathode and anode wells, respectively. The plate was run for 30 min at 120 V at room temperature, washed overnight in 1 mol/L of saline, pressed under three layers of Whatman 3 paper, soaked for 5 min in distilled water, pressed again, and dried at 37°C. The dehydrated gel was stained with 0.1% coomassie blue and destained with an acetic acid-water-methanol mixture (10:50:50).

With the exception of a weak band close to the antiserum well, a precipitin band was interpreted as a positive p30 result.

Enzyme-Linked Immunosorbent Assay (ELISA)

Five seminal stains, four liquid semen samples, and one vaginal swab were tested by indirect enzyme-linked immunosorbent assay (ELISA) [*24*]. Stains were extracted for ½ h in a minimum quantity of 0.0005% Tween 20 in phosphate buffered saline (PBS), were diluted to 1/10 000 in this buffer and a 40-μL aliquot was allowed to absorb in an Immulon 2 microtitre plate (Dynatech) at 22°C for 3 h. The plates were washed three times with 0.0025% Tween 20 in PBS. Forty microlitres of anti-p30 diluted 1/1000 in 1% bovine serum albumin (BSA) −0.0005% Tween 20 in PBS was added and the plates were incubated for 2 h at 22°C. After washing again as described, 40 μL of antirabbit $F(ab^1)_2$ (sheep) conjugated with alkaline phosphatase (Sigma) diluted 1/1000 in BSA-Tween-PBS was added and allowed to react at 22°C for 3 h. After washing again as described, 40μL of paranitrophenyl phosphate 1 mg/mL in diethanolamine (0.9 mol/L pH 9.8 with 0.5 mmol/L magnesium chloride) was added and the plate was incubated at 37°C for 1 h. The reaction was stopped by the addition of 40 μL of 2 mol/L sodium hydroxide and the color development was assessed visually on a scale of 1 to 10 by comparison with the positive and negative controls.

Acid Phosphatase

Vaginal swabs were actively extracted in 0.45 mL of distilled water. One drop of fast blue working solution [*25*] was added to a fraction of a drop of extract on filter paper. After exactly 30 s the color development was compared to a standardized Munsell color chart [*26*] and recorded only as strong (Munsell #, 75P 7/8, 6/8, or 5/8), medium (Munsell # 10P 8/4 or 75P 8/6), or weak (Munsell # 10P 9/1, 75P 9/2). Reactions that were at least medium at 30 s were recorded as positive acid phosphatase. To quantitate this test for academic purposes, 20 liquid semen samples which had been frozen were pooled. Doubling dilutions in distilled water up to 1/2048 were tested as described. A positive reading was obtained at 1/512 (0.04 μL of semen). Liquid semen samples from known low and high level acid phosphates donors gave positives at 1/32 (0.6 μL of semen) and 1/1024 (0.02 μL of semen), respectively. To simulate case work conditions, swabs were saturated in the diluted pooled liquid semens, dried overnight at room temperature, and extracted as described. The last dilution that gave a positive reading was 1/64 and since the average swab absorbs about 350 μL, this dilution represents approximately 10 μL of semen.

Spermatozoa

Extracts from vaginal swabs prepared as described were centrifuged. The pellet was smeared on a glass slide, fixed, stained with Christmas tree stain [*27*], and examined microscopically at ×1000.

Results

GGT

When a single dried semen sample was tested five times, the 10-min absorbance values ranged from 0.132 to 0.185 (81 to 114 IU). The maximum difference in absorbance observed with seven duplicated samples was 0.095.

Population Variation—As can be seen in Fig. 1, the range of GGT in semen was unaffected by the number of spermatozoa in the ejaculate. The absorbance values for 54 normal semen samples ranged from 0.110 to 0.660, for 20 oligospermic samples from 0.053 to 0.510, and for 8 azoospermic samples from 0.110 to 0.850. Vasectomy did not affect the GGT levels as absorbance values for twelve post-vasectomy samples ranged from 0.160 to 0.710.

When six separate ejaculates from one person were tested, the absorbance values ranged from 0.062 to 1.02, exceeding our observed population variation. Consequently, the lowest recorded value for normal males, 0.062, was chosen as the minimum quantity of GGT characteristic of semen. This figure was used in the interpretation of data from postcoital swabs.

To establish the variation in GGT values within a seminal stain, six to eight random samples from each of three stains were assessed. The absorbance varied from 0.42 to 0.84 in one stain.

Stability—Dried seminal stains from five donors were aged at 4, 22, and 37°C for ten weeks and then tested. The original GGT activity was maintained in stains stored at 4°C (Fig. 2) but approached uninterpretable values within two to three weeks in stains stored at 22 (Fig. 3) and 37°C (Fig. 4).

Specificity—The specificity of GGT to seminal fluid was tested by examining other body fluids (Fig. 5). Ten urine samples and ten saliva samples gave no absorbance readings. Thirty samples of semen-free vaginal secretion yielded absorbance values from 0 to 0.045 and seven samples of human milk gave absorbance values from 0.065 to 0.620.

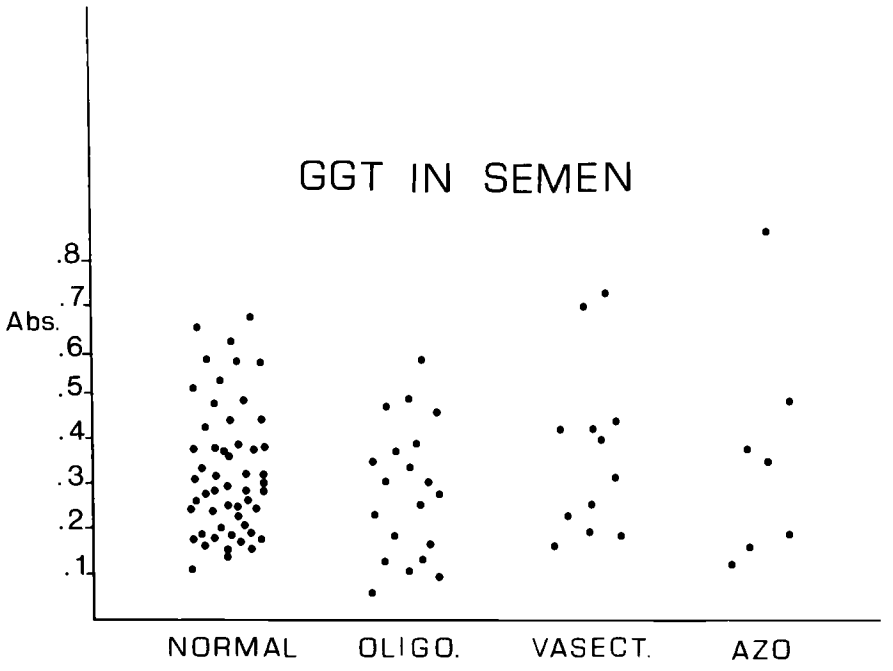

FIG. 1—*GGT activity in normal and abnormal semen samples.*

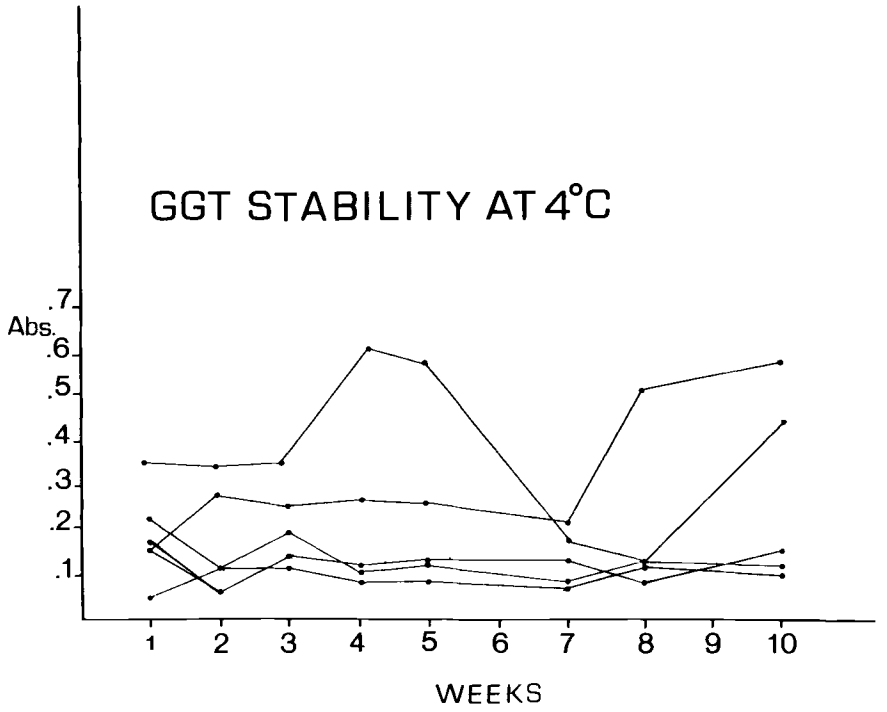

FIG. 2—*The stability of GGT in five semen samples stored at 4°C.*

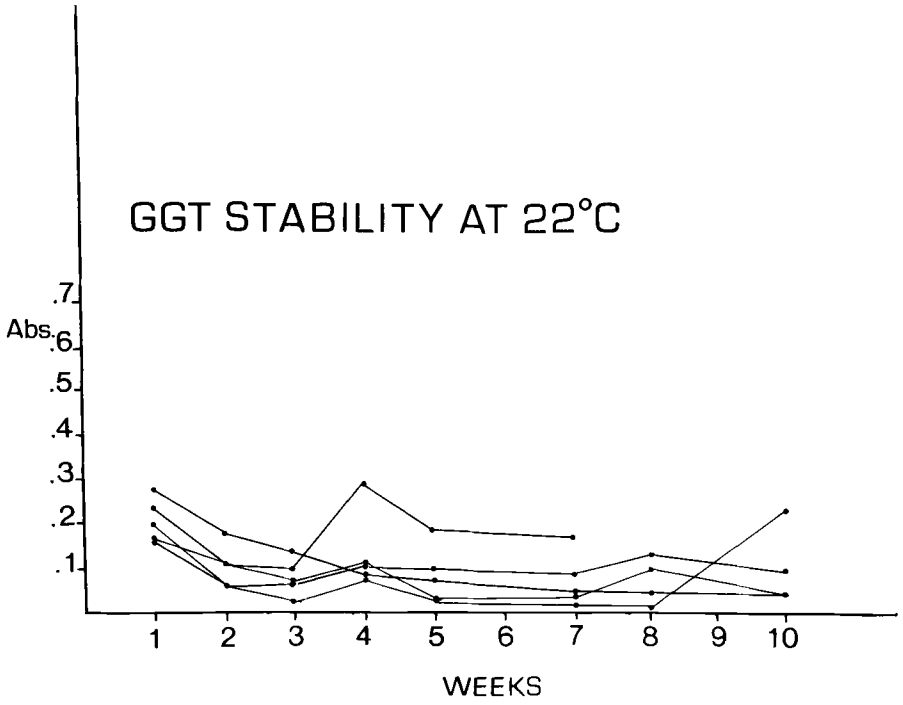

FIG. 3—*The stability of GGT in five semen samples stored at 22°C.*

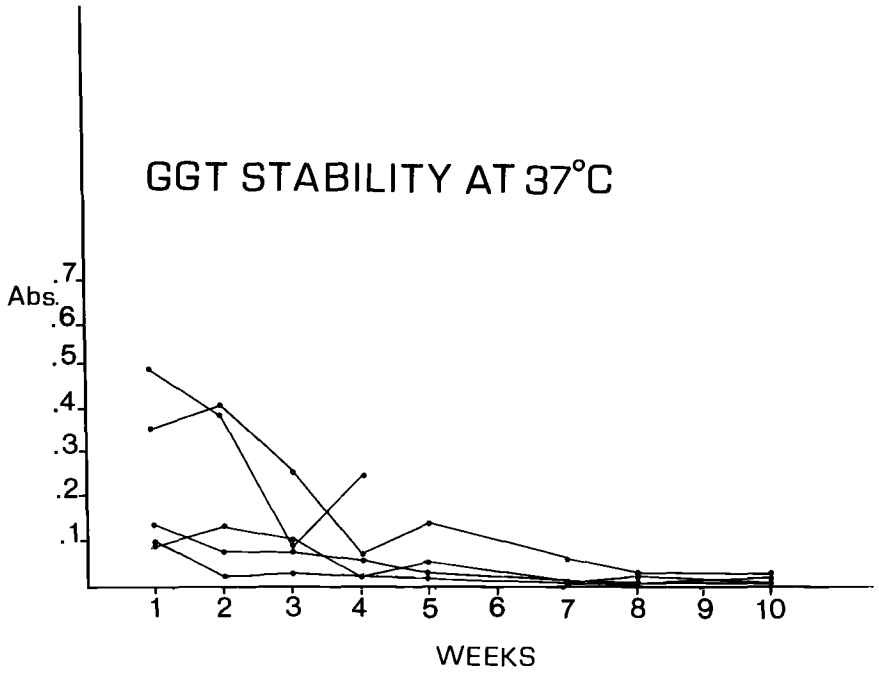

FIG. 4—*The stability of GGT in five semen samples stored at 37°C.*

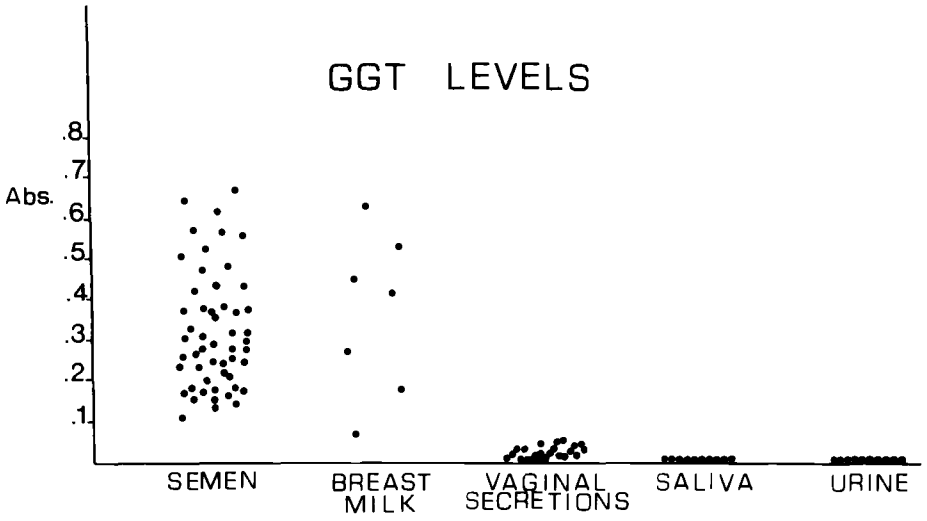

FIG. 5—*GGT activity in body fluids.*

On the basis of GGT alone, semen and human milk could not be distinguished. As well, high absorbance values from some vaginal secretions approached low values obtained from some semens. Statistical analysis of this marker, in view of its nonspecificity, was considered to be pointless.

Postcoital Swabs—Thirty-one postcoital swabs from four couples were examined (Fig. 6). After an interval of 2 h, only 5 of the 22 swabs examined gave absorbance values above 0.062.

p30—Cross-Over Electrophoresis

Sensitivity—An interpretable precipitin arc was obtained with all 94 semen samples regardless of the number of spermatozoa. The final dilution at which liquid semen samples gave positive results ranged from 1/100 to 1/3000, a reflection of the reported p30 population range [*17*] of 145 to 5900 μg/mL with a mean of 800 μg/mL.

Stability—p30 was identified in five-year-old semen stains stored at 22°C and in ten-week-old stains stored at either 4 or 37°C. p30 identification was affected neither by in vitro incubation at 37°C for 48 h with body fluids such as urine, saliva, or perspiration, nor by incubation at 37°C for eight days with vaginal secretion.

Specificity—With the exception of five samples of post-ejaculate urine, p30 was not detected in any body fluid other than semen. However, a weak band close to the antibody well was detected with a semen-free vaginal swab from a 20-year-old woman. SERI[2] reported similar findings with vaginal swabs from a 75-year-old woman and suggested they were due to an unusually high protein concentration in the samples.

Twenty-two of the forty-one postcoital swabs donated by four staff members gave positive p30 results. All swabs were positive up to 6 h postcoitus. Swabs from two donors were positive up to 11 and 16 h, respectively.

Casework Swabs—Vaginal swabs from 144 cases of alleged sexual assault were assessed in three ways: qualitative acid phosphatase, presence of spermatozoa, and presence of p30. The time interval between the assault and the examination as stated by the complainant was recorded.

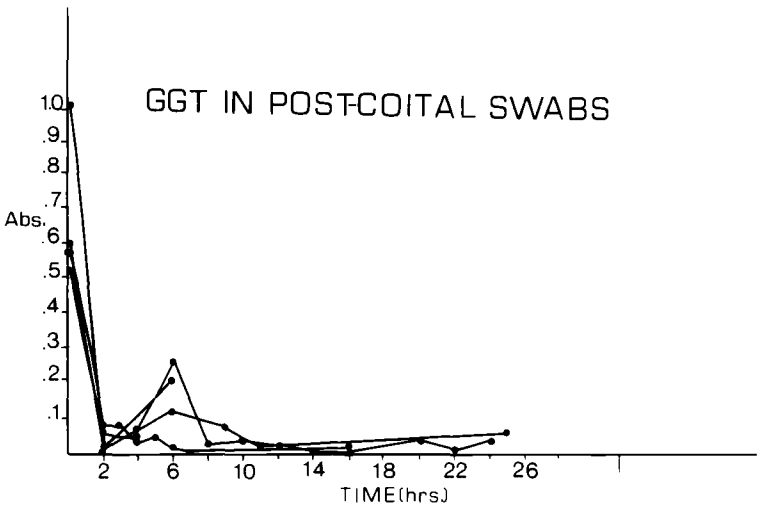

FIG. 6—*Stability of GGT in postcoital swabs.*

[2]Personal communication, Serological Research Institute, Emeryville, CA.

As can be seen in Table 1, almost 32% of the swabs examined were negative for all three markers. Perhaps ejaculation did not occur as 77% of the swabs were taken within 8 h of the alleged assault. Considering the remaining 98 swabs for which at least 1 marker was positive, 63% gave positive p30 results, 59% were positive for both acid phosphatase and p30, and 86% were positive for either acid phosphatase or p30. The value of p30 is illustrated by the one sample that was positive for p30 alone and a second sample, probably azoospermic, that was positive for p30 and acid phosphatase. Excluding the all negative category, 95% of the swabs fall into one of three categories: all three markers identified, only spermatozoa identified, or spermatozoa and acid phosphatase identified. It was not surprising to find that spermatozoa were the most stable markers of semen. p30 seems to be the least stable as shown by the observation that ten of the swabs were negative only for p30 including eight swabs taken within 8 h of the alleged assault. With the exception of 14 swabs (the 10 negative for p30, the 3 negative for only acid phosphatase, and the single swab negative for acid phosphatase and sperm) there was excellent correlation between p30 and acid phosphatase results whether positive or negative.

Although p30 was most often identified on vaginal swabs within 8 h of intercourse, positive results have been obtained both with donor swabs and case swabs up to 16 h postcoitus.

p30—ELISA—This preliminary study gave very interesting results. All nine semen samples contained readily detectable p30 at diluations up to 1/10 000. A single vaginal swab taken three days postcoitus gave indications of p30 at a dilution of 1/500. It appears as though the vaginal stability of p30 determined by this technique may approach the known vaginal stability of spermatozoa, however more work must be done.

Discussion

In our hands, GGT determination was not suitable for use with forensic science case material. The quantitative overlap between semen and vaginal secretion would make GGT interpretation with case material difficult. Even more important, the limited vaginal stability of 2 h as demonstrated on postcoital lab staff swabs indicates that the test would have very limited practical application. p30 on the other hand was almost totally specific to semen, and its identification in post-ejaculate urine is a characteristic shared by spermatozoa. There was no indication in this study that a positive p30 precipitin band in the correct position could result from any substance other than semen. Poyntz [*22*] has demonstrated that p30 is not found in semen-free postcoital swabs unlike the observation of elevated acid phosphatase levels in

TABLE 1—*Correlation of spermatozoa, p30, and acid phosphatase results from 144 case work vaginal swabs with reported time after intercourse.*

	Reported Time After Intercourse, hours			
	0-8	8½-45	Total	
	No.	No.	No.	%
Positive sperm, p30, A.P.[a]	52	5	57	39.6
Negative sperm, p30, A.P.	30	16	46	31.9
Positive sperm; negative p30, A.P.	16	10	26	18.1
Positive p30; negative sperm, A.P.	1	0	1	0.7
Negative sperm; positive p30, A.P.	1	0	1	0.7
Negative p30; positive sperm, A.P.	8	2	10	6.9
Negative A.P.; positive sperm, p30	3	0	3	2.1
Total	111(77.1%)	33(22.9%)	144	100

[a]A.P. = acid phosphatase.

these samples. However, the absence of a p30 precipitin arc does not necessarily imply the absence of semen since we have shown that by crossed electrophoresis p30 is the least stable marker in vaginal samples, not usually found after 8 h postcoitus and never identified beyond 16 h with either donor swabs or case material. Of course the demonstrated stability of a marker is a function of the sensitivity of the assay used, and ELISA is 100 times more sensitive than crossed electrophoresis. The increased sensitivity offered by ELISA would require semiquantitative interpretation particularly of results on vaginal swabs. If the majority of sexual assault victims are examined within 8 h of the offense, perhaps extending the potential identification of p30 beyond 24 h would confuse rather than clarify the issue.

It was not surprising to find that spermatozoa, known to survive up to five days [*9*], were the most stable element of semen. Their identification alone on case swabs whether as a result of a previous intercourse or a small quantity of semen from a recent assault was not unexpected.

Vast quantitative differences in acid phosphatase levels in semen combined with the first-order intravaginal decay over time as demonstrated by Sensabaugh [*12*], have contributed to the lack of confidence in this marker as a sole indicator of semen.

There are problems inherent in the analysis of postcoital swabs from case material. For example, since the information reported by the complainant cannot be considered infallible, the initial source and quantity of semen on the swab is unknown. As well, each semen marker is subject to both physical loss and intravaginal enzymatic degradation, although not at the same rate. Also, after the swab is taken the deterioration of the proteins in the sample is dependent on the humidity, temperature, and time involved in the drying procedure. Finally, optimal extraction methods must be used to ensure an accurate comparison of the relative sensitivities of three different techniques to identify three different markers.

Our data indicate quite close correlation of acid phosphatase and p30 stability on vaginal swabs from case material. In contrast to Poyntz and Martin's data [*22*], our increased success in detecting p30 is probably due to technical differences such as the concentration of the swab extract, differing buffer system, and the length of time of the washing stage.

Although only 1 of the 144 case swabs examined gave a positive p30 result in the absence of spermatozoa, this finding demonstrates the value of p30 determinations.

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