(5) Blanton, C. D., and Nobles, W. L., *J. Pharm. Sci.*, 51, 878(1962).
(6) Nobles, W. L., Dissertation, University of Kansas, Lawrence, Kan., 1952, p. 42.
(7) Chaikin, S. W., and Brown, W. G., *J. Am. Chem. Soc.*, 71, 122(1949).
(8) Nobles, W. L., Dissertation, University of Kansas, Lawrence, Kan., 1952, p. 86.

Estimation of Amphetamine in Urine of Race Horses

By M. S. KARAWYA, M. A. EL-KIEY, S. K. WAHBA, and A. R. KOZMAN

Separation of amphetamine from urine of doped horses was achieved on anion-exchange resin (Na^+) and Silica Gel G plates. A micro method for the estimation of the free base was performed by measuring the violet color developed after nitration of the amphetamine eluted from either an ion-exchange resin column or Silica Gel G plates.

In Egypt in 1961, the menace of doping with amphetamine hovered over the horseraces and seriously threatened the results of the race. Since then, the race authorities have decided to stand against this new doping invader.

The method of analysis of amphetamine adopted by the B.P. (1) depends on steam distillation, while that of the E.P. (2) makes use of the direct extraction with ether.

The biological fluids contain normally some steam volatile and ether extractable bases (3) which may interfere in the determination of amphetamine. This fact renders the official methods not applicable for the separation and determination of amphetamine in urine of doped animals.

In an attempt to eliminate these interfering substances, ion-exchange and thin-layer chromatographic techniques were adopted.

Tampsett (4) was able to elute amphetamine on a cationic ion-exchange resin[1] column. He used 400 ml. of 8.0 *N* hydrochloric acid to achieve complete recovery. However, on trying this method, the eluate was found to be highly colored, and the colorimetric assay of the eluted amphetamine was almost impossible.

Materials—The materials used in this experiment were horse urine containing amphetamine (100 mcg./100 ml.); a column of ion-exchange resin[2] (Na^+) (1 × 15 cm.); and eluents used in the following succession: (*a*) 0.5% ammonium hydroxide, (*b*) acetone–water (3:7), (*c*) distilled water, (*d*) 10% aqueous solution of potassium chloride–1 *N* hydrochloric acid (1:1).

EXPERIMENTAL

Separation of Amphetamine on Ion-Exchange Resin (Na^+)

In the present work an anion-exchange resin[2] (Na^+) was chosen for the quantitative elution of amphetamine from horse urine.

Procedure—Add the urine sample to the resin column. Remove the impurities by washing successively with 50 ml. of each of the above-mentioned eluents (*a*), (*b*), and (*c*), and then elute the amphetamine with 150 ml. of eluent (*d*). Render the eluate alkaline with ammonium hydroxide T.S., extract the free base with chloroform (4 × 25 ml.), evaporate the chloroform, and keep the residue for the colorimetric estimation as described later. The results in Table I show the following observations.

A—The successive washing of the column with 0.5% ammonium hydroxide, acetone–water (3:7), and distilled water achieves the removal of most of the urine pigments and other impurities but not amphetamine.

B—Complete recovery of amphetamine is effected by washing the ion-exchange resin column with 150 ml. of 10% potassium chloride–1 *N* hydrochloric acid (1:1).

Separation of Amphetamine on Silica Gel G Plates

Separation of amphetamine from the saliva on silica gel plates was performed by Baeumler *et al.* (5) using triethanolamine–acetone–methanol (0.03:1:1). However, it was found difficult to separate amphetamine from urine on adopting the same procedure.

Other systems were tried and the system *n*-butanol–glacial acetic acid–water (4:1:5) proved to effect good separation of amphetamine from urine pigments, R_f values being 0.65 and 0.95, respectively.

Amphetamine is located with bromocresol blue T.S. when a blue color is obtained.

Procedure—Amphetamine is first extracted from urine before its application on the plate as follows.

Table I—Recovery of Amphetamine from Horse Urine by Columns of Ion-Exchange Resin (Na^+)

Fraction of Effluent	100 mcg./100 ml. Urine: Ion-Exchange Resin	100 mcg./100 ml. Urine: Color of Effluent
Eluent (*a*) (50 ml.)	...	Yellow
Eluent (*b*) (50 ml.)	...	Yellow
Eluent (*c*) (50 ml.)	...	Yellow
Eluent (*d*) (50 ml.)	58.20	Colorless
Eluent (*d*) (50 ml.)	30.15	Colorless
Eluent (*d*) (50 ml.)	13.20	Colorless
8 *N* HCl (400 ml.)	...	Colorless
Total	101.55	

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[1] Dowex-50, Dow Chemical Co., Midland, Mich.
[2] Amberlite IRC-50, Rohm & Haas, Philadelphia, Pa.

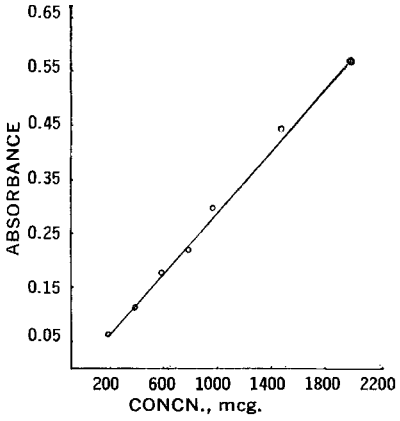

Fig. 1—Absorbance/concentration curve of amphetamine.

Introduce 50 ml. of urine to a separator and render it alkaline to litmus by the gradual addition of ammonium hydroxide T.S. solution. Extract the liberated base with chloroform (4 × 20 ml.), washing each portion with the same 5 ml. of water. Dehydrate the combined chloroformic extract with anhydrous sodium sulfate, washing the sodium sulfate with fresh chloroform and add the washing to the mother extract. Adjust the combined chloroformic extract to a suitable known volume according to the concentration of amphetamine. Another sample of urine free of amphetamine was treated and extracted as described above to be used as a blank.

By means of an Agla micrometer syringe apply the chloroform extracts of both experiment and blank on the starting line of a Silica Gel G plate, 2 cm. apart. Apply on each side of the starting line a pilot spot of a chloroformic solution of authentic amphetamine. The amphetamine content of any spot lies between 50–250 mcg. Insert the plate in the chromatographic jar and develop by the organic layer of the solvent system. In order to locate the amphetamine, spray the two peripheral pilot spots with bromocresol T.S. after covering the central part of the plate with a sheet of glass. Scrape the nonsprayed areas enclosed between and at the level of the located pilot spots with a thin spatula. Collect the powder obtained from each area on a glazed paper and transfer it quantitatively to a micro column (0.4 × 10 cm.). Wash each column with 10 ml. of ethanol.

Evaporate the ethanol cautiously, and keep the residues corresponding to experiment and blank for the colorimetric estimation.

Colorimetric Determination of the Eluted Amphetamine from Resin Columns and Silica Gel Plates

The nitration test (6) is used as the basis of the colorimetric estimation of amphetamine after making the following modification.

A 0.5% amount of potassium hydroxide in methanol–acetone (97:3) is used instead of 10% aqueous sodium hydroxide which does not allow stability of color. The procedure is run as follows.

To the residue in a test tube, add 0.1 Gm. of potassium nitrate and 2 ml. of sulfuric acid. Heat in a boiling water bath for 15 min., then cool. Transfer the liquid quantitatively with distilled water to a separator, wash the liquid with chloroform, reject the chloroform, make the aqueous solution alkaline with the gradual addition of ammonium hydroxide, and extract with chloroform. Evaporate the chloroformic extract in an evaporating dish and dissolve the residue in 10 ml. of acetone. Transfer the acetone solution to a test tube, add 2 drops of 0.5% potassium hydroxide in methanol–acetone (97:3), and allow to stand for 10 min. Measure the developed purple color in a Unicam SP 1300 colorimeter using No. 1 filter (370–515 mμ) against the blank experiment. The results are deduced from an absorbance/concentration curve (Fig. 1), from which it is clear that the color obeys Beer's law in the range of 50–250 mcg. of amphetamine.

The color reaches its maximum after 10 min. and remains stable for another 15 min.

The colorimetric method was applied to residues obtained from both ion-exchange column and chromatoplates using urine samples containing variable amounts of amphetamine. The results are shown in Table II.

From Table II, it is clear that amphetamine can be recovered quantitatively from either the ion-exchange resin (Na^+) column or Silica Gel G plate, with an error of +3.6 to +5.0 and −0.8 to +2.5%, respectively.

Tracing of Amphetamine in Urine of Doped Horses

In order to trace amphetamine in urine of doped

Table II—Recovery of Amphetamine from Ion-Exchange Column and Silica Plates

Quantity Added, mcg.	Quantity Recovered from Column	Error, %	Quantity Recovered from TLC	Error, %
100	105	+5.0	100	0.0
150	155	+3.6	152	+1.3
200	210	+5.0	205	+2.5
250	260	+4.0	248	−0.8

Table III—Excretion Rate of Amphetamine in Horse Urine

Time of Collection of Urine	Mean Vol. of Collected Urine, ml.	% with Respect to Injected Dose, 100 mg. 1st Horse	2nd Horse	3rd Horse	Mean
24 hr. before drugging	3000	..	..	..	...
3 hr. after drugging	300	16	18	17	17.0
12 hr. after drugging	400	14	15	12	13.6
24 hr. after drugging	1350	18	17	19	18.0
48 hr. after drugging	2300	6	7	8	7.0
72 hr. after drugging	3400	..	..	..	...
Total		54	57	56	55.6

horses three horses weighing between 250 and 300 Kg. were chosen. The animals were kept in a clean stable under veterinary medical observation[3] for 3 days before the administration of the drug and were examined constantly to ascertain their normal state of health. During the 24 hr. prior to administration of amphetamine, urine was collected from the three horses in leather bags tied under their bodies. Each animal was then injected subcutaneously with 100 mg. amphetamine sulfate. The urine of each animal was collected periodically after 3, 12, 24, 48, and 72 hr. by means of a sterile catheter. In addition, a clean leather bag was tied under each horse to collect any urine which might pass off between the specified collection times. The urine samples were pale yellow, turbid, and possessed a pH 5.9–6.3.

The urine samples were made alkaline with ammonium hydroxide T.S., shaken with chloroform, the extract chromatographed by TLC, and the separated amphetamine was colorimetrically assayed as described above.

The urine collected before the administration of amphetamine was used as a blank. The results are shown in Table III, from which it is clear that about 55.6% of the administered amphetamine was excreted in the urine of horses within 48 hr. after the administration of amphetamine. During this period, amphetamine could be detected by thin-layer chromatography; after 48 hr. it was hardly detectable and had almost disappeared after 72 hr.

[3] The authors thank Dr. A. Hanna, Faculty of Veterinary Medicine, Cairo University, for his valuable help.

REFERENCES

(1) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1963, p. 43.
(2) "Egyptian Pharmacopoeia," Cairo University, Cairo, Egypt, U. A. R., 1953, p. 126.
(3) "Clinical Biochemistry," 6th ed., W. B. Saunders and Co., London, England, 1962, pp. 383–394.
(4) Tompsett, S. L., *Acta Pharmacol. Toxicol.*, **17**, 295 (1960).
(5) Baeumler, J., Brault, A. L., and Obersteg, J. I., *Schweiz. Arch. Tierheilk.*, **106**, 346(1964).
(6) Rathenasinkam, E., *Analyst*, **76**, 155(1951).

Antibacterial Activity of Some Substituted Benzofurans. Preliminary Study of Structure Activity Relationship

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Sensitivity of three substituted nitrobenzofurans, 4-methyl-6-phenylbenzofuran, 2(*p*-nitrophenyl)benzofuran, and 2-aceto-5-methyl-7-nitrophenylbenzofuran, against *Staphylococci, Pyocyanea,* and *B. coli* was studied. A possible structure activity relationship has been worked out, which suggested that the sensitivity of benzofurans is due to nitro substitution. Sensitivity is reduced by substitution of aceto and methyl groups.

NITROFURANS have been widely studied for their antibacterial activity (1–3). A series of substituted benzofurans and nitrobenzofurans were synthesized and tested for their antibacterial activity.

The reaction of *p*-nitrobenzyl bromide with an *O*-hydroxy carbonyl compound in presence of anhydrous potassium carbonate in methanol yields a 2-(*p*-nitrophenyl) benzofuran (4, 5). This reaction was utilized to prepare a few benzofurans required for pharmacological studies.

The reaction of benzyl bromide with hydroxymethyl propiophenone and *p*-nitrobenzyl bromide with 2-hydroxypropiophenone and 2-hydroxy-3-aceto-6-methylpropiophenone in methanol, in the presence of anhydrous potassium carbonate from 45 min. to 1 hr., furnishes the respective benzyloxy and *p*-nitrobenzyloxy derivatives. When allowed to react further with anhydrous potassium carbonate in methanol under reflux (time varying from 6–9 hr.), they yield the respective benzofurans—*viz.*, 4-methyl-6-phenylbenzofuran (a pasty substance) (I), 2-(*p*-nitrophenyl) benzofuran (m.p. 193°) (II), and 2-aceto-5-methyl-7-nitrophenylbenzofuran (m.p. 68°) (III). All the three compounds are completely soluble in alcohol, acetone, and ether.

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EXPERIMENTAL

Aqueous suspensions of these compounds were prepared in 1% polysorbate 80,[1] the wetting agent being used to compensate for low aqueous solubility. Control experiments were also performed with the same solvent.

The bactericidal property of the test agents (I, II, and III) was studied against *Staphylococci, Pyocyanea,* using nutrient agar media and *B. coli* on MacConkey's agar media, by the use of impregnated filter paper disks to determine the bacterial sensitivity for the compounds under study.

The disks were soaked in the solutions, the activity of which was to be tested, placed on the media, and incubated overnight at 37°. The diameters of the circular areas of inhibited growth were observed and percentage of inhibition was calculated.

RESULTS AND DISCUSSION

The results of these observations are presented in Table I. From the results it is evident that compound II is the most active against all three test

[1] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.