g. (63.8%); m.p. 210–212° (dec.). For analysis, the product was recrystallized from 90% aqueous methanol with light charcoaling, and again from 80% aqueous methanol; m.p. 212–213°; $\lambda_{max.\ (m\mu)}^{pH\ 1}$ 257 (ε 15,200), $\lambda_{max.\ (m\mu)}^{pH\ 13}$ 260 (ε 15,200), $\lambda_{max.\ (m\mu)}^{H_2O}$ 259 (ε 15,000); $\bar{\nu}_{max.(cm.^{-1})}^{KBr}$ 3,400–3,100 (broad OH, NH), 1,610, 1,575 (C=C and C=N); $[\alpha]_D^{20}$ −53° (c 1.01 H_2O).

Anal.—Calcd. for $C_{11}H_{15}N_5O_4$: C, 46.97; H, 5.38; N, 24.90. Found: C, 46.84; H, 5.36; N, 25.50.

REFERENCES

(1) Prokop, J., and Murray, D. H., *J. Pharm. Sci.*, **54**, 359(1965).
(2) Murray, D. H., and Prokop, J., *ibid.*, **54**, 1468(1965).
(3) *Ibid.*, **56**, 865(1967).
(4) *Ibid.*, **54**, 1637(1965).
(5) Baker, B. R., in "Chemistry and Biology of Purines," Ciba Foundation Symposium, Little, Brown, Boston, Mass., 1957, p. 120.
(6) Montgomery, J. A., and Thomas, H. J., *Adv. Carb. Chem.*, **17**, 301(1962).
(7) Rembarz, G., *J. Prakt. Chem.*, **19**, 315(1963); through *Chem. Abstr.*, **60**, 1825g(1964).

Keyphrases

9-(3-Deoxyaldofuranosyl) adenines—synthesis
IR spectrophotometry—structure
Optical rotation—identity

Determination of Caffeine in Plasma by Gas Chromatography

By F. L. GRAB* and J. A. REINSTEIN†

A gas chromatographic method has been developed for the rapid, precise, and specific determination of caffeine in plasma. The method overcomes the major drawbacks of previous methods for the determination of caffeine in body fluids, which were: difficult isolation from interfering materials, a substantial blank error and low sensitivity. A standard response curve relating the signal-height ratio of caffeine to that of an internal standard, hexobarbital, permits quantitation of the amount of caffeine present. The method involves extraction of caffeine from plasma with chloroform, after the aqueous phase was adjusted to pH 11.5–12.0. The chloroform extract was evaporated to dryness and the sample was redissolved in carbon disulfide. Two milliliters of plasma was used and caffeine was determined at a concentration of 0.25 mcg./ml.

CAFFEINE, due to its widespread occurrence in beverages, is probably the drug consumed most today. There is, however, no rapid and sensitive method available for measuring this drug in biological fluids. Connors (1) reviewed many methods for the detection of xanthines, but these were generally not specific for caffeine. The older methods involved extraction of caffeine followed by a gravimetric or volumetric assay (4). These were succeeded by the colorimetric murexide reaction of Tanaka and Ohkubo (5). In addition a number of reagents that form colors have been employed by the authors and by others in an attempt to develop more specific tests for the xanthines. These were used in conjunction with paper (1–3, 13) and thin-layer chromatography (17). The reagents include mercuric chloride, potassium ferrocyanide, mercuric acetate, Dragendorff's reagent, and an alkaline phosphotungstate reagent. Visualization of the spots has also been achieved under UV light at 254 mμ using fluorescent thin-layer plates (10, 12, 17). Other methods include argentimetric (2), iodometric (1), solvent extraction (2, 8), ion exchange (2, 12), nonaqueous titrations (1), Kjeldahl (1), and spectrophotometric (2, 6–9, 11, 14, 15) methods. These have proved successful in measuring qualitatively, and in some cases

Received May 22, 1968, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122
Accepted for publication June 18, 1968.
Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Abstracted from a dissertation presented to the Graduate School of the University of California, San Francisco Medical Center, by Frederick L. Grab in partial fulfillment of Doctor of Philosophy degree requirements.
This investigation was supported by Public Health Service training grant No. 5 TI GM 0728 from the Research Training Grants Branch, National Institute of General Medical Sciences, Bethesda, Md.
The authors wish to express their appreciation to Dr. Malcolm Rowland for his valuable discussion, criticism, and recommendations. Thanks also to Miss Lotte Kuhn for drawing blood samples and Mr. Howard Darvey for his general assistance. Hexobarbital was supplied by Winthrop Laboratories.
* American Foundation for Pharmaceutical Education Fellow 1964–1968. Pharmaceutical Manufacturers Association Fellow 1967–1968.
† Present address: Syntex, S.A., Mexico 10, D. F., Mexico.

quantitatively, pure xanthines. They were used in detecting mixtures of some xanthines and xanthines mixed with a number of other drugs.

Previously, the analytical method of choice in determining caffeine in biological systems has been spectrophotometry. The original development was made by Ishler *et al.* (6) and since then most of the determinations of caffeine in blood, tissue, and urine were based on this approach (2, 7–9, 21–23). However, these methods do not possess the sensitivity or specificity required for estimating concentrations of this drug produced by the ingestion of normal amounts of caffeine-containing beverages (2, 8, 21, 23). The authors have attempted to use a spectrophotometric assay but meaningful results could not be obtained at the concentration range of interest. The blank values were too high (relative to caffeine blood levels) and variable.

Gas chromatographic assays have been reported for many drugs in biological fluids (16), and xanthines have been examined in a number of GC systems (18, 19, 24–29). Such systems combine separation and quantitation of components and appear to offer distinct advantages over other methods of analysis.

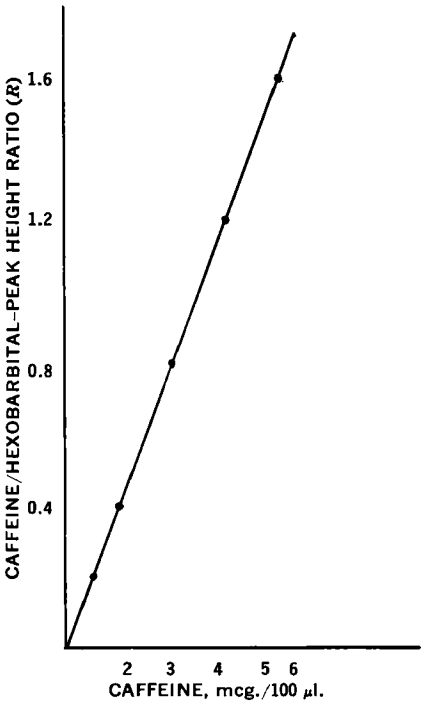

Fig. 2—Standard gas chromatogram response curve caffeine; internal marker, hexobarbital.

EXPERIMENTAL

Equipment—A Varian Aerograph series 1200 gas chromatograph with a flame-ionization detector and a Varian model 20 recorder set at 20 in. $hr.^{-1}$ was used. The column employed was a 1.9 m. (6 ft.) stainless steel helix, 0.32 cm. ($^1/_8$ in.) o.d. packed with 3% OV 17 on Chromosorb W 100–120 mesh AW/-DMCS H.P. (Applied Science lot 4223). The N_2 gas flow rate was 30 ml./min. Operating temperatures were: oven, 200°; injection port, 260°; and detector, 260°. Attenuations from 0.1 × 8 to 0.1 × 64 were used.

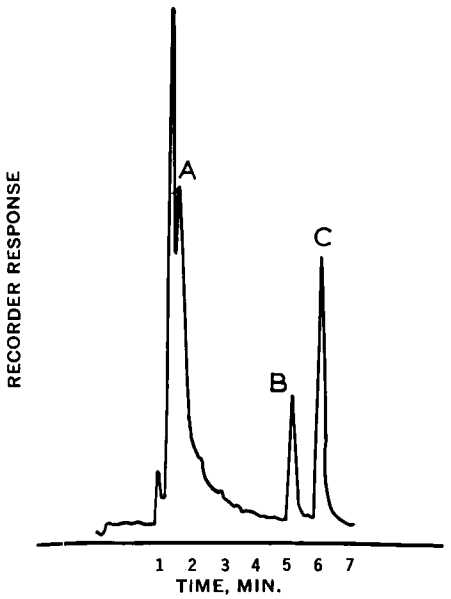

Fig. 1—Typical chromatogram. Key: A, carbon disulfide; B, hexobarbital (R_t = *4.3); C, caffeine* (R_t = *5.2). Column 3% OV 17.*

Reagents—(*a*) Caffeine (Eastman Organic) purified by vacuum sublimation; (*b*) hexobarbital (Winthrop), the internal standard; (*c*) reagent grade carbon disulfide (MCB) further purified by distillation; (*d*) heparin solution, 1,000 units/ml. (free of preservatives); (*e*) 2.5 *N* sodium hydroxide (Fisher Certified Reagent); (*f*) technical grade chloroform (Baker) freshly distilled.

Standard Curve—Standard curves were obtained by preparing solutions of caffeine 0.75–6.0 mcg./100 µl. and hexobarbital 2.0 mcg./100 µl. in carbon disulfide (20). Both caffeine and hexobarbital give sharp peaks with some slight tailing (Fig. 1). The standard curve given by the peak-height ratio of caffeine/hexobarbital *versus* caffeine concentration was linear and reproducible (Fig. 2).

Preparation of Plasma Samples—A 5-ml. sample of blood was taken, added to 2 drops of heparin, and spun down in a centrifuge (IEC International Clinical Centrifuge) for 10 min. at 2500–3000 r.p.m. Two milliliters of plasma was pipeted into a 25-ml. round-bottom screw-cap Pyrex tube with a Teflon-lined cap. The pH was adjusted to 11.5–12.0 with 2.5 *N* sodium hydroxide. To this 10 ml. of chloroform was added. The tubes were then agitated for 15 min. and then centrifuged (IEC International Centrifuge series 2) for 10 min. at 1,000 r.p.m. Using a 10-ml. hypodermic syringe with a 10-cm. (4 in.) cannula, 75% of the chloroform layer was withdrawn and placed into a conical ground-glass

stoppered 15-ml. centrifuge tube. The chloroform extract was then evaporated to dryness in a 60–80° water bath, mounted on a steam cone. Bumping was prevented by placing an antibumping chip, previously boiled in carbon disulfide, into the tube, just prior to driving off the chloroform. One milliliter of hexobarbital solution (2 mcg./ml.) in carbon disulfide was pipeted into the tube. This solution was then concentrated to approximately 100 µl. by heating in the water bath at 40–60°. A 2-µl. sample of this concentrate was then injected onto the column.

RESULTS AND DISCUSSION

Prior to using the present assay to determine plasma levels of caffeine after the ingestion of a cup of coffee, attempts were made to measure the concentration of a known and unknown amount of caffeine in plasma. An accurately measured amount of caffeine was added to two plasma samples, one containing an unknown amount of caffeine and the other containing no caffeine. The plasma samples were treated as described under *Experimental*. A linear plot was obtained for both plasma samples when the caffeine/hexobarbital-peak height ratio was plotted against caffeine concentration (Fig. 3). Theoretically both graphs should be parallel, differing only in the intercept with the y axis. This was the case. The intercept was equal to the concentration of caffeine present in the plasma samples before the additions were made. The sample which contained no caffeine originally passes through the origin, while the other intercepts the y axis at a peak-height ratio of 0.2. This corresponds to a caffeine concentration of 0.5 mcg./ml.

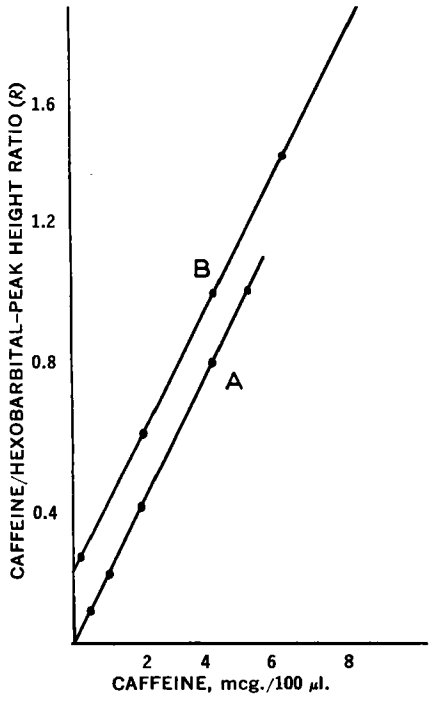

Fig. 3—Calibration curve of caffeine extracted from 2 ml. of plasma. Key: A, no caffeine present in original plasma sample; B, unknown amount of caffeine present in original plasma sample.

TABLE I—RECOVERY OF CAFFEINE ADDED TO PLASMA

Caffeine Added, mcg./ml.	Caffeine Found, mcg./ml.	Recovery, %
0.0	0.00	—
0.5	0.49	98
1.0	1.01	101
2.0	1.95	98
2.5	2.56	102

The standard curve (Fig. 2) can be compared to the calibration curve (Fig. 3A), since the two graphs share a common abscissa. The slope of the latter is 75% of the former. Taking into account that a 75% aliquot of the plasma extract was withdrawn, the values at each level of caffeine are identical for both curves. This indicates that the caffeine was quantitatively extracted (Table I).

The above procedure was repeated in five other subjects who had abstained from caffeine-containing beverages for 36 hr. Plasma samples were obtained and a calibration curve was determined for each. The curves passed through the origin and possessed the same slope. This indicates that the method is reliable and may be used for estimating caffeine plasma levels.

Absorption of Caffeine—Using the assay procedure, the absorption of caffeine, following the ingestion by three subjects of coffee containing 100 mg. of the drug, was studied. Caffeine was detected within 5 min. after ingestion. Peak plasma levels of 1.5–1.8 mcg./ml. were seen 50–75 min. after the dose. It was found that absorption could be described by a pseudo first-order process. The intersubject variation in the half-life of absorption was 8–17 min., while the intrasubject variation was much smaller. Apparent metabolic half-lives varied from 3.5–6 hr. in two subjects and from 6–10.5 in a third (*e.g.*, Fig. 4). Detailed data on the absorption and metabolism of caffeine in the body will be reported in a subsequent paper.

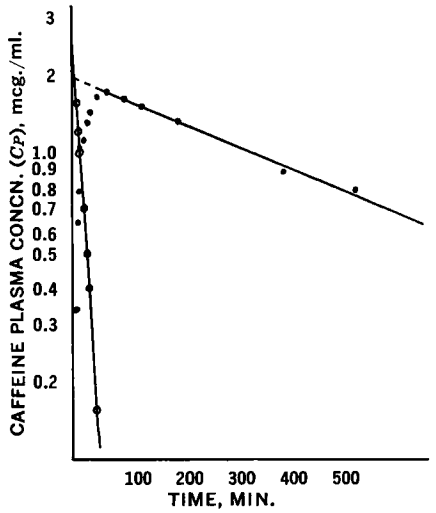

Fig. 4—Caffeine concentration after the ingestion of coffee containing 100 mg. of the drug. A graphical estimation of the metabolic and absorption (from the residual plot) half-lives are shown. Key: ●, observed data, $t_{1/2}^{m}$ = 6.0 hr.; ○, residual data, $t_{1/2}^{a}$ = 10.4 min.

SUMMARY

A gas chromatographic method has been developed for the determination of caffeine in plasma. A standard response curve relating signal-height ratios of caffeine to hexobarbital permits a quantitative estimation of the amount of caffeine present. The method is more rapid and sensitive than any previously reported. Plasma levels of caffeine after the ingestion of one cup of coffee have been studied and concentrations down to 0.25 mcg./ml. have been accurately determined.

REFERENCES

(1) Connors, K. A., "Pharmaceutical Analysis," Higuchi, T., and Brochmann-Hanssen, E., Eds., Interscience, New York, N. Y., 1961, pp. 240–249.
(2) Cornish, H. H., and Christman, A. A., *J. Biol. Chem.*, **228**, 315(1957).
(3) Abelson, D., and Borcherds, D., *Nature*, **179**, 1135 (1957).
(4) Salant, W., and Rieger, J. B., *U. S. Dept. Agr. Bur. Chem. Bull.*, **1912**, 157.
(5) Tanaka, U., and Ohkubo, Y., *J. Coll. Agr. Tokyo Imp. Univ.*, **14**, 153(1937).
(6) Ishler, N. H., Finucane, T. P., and Borker, E., *Anal. Chem.*, **20**, 1162(1948).
(7) Fisher, R. S., Algeri, E. J., and Walker, J. T., *J. Biol. Chem.*, **179**, 71(1949).
(8) Axelrod, J., and Reichenthal, J., *J. Pharmacol. Exptl. Therap.*, **107**, 519(1953).
(9) Sant'Ambrogio, G., Mognoni, P., and Ventrella, L., *Arch. Intern. Pharmacodyn.*, **150**, 259(1964).
(10) Holiday, E., and Johnson, E., *Nature*, **163**, 216(1949).
(11) Grab, F., and Reinstein, J., presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
(12) Weissmann, B., Bromberg, P., and Gutman, A., *Proc. Soc. Exptl. Biol. Med.*, **87**, 257(1954).
(13) Dikstein, S., Bergmann, F., and Chaimovitz, M., *J. Biol. Chem.*, **221**, 239(1956).
(14) Smith, R., and Rees, D., *Analyst*, **88**, 310(1963).
(15) Bower, R., Anderson, A., and Titus, R., *Anal. Chem.*, **22**, 1056(1950).
(16) Burchfield, H., and Storrs, E., "Biochemical Applications of Gas Chromatography," Academic Press, New York, N. Y., 1962.
(17) Stahl, E., "Thin Layer Chromatography," Academic Press, New York, N. Y., 1964.
(18) Elefant, M., Chafetz, L., and Talmage, J., *J. Pharm. Sci.*, **56**, 1181(1967).
(19) Svendsen, A., and Brochmann-Hanssen, E., *ibid.*, **51**, 414(1962).
(20) Rowland, M., and Riegelman, S., *Anal. Biochem.*, **20**, 463(1967).
(21) Goldstein, A., Warren, R., and Kaizer, S., *J. Pharmacol. Exptl. Therap.*, **149**, 156(1965).
(22) Colton, T., Gosselin, R., and Smith, R., *Clin. Pharmacol. Therap.*, **9**, 31(1968).
(23) Nash, H., "Alcohol and Caffeine," Charles C Thomas, Springfield, Ill., 1962.
(24) Lloyd, H., Fales, H., Highet, P., Vanden-Heuvel, W., and Wildman, W., *J. Am. Chem. Soc.*, **82**, 3791(1960).
(25) Massingill, J., and Hodkins, J., *Anal. Chem.*, **37**, 952 (1965).
(26) Parker, K., Fontan, C., and Kirk, P., *ibid.*, **85**, 356 (1963).
(27) Hoffman, A., and Mitchell, H., *J. Pharm. Sci.*, **52**, 305(1963).
(28) Brochmann-Hanssen, E., and Fontan, C., *J. Chromatog.*, **19**, 296(1965).
(29) Wisniewski, J., F & M Scientific Applications Lab. Rept. 1001, 1965.

Keyphrases

Caffeine in plasma–analysis
Plasma analysis–caffeine
GLC–analysis
Hexobarbital–internal standard

Oxidation of Organic Compounds with Xenon Trioxide I

Stoichiometric and Kinetic Studies Involving Cinnamyl Alcohol, Cinnamaldehyde, and Cinnamic Acid

By HAROLD J. RHODES, REGINA P. SHIAU, and MARTIN I. BLAKE

The stoichiometry involved in the xenon trioxide oxidation of *cis*- and *trans*-cinnamyl alcohol, *trans*-cinnamaldehyde, and *trans*-cinnamic acid to carbon dioxide and water was determined using an excess of xenon trioxide. The rate of oxidation of *trans*-cinnamaldehyde with xenon trioxide within the concentration range studied, was found to be first order with respect to xenon trioxide and zero order with respect to *trans*-cinnamaldehyde. The reaction rate is pH-dependent and inversely related to the ionic strength of phosphate buffer.

STUDIES INVOLVING the preparation and oxidizing properties of xenon trioxide in aqueous solution have been cited in an earlier paper (1), which described the determination and stoichiometry involved in the oxidation of certain primary, secondary, and tertiary aliphatic and aromatic alcohols.

The present investigation concerns the xenon

Received May 22, 1968, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

Accepted for publication July 23, 1968.

The authors gratefully acknowledge E. H. Appelman, Argonne National Laboratory, Argonne, Ill., for supplying the xenon trioxide and highly purified water used in this study.