of a crystalline product, m.p. 162–163°, which did not depress the melting point of the product isolated from the sapogenin mixture of fenugreek seeds. The infrared spectra were identical in all details. Elution with 2% methanol in benzene recovered most of the unchanged diosgenin.

Diosgenin Methanesulfonate.—This was prepared by treatment of a solution of diosgenin (0.34 Gm.) in pyridine (5 ml.) with methanesulfonyl chloride (2 ml.) at room temperature for 18 hours. Working up in the usual manner afforded 0.22 Gm. of the desired product which, after repeated crystallization from methanol, gave a pure sample, m.p. 162–163°, $[\alpha]_D$ − 108°.

Anal.—Calcd. for $C_{28}H_{44}O_5S$: C, 68.30; H, 8.94. Found: C, 68.27; H, 9.00.

A solution of this substance (0.1 Gm.) in pyridine (3 ml.) was refluxed for 10 hours. After dilution with water and isolation of the product, chromatography on a column of 4 Gm. of alumina gave a fraction (30 mg.) recovered with light petroleum. This was repeatedly crystallized from light petroleum-methanol to give a pure sample of 25D-spirosta-3,5-diene, melting point and mixed m.p. 160–162°, which was identical in all respects with the preparations described previously.

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Study of the Polyvinyl Alcohol-Borate-Iodine Complex III. Detection of Borates in Urine

By ANTHONY J. MONTE-BOVI, JOHN J. SCIARRA, and VINCENT DE PAUL LYNCH

A survey of the prevalence of boric acid toxicity indicates the difficulty encountered in detecting cases of poisoning by this commonly used substance. Because of this situation, an investigation was carried out utilizing a previously reported method for the detection of borates by the polyvinyl alcohol-borate-iodine reaction. The test was extended in this study to permit the detection of boric acid in the urine of poisoned animals. Rabbits were fed varying quantities of boric acid, and urine samples were collected. After concentration of the urine sample, the polyvinyl alcohol-iodine reagent was applied to the residue. Results of both *in vitro* and *in vivo* studies indicate that this method is capable of detecting as little as 0.3 mg. of boric acid (0.05 mg. B).

THE CHARACTERISTIC blue produced when solutions of polyvinyl alcohol (PVA) and iodine were brought into contact with boric acid has been reported in several diverse areas (1–5). Since most of these applications involved macroquantities of boric acid, there was need to determine the suitability of this method for the detection of smaller quantities and to extend the investigation further for the purpose of determining the possibility of utilizing this test for the detection of boric acid in animal fluids and tissues.

That such a test is desirable becomes apparent when one surveys the literature concerned with case histories of poisoning by boric acid. As early as 1904 (6) and as recently as March 1963 (7), attention was called to the toxic nature of boric acid. In a previous study, Sciarra (8) summarized the prevalence of poisonings due to boron compounds. Most cases reported in the literature involved children ranging in age from a few weeks to 12 years. In many of these instances it was noted that several days elapsed between the time of admission to the hospital and the time at which boric acid poisoning was first suspected. This problem is further complicated by the fact that the afflicted children usually were not given medical attention during the critical early stages of poisoning—apparently because the toxicity symptoms are not characteristic.

Many investigators have called attention to this problem. McNally and Rukstinat (9) commented on 58 cases, 28 of which proved fatal. Pfeiffer (10) revealed 86 cases, of which 42 were fatal. Goldbloom and Goldbloom (11) reported on 109 poisonings by boric acid. Valdes-Dapena and Arey (12) analyzed 175 cases, of which 86 resulted in death. It may be that there is some duplication in reporting these cases. Nevertheless, the records clearly indicate the prevalence of boric acid poisoning; and in spite of the many articles and warnings relative to the toxic nature of the boron compounds, poisoning continues. As late as 1962 (12) and 1963 (13, 18), accidental deaths due to the erroneous use of these compounds have been reported. It should also be noted that many of these investigators mention the probability that many such cases of poisoning go undetected, since the symptoms produced may be confused with those produced by other illnesses. This is especially true in cases of chronic toxicity; for acute poisoning usually demands rapid medical treatment which, while it may not be specific for boron compounds, will at least counteract the poison and prevent further damage to the victim. The problem is complicated further because, through the ingestion of certain foods boron can be found in the blood. A range of 0–0.72 mg./100 ml. of blood was reported (12). In addition, the expanding use of boron industrially

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TABLE I.—DETECTION OF BORIC ACID ADDED TO AQUEOUS AND HUMAN URINE SOLUTIONS

Sample	H_3BO_3, mg.	B, mg.	Results[a] Aqueous Soln.	Results[a] Urine Soln.
1	0	0	−	−
2	50.0	8.75	++	++
3	25.0	4.40	++	++
4	12.5	2.20	++	++
5	6.3	1.10	++	++
6	3.2	0.56	++	++
7	2.5	0.44	++	++
8	1.3	0.23	+	+
9	0.6	0.11	+	+
10	0.3	0.05	+	+
11	0.15	0.03	*	*
12	0.03	0.005	−	−

[a] −, Negative test, as shown by the formation of rose-brown to yellow-brown; ++, strongly positive test, as shown by the formation of distinct blue to blue-black; +, weakly positive test, as shown by the formation of faint blue-red solutions leaving a distinctly blue ring around the edges of the dish as it stands for 10–15 minutes; *, inconclusive test, as shown by barely perceptible blue-red.

and in household products has increased the possibility of occupational poisoning by such substances (15).

Many tests have been developed for the purpose of detecting such boron compounds. The commonly known turmeric paper test has been unreliable (1) and unless carefully controlled will give false or confusing results. Smith *et al.* (15) report good results by using carminic acid to produce a color and measuring its intensity in a DU spectrophotometer. Draize and Kelley (16) also used a carminic acid reagent for a series of studies and reported good results.

Since the previously mentioned PVA-borate-iodine reaction gave reliable, accurate, and reproducible results on a macroscale, it was decided to investigate the possibility of adapting this test to the detection of lesser quantities of boric acid in both *in vitro* and *in vivo* conditions. The problems associated with the methods of detecting boron compounds in body fluids have already been reported (14–17).

EXPERIMENTAL

Detection of Boric Acid in Aqueous Solution.—A solution containing 50 mg. of boric acid U.S.P. in 10 ml. of water was prepared. Varying quantities of this solution were diluted as necessary, then placed into small porcelain casseroles in such volumes to yield a range of boric acid from 0.03 to 50 mg. as shown in Table I. A few drops of a 5% sodium hydroxide solution was added to render the solution slightly alkaline to litmus paper. This procedure decreases the volatility of boric acid by converting it to sodium borate. The resulting solution is then evaporated to dryness over a steam bath and re-acidified with 3–4 drops of hydrochloric acid to make the solution slightly acid to litmus; 3–4 drops of a 2% PVA solution[1] (51–05 or equivalent) and 1 drop of approximately 0.1 *N* iodine solution are added. A positive test is indicated by the appearance of a blue-black or blue color, the intensity of which depends upon the concentration of boric acid. A negative test produces a yellow, yellow-brown, or red-brown color. The results obtained are shown in Table I and represent the average of five determinations for each concentration of boric acid.

Detection of Boric Acid in Human Urine.—The above procedure was then adapted to detect the presence of boric acid in human urine and to determine whether the normal constituents found in human urine would interfere with the test and possibly lead to false results.

Samples of human urine were collected and tested for the presence of boric acid utilizing the procedure described under *Detection of Boric Acid in Aqueous Solution.* All samples gave negative results. Other samples of this same urine were then mixed with varying amounts of a solution containing 50 mg. of boric acid U.S.P. in 10 ml. of urine and diluted with urine if necessary to give the concentrations of boric acid as shown in Table I. The urine samples were then treated as for the aqueous solutions. The results are also shown in Table I. From this table, it can be seen that as little as 0.3 mg. of H_3BO_3 (0.05 mg. B) can be detected by this method.

Detection of Boric Acid in Animal Urine.—Since the addition of boric acid to voided human urine indicated the absence of naturally occurring substances which might interfere with the test, further investigation of its applicability to the detection of boron compounds in animal tissues and fluids was carried out.

Rabbits were chosen as the test animals for this study, mainly because of their ease of handling and because other investigators have used these animals for similar studies.

Initially, samples of urine were obtained by catheterization from normal animals maintained on similar routine diets. These samples were evaporated and treated as previously described under *Detection of Boric Acid in Human Urine.* All tests performed on these samples were negative and demonstrated that normal rabbit urine did not contain a substance which might interfere with this test.

Test animals were then given boric acid solutions by the oral and i. v. routes in concentrations as shown in Table II. Urine samples were collected by

TABLE II.—DETECTION OF BORIC ACID IN URINE OF RABBITS GIVEN BORIC ACID[a]

Dose of H_3BO_3 mg./Kg. of Body Wt.	Route of Administration	Time Period over which Urine was Collected, Hr.	Results[c]
50	I.V.	2	+
100	I.V.	1.5	++
50	oral	5	+
75	oral	5	+
110	oral	3	+
110	oral	4.5	+
150	oral	3	++
150	oral	4.5	+
215	oral	4	++
300	oral	4	++
300	oral	6	+
440	oral	4	++
570	oral	5	++
750	oral	5	++
1000[b]	oral	3	++

[a] Table II represents a minimum of three specimens at each dose level. [b] Lethal dose. [c] ++, Indicates a strongly positive test, as shown by the formation of distinct blue or blue-black; +, indicates a weakly positive test, as shown by the formation of faint blue-red solutions leaving a distinctly colored ring around the edges of the dish as it stands for 10–15 minutes; −, negative test would show as a rose-brown to yellow-brown.

[1] Marketed as Elvanol by E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.

catheterization. As shown in Table II, positive tests were obtained with the PVA-iodine reagent.

By a series of experiments which followed, rabbits were fed boric acid solutions of various concentrations (as shown in Table II) *via* a stomach tube. Samples of urine were collected by catheterization and tested for boron content. In all instances, control samples were collected from untreated animals. The results from these tests are shown in Table II.

DISCUSSION

As can be seen from Table I, as little as 0.3 mg. of H_3BO_3 (0.05 mg. B) can be detected in both aqueous and urine solutions with the PVA-borate-iodine reaction. The constituents normally found in urine did not interfere with the test, and quantities of boric acid above 0.3 mg. could easily be detected.

Starch, as an environmental contaminant, will give a positive test; therefore, its presence, if suspected, must be ruled out as suggested in a previous paper by Monte-Bovi and Sciarra (3).

The results of the PVA-iodine test when applied to samples of urine collected from animals treated or fed boric acid indicate the suitability of this test for these purposes. While we did not repeat these experiments on humans for obvious reasons, others (17, 19) have cited that boron compounds when absorbed by humans are found in body fluids and tissues.

While there are several other tests available for the detection of small quantities of boric acid as cited in the literature, the PVA-borate-iodine complex offers another accurate and sensitive method. This test can also be used as a confirmatory test when other methods give inconclusive results.

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Chromatographic Method for the Simultaneous Determination of Aspirin, Caffeine, and Acetaminophen

By K. THOMAS KOSHY

A procedure for the quantitative determination of aspirin, caffeine, and acetaminophen in a mixture is described using a modified chromatographic technique developed by Levine. In addition, this procedure can be used for the estimation of salicylic acid, if present in the mixture.

A REMARKABLE chromatographic technique for the analysis of mixtures of aspirin, caffeine, and phenacetin was developed by Levine (1) using a duplex column having aqueous solutions of sodium bicarbonate and sulfuric acid as immobile phases on a Celite support. The technique was extended by Heuermann and Levine (2) to the analysis of more complex mixtures. In principle, this technique is adaptable to the separation of acidic, basic, and neutral components in a mixture, provided suitable solvents are available. Since there are many formulations containing aspirin, caffeine, and acetaminophen (APAP), the purpose of this study was to determine these constituents in such a mixture. The study was further extended to the determination of *p*-aminophenol and salicylic acid in the presence of these components. The latter two are hydrolytic degradation products of APAP and aspirin, respectively.

EXPERIMENTAL

Preparation of Chromatographic Column.—Commercial acid-washed kieselguhr (Celite 545 Johns-Manville Corp.) was used in this study. It contained impurities which interfered in the analysis of *p*-aminophenol and was purified by boiling for 1 hour with concentrated hydrochloric acid, washing with water, and drying at 100°. The column was prepared as described by Heuermann and Levine (2). However, column arrangement was reversed. The column containing sulfuric acid as the immobile phase (column A) was mounted in such a manner that the effluent would flow into the column containing sodium bicarbonate as the immobile phase (column B). This was done to avoid contact of *p*-aminophenol with the sodium bicarbonate column since it was observed that this compound was not stable in the presence of bases.

Procedure.—Water-washed solvents were used throughout the chromatographic separation of the constituents. The sample size could be varied within wide limits depending on the composition of the material to be analyzed. Ethyl acetate was used as solvent for the sample. Chloroform could be used, provided the sample was first dissolved in a few milliliters of alcohol. However, excess alcohol should be avoided, as this would remove water from the column.

The columns mounted as described above were washed with 50 ml. of ethyl ether. The accurately

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