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Anabolic Steroids—Analysis of Dosage Forms from Selected Case Studies from the Los Angeles County Sheriff's Scientific Services Bureau

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ABSTRACT: Four selected case studies are described to demonstrate the detection and identification of anabolic steroids in dosage forms as typically encountered in the forensic laboratory. General analytical schemes are presented to provide conclusive identification of anabolic steroids and human chorionic gonadotropin (HCG) as required by the California Uniform Controlled Substances Act. Methodology designed to simplify and reduce analysis time, and yet comply with the legal reporting requirements for anabolic steroids, is reviewed.

KEYWORDS: toxicology, steroids, human chorionic gonadotropin, anabolic steroids, forensic science analysis, controlled substances

Anabolic steroids under present California law are controlled substances as listed under Schedule III of the California Uniform Controlled Substances Act.[2] Steroids present a timely, unique, and challenging analytical demand upon the forensic laboratory accustomed to the analysis and identification of narcotics and other dangerous drugs. Including veterinary products, it is estimated that there are as many as 80 anabolic/androgenic steroids marketed worldwide [*1*].

The expanding numbers of compounds encountered including numerous pharmaceutical derivatives and synthetic modifications, coupled with the complexities of both structure and nomenclature, and the unavailability of certain reference standards, may seem discouraging. In addition, illicit as well as legitimate foreign sources of pharmaceutical and veterinary steroids, often not readily identified by label, or purposely mislabeled, can further exasperate the forensic chemist attempting to develop a routine laboratory method for "all" anabolic steroids.

In this communication, general analytical schemes are offered to initiate the analysis of anabolic steroids. Four selected case studies are presented as examples of the following: (1) the mismarked tablet, (2) the unmarked tablet, (3) a time-saving analytical stratagem for steroids dissolved in oil, and (4) the detection and identification of human chorionic gonadotropin (HCG). For recent reviews pertaining to the analysis of anabolic steroids

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[2]Assembly Bill No. 1591, Chapter 567, Section 11056 of the Health and Safety Code of the California Uniform Controlled Substances Act, as amended 21 Sept. 1989.

refer to Refs *2* and *3*. Reference *1* is useful as a concise and comprehensive collection of analytical data.

Materials and Methods

Cases of Solid Dose Steroid Tablets

Several tablets are pulverized with mortar and pestle and extracted by vigorous mixing with approximately 2 mL of chloroform ($CHCl_3$) in a 13 by 100 mm, 10-mL glass test tube. The undissolved excipient is removed by centrifugation, and the supernatant is filtered through Whatman No. 2 filter paper. The filtrate is dried with crystals of sodium sulfate (Na_2SO_4), evaporated under vacuum, and dissolved in methanol (MeOH); it is then suitable for GC/MSD (gas chromatography coupled with a mass selective detector) analysis, or dissolved in $CHCl_3$, for analysis by infrared (IR) or potassium bromide (KBr) disks.

Cases of Steroids Dissolved in Injectable Vegetable Oils

Approximately 2 mL of oil is first extracted by vigorous shaking with MeOH [2:1 (v/v) MeOH/oil in a 13 by 100-mm, 10-mL glass test tube for at least 2 min]. The MeOH extract, after separation by centrifugation, is decanted and placed in the freezer compartment of a refrigerator for 15 min in order to precipitate coextracted components of the oil. After 15 min at −20°C, the MeOH solution becomes cloudy and turbid and is immediately centrifuged to remove the cold-induced precipitation. The remaining clarified supernatant is adequate for analysis by GC/MSD.

Alkaline Hydrolysis of C17-Alkyl Esters

The California Controlled Substances Act, as recently amended, requires the identification of the anabolic steroid moiety without regard to the presence of ester linkages at the C17 position. Consequently, a convenient time-saving analytical procedure involves cleavage of any C17-ester linkage to liberate the parent steroid. Typically, injectable vegetable oils contain the anabolic steroid as an alkylated ester at the C17 position.

A simple and rapid procedure involves the addition of approximately five solid potassium hydroxide (KOH) pellets to approximately 2 mL of the MeOH extract and allowing the hydrolysis to proceed at room temperature for 15 min. The MeOH is dried under vacuum, and the residue dissolved in diethyl ether. The ether solution is washed with water, dried with anhydrous Na_2SO_4, and evaporated under vacuum; the residue dissolved in MeOH is suitable for GC/MSD analysis.

Instrumental Analysis

IR spectral analysis was carried out using a Beckman FT-IR Model FT1200 on KBr disks.

A Hewlett-Packard 5890A gas chromatograph coupled to a HP 5970 mass selective detector was utilized for GC/MSD analyses. The gas chromatograph was equipped with an HP-1 fused silica capillary column, cross linked with 100% dimethylpolysiloxane (12 m by 0.2-mm inside diameter; 0.33 μm film thickness). The injection ports were capillary split injectors with split silanized glass inserts.

The GC/MSD, operated in the scan mode from 40 to 500 atomic mass units, utilized helium as the carrier gas at a flow rate of 1 mL/min; oven temperature was 150°C with a split ratio of 20:1. The septum purge was 2.0 mL/min. The injector and interface

temperatures were 250 and 280°C, respectively. The temperature program was set at an initial temperature of 180°C to increase 15°C/min, to reach a final temperature of 265°C.

A Hewlett-Packard Model 5890A gas chromatograph equipped with flame ionization detector (FID) was utilized as a preliminary screen for evaluating the possible presence of a steroid in either tablet or oil extracts. The chromatograph was fitted with a 10 m by 0.53 mm inside diameter (2.65 μm film thickness) HP-1 fused silica capillary column, cross linked with 100% dimethylpolysiloxane. Injector and detector temperatures were maintained at 225 and 250°C, respectively. Steroids were chromatographed at an initial temperature of 210°C to increase at a rate of 20°C/min to reach a final temperature of 250°C.

Immunoassay for Human Chorionic Gonadotropin

Double-diffusion Ouchterlony plates were utilized for the detection and identification of HCG. Rabbit anti-serum to HCG and HCG were purchased from Sigma Chemical Co., Lot Numbers 28F4843 and 28F0499, respectively. The analyses were carried out in 1% Type I agarose gels formed on gel-coat backing. After overnight diffusion at room temperature, the gels were soaked for several hours in 1*M* saline, rinsed with deionized water, pressed, and oven dried. The protein precipitin bands were visualized by staining with Comassie blue.

The standard HCG stock solution was prepared by dissolving the lyophilized powder in deionized water to produce a concentration of 1000 international units (IU)/mL; it was stored frozen. A working solution of standard antigen (500 IU/mL) was utilized in the assay. The stock antibody was prepared by diluting, according to manufactures instructions, the antiserum 1:5 with 10m*M* (NaP_i), pH 7.8, 150m*M* sodium chloride (NaCl), and 0.1% sodium azide (NaN_3); it was stored frozen. The sensitivity of the method was such that 10 μL of 500 IU/mL HCG could be detected with 10 μL of the 1:5 diluted stock antibody.

Reagents

All reagents and solvents were of analytical grade. Standard reference steroids were purchased from Sigma Chemical Co., St. Louis, MO.

Results and Discussion

Case Study I

This case contained white single-scored oval tablets with the "Searle" logo and the inscription 1401. Both the National Drug Code [*4*] and the Physicians' Desk Reference [*5*] identified this tablet as Anavar, containing 2.5 mg of oxandrolone. Figure 1 is the IR spectrum of the $CHCl_3$ extract of four to these tablets. Figure 2 is the IR spectrum of 17-alpha-methyltestosterone. As a final confirmation of structural identity, GC/MSD analysis of the extract was performed as shown in Fig. 3. The retention time and mass spectral data matched that of authentic 17-alpha-methyltestosterone.

This case represents an example of the mismarked steroid tablet. Oxandrolone was not detected in the $CHCl_3$ extract; whereas, 17-alpha-methyltestosterone was the only steroid detected. It should be noted that these tablets were not accompanied by, nor contained in, a manufacturer's sealed and labeled container. Consequently, the possibility remains that these tablets are the product of a clandestine manufacturer intent on producing a "look-alike" counterfeit, or substitute, for an otherwise approved legitimate

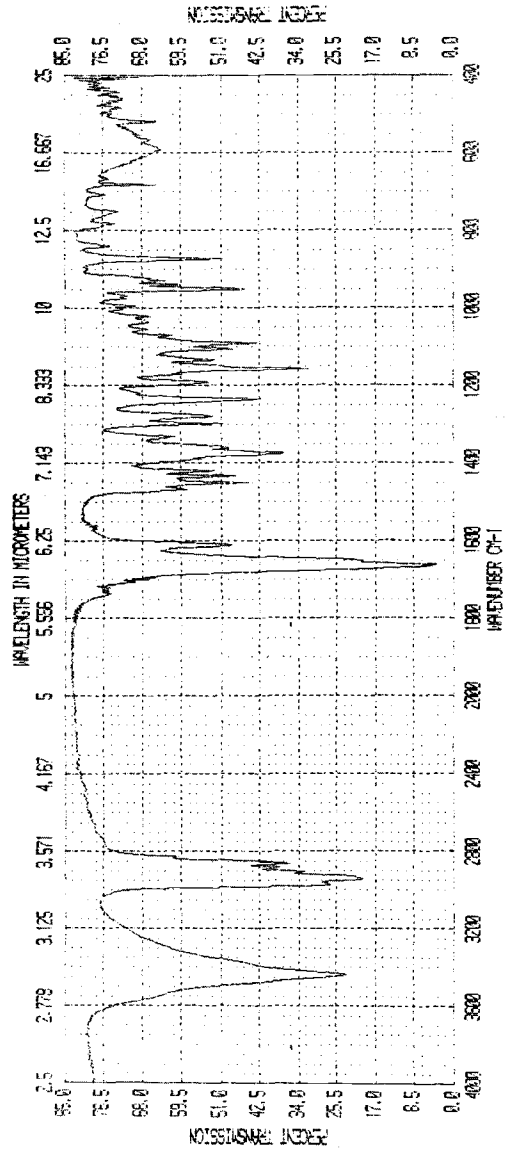

FIG. 1—*IR spectrum of $CHCl_3$ extract of "Searle" tablets. Sample run as KBr disk.*

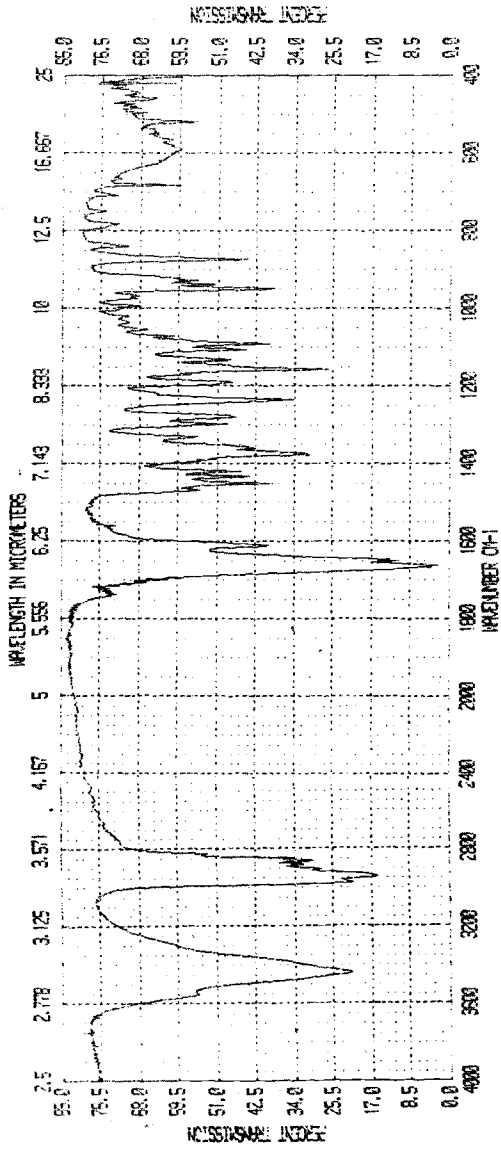

FIG. 2—*IR spectrum of 17-alpha-methyltestosterone (Sigma standard). Sample run as KBr disk.*

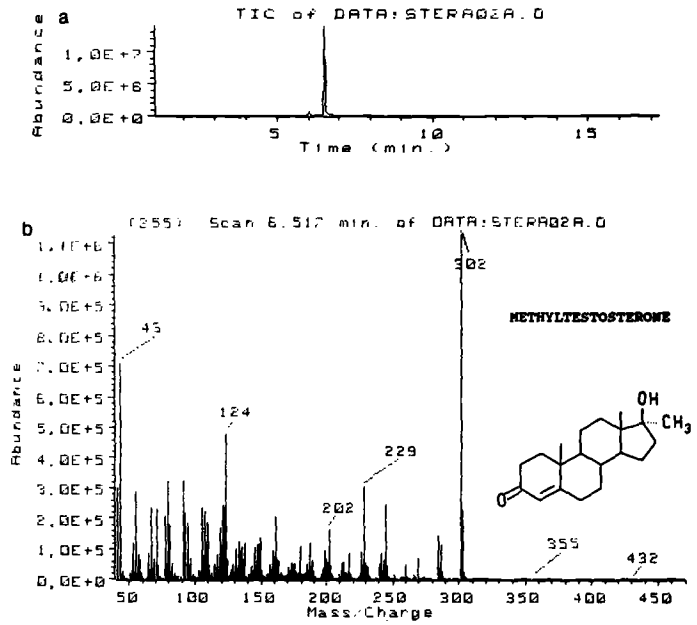

FIG. 3—*Total ion chromatogram* (a) *and mass spectrum* (b) *of* $CHCl_3$ *extract of "Searle" tablets.*

anabolic steroid. Or, alternatively, these tablets may have resulted from a substitution, intentional or otherwise, by the manufacturer of one active anabolic steroid for another.

Representatives of the Searle Company have acknowledged awareness of look-alike tablets inscribed with the "Searle" logo and 1401. Generally, these look-alike tablets are associated with marked pharmaceutical vials that can be distinguished from legitimate Searle vials manufactured for oxandrolone tablets.[3]

Case Study II

In this case a large number of white round double-scored tablets was received with additional exhibits of anabolic steroids. These tablets were otherwise unmarked and closely resembled "mini-bennies." However, the assortment of other exhibits contained in the case suggested an analysis for the possible presence of an anabolic steroid. Figures 4, 5, and 6 presents the GC/MSD data obtained from a $CHCl_3$ extract of these unmarked tablets. Two anabolic steroids, eluting at retention times of 6.4 and 6.6 min were detected, and their structural identities were confirmed by computerized matching of each mass spectrum to those of authentic reference steroids. The two steroids detected were 17-alpha-methyltestosterone and methandrostenolone.

Recently, Johnson et al. [6] communicated a report describing "cross-tops" as tablets seized among other anabolic steroids and submitted to the California Criminalistics Institute for analysis. Analysis confirmed the presence of both methandrostenolone and 17-alpha-methyltestosterone. These tablets were described as "visually indistinguishable from the ephedrine/caffeine containing double-scored tablets," often referred to as a look-alike mini-bennie. A recent report from The Orange County Crime Laboratory, California [7] described mini-bennie tablets of methandrostenolone and 17-alpha-methyltestosterone.

[3]Personal communication from Searle to Erin A'Hearn.

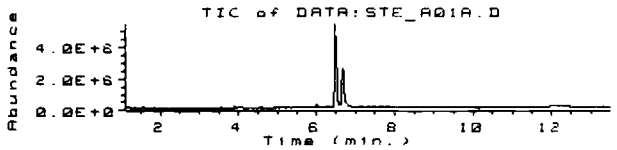

FIG. 4—*Total ion chromatogram of $CHCl_3$ extract of "mini-bennie" tablets.*

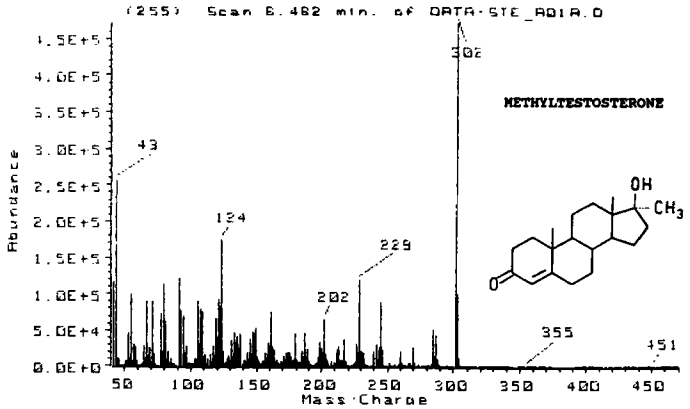

FIG. 5—*Mass spectrum of first eluting GC peak from $CHCl_3$ extract of "mini-bennie" tablets.*

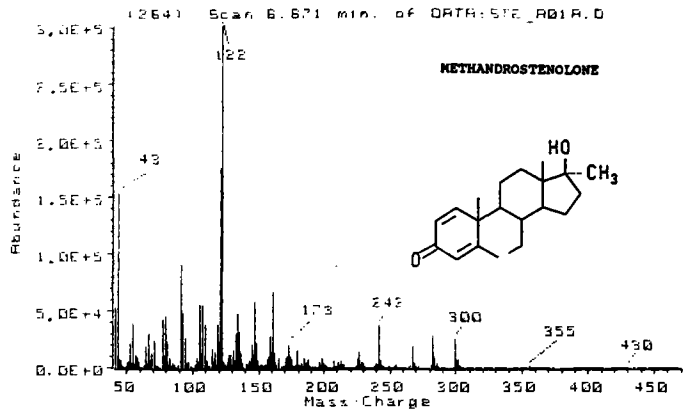

FIG. 6—*Mass spectrum of second eluting GC peak from $CHCl_3$ extract of "mini-bennie" tablets.*

Case Study III

This case contained, among other exhibits, a bottle labeled Equipoise with the brand label of Squibb. This label displayed the following information: made in Mexico; Lot. No. 8433-88523; Boldenone Undecylenate, 25 mg/mL, in an injectable vegetable oil; for animal use only.

The oil was extracted with MeOH as previously described. The total ion chromatogram (TIC) of the MeOH extract, after injection into the GC/MSD, is displayed in Fig. 7*a*. The TIC of the MeOH extract after alkaline hydrolysis, as previously described, is presented in Fig. 7*b*. Figures 8 and 9 report the corresponding mass spectra for the GC peaks eluting at retention times of 6 and 32 min.

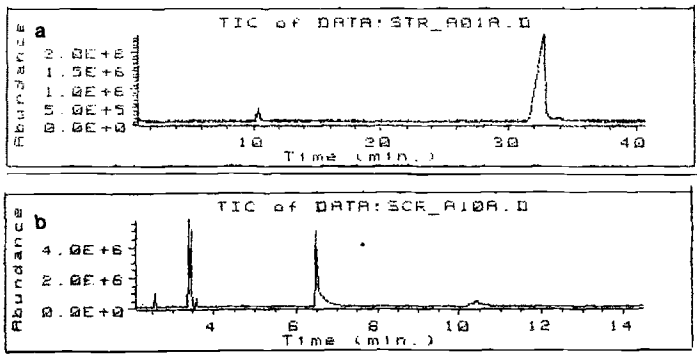

FIG. 7—*Total ion chromatogram of MeOH extract of Equipoise before* (a) *and after* (b) *alkaline hydrolysis.*

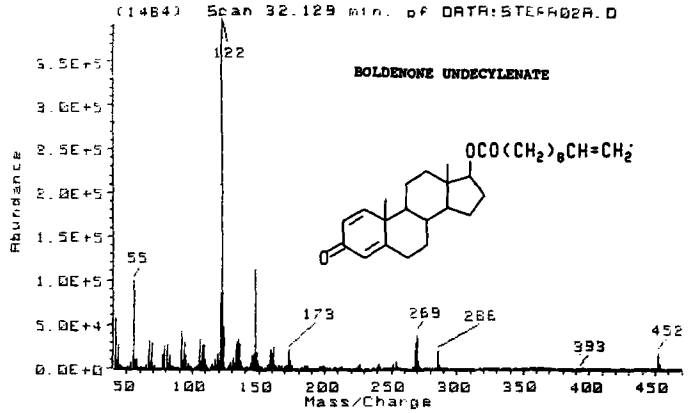

FIG. 8—*Mass spectrum of Boldenone Undecylenate derived from MeOH extract of Equipoise.*

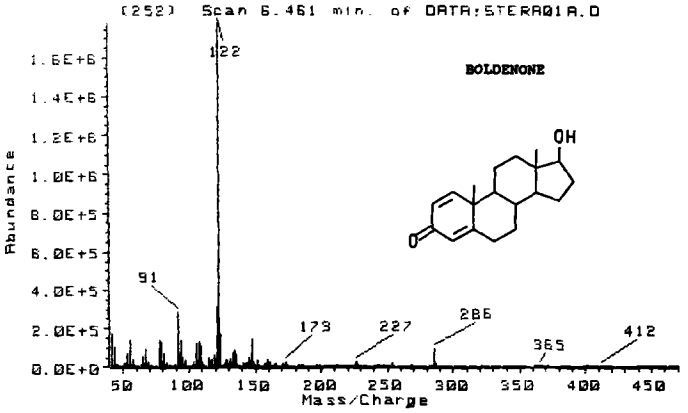

FIG. 9—*Mass spectrum of Boldenone derived from MeOH extract of Equipoise after alkaline hydrolysis.*

Boldenone Undecylenate elutes on the gas chromatograph (GC/MSD) at a retention time of approximately 32 min, and Boldenone elutes at approximately 6 min when run under the same conditions. These retention times, and associated mass spectra, match authentic standards of Boldenone and Boldenone Undecylenate. Consequently, the analysis time is considerably reduced, and the gas chromatography significantly improved after alkaline hydrolytic cleavage of the C17 ester linkage.

The current version of the California Health and Safety Code, Chapter 567, as amended in Section 11056 with reference to the definition of controlled substances within Schedule III, Section I, Subheading f, reads as follows:

> *Anabolic steroids and chorionic gonadotropin. Any material, compound, mixture, or preparation containing chorionic gonadotropin or an anabolic steroid, including, but not limited to, the following:*

This paragraph then continues and proceeds to itemize 31 specifically identified anabolic steroids in addition to chorionic gonadotropin. It is clear from this amended definition that any "compound" containing, without regard to chemical or synthetic alteration, modification, combination, or derivatization, any of the aforementioned listed steroids, and any and all unlisted anabolic steroids, is a Schedule III controlled substance under current California law.

In the authors' opinion, acceptance of the above interpretation allows for the alkaline hydrolysis of C17 esterified steroids to liberate the free steroid; the subsequent identification of the derived steroid moiety is the minimum requirement to satisfy the letter and intent of the amended version of Section 11056.

Cases Containing Human Chorionic Gonadotropin

In addition to anabolic steroids, human chorionic gonadotropin (HCG), a glycoprotein hormone, is presently controlled as a Schedule III substance. Several cases have been received in our laboratory that consist of exhibits of anabolic steroids often including and accompanied by pharmaceutical injection vials of HCG. Occasionally, exhibits of HCG not associated with steroids have been seized and submitted for analysis.

Figure 10 presents the results of two such cases analyzed by Ouchterlony double-diffusion immunoassay. This is a sensitive and specific assay for the detection of antigen-antibody complexes, such as that produced from HCG and its rabbit antibody.

Typically, HCG manufactured for subcutaneous injection is prepared by mixing a lyophilized powder with sterile water to produce a solution containing HCG at 1000 IU/mL. The immunoassay, as routinely carried out in our laboratory, has sufficient sensitivity to allow detection of 5 μL of such a hypodermic preparation.

Conclusions

In summary, the forensic analysis for anabolic steroids and human chorionic gonadotropin does not require any new technology or expensive additional instrumentation. Crime laboratories equipped with a gas chromatograph and mass spectrometer have the essential instruments required. The methods of sample preparation are not complex and can be carried out in about the same time as required for the analysis of many other controlled substances.

Alkaline hydrolysis of 17-esterified steroids, enabling the identification of a prototype anabolic steroid, is concluded to be permissible for reporting under The California Uniform Controlled Substances Act.

The application of an immunochemical procedure for the detection and identification of human chorionic gonadotropin provides a simple and inexpensive assay, with sufficient specificity and sensitivity to be of value in the forensic laboratory.

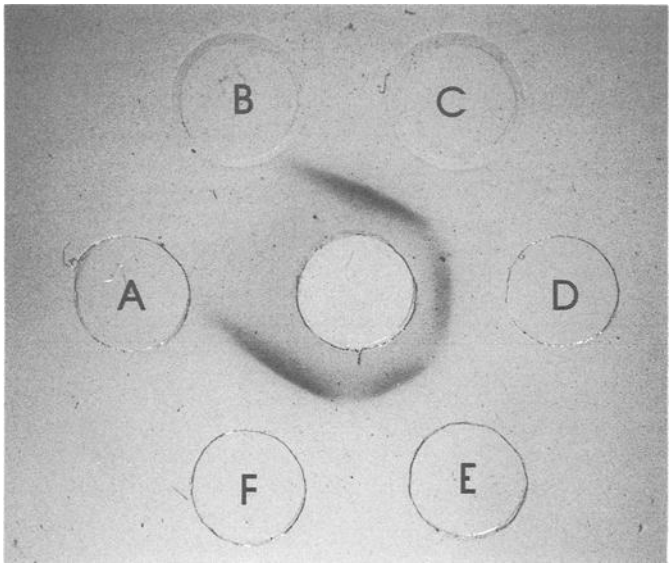

FIG. 10—*Double-diffusion Ouchterlony plate with rabbit anti-serum to HCG in center well with 10 μL of 1:5 dilution of stock antibody surrounded by* (a,b) *blanks of deionized water,* (c,f) *10 μL of 500 IU/mL of standard HCG, and* (d,e) *10 μL of two separate case submissions of HCG, each prepared to be 500 IU/mL.*

Acknowledgment

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