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CAFFEINE IN PLASMA AND SALIVA BY A RADIOIMMUNOASSAY PROCEDURE

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ABSTRACT

COOK, C. E., C. RAY TALLENT, ELLEN W. AMERSON, MARY W. MYERS, JOHN A. KEPLER, GEORGE F. TAYLOR AND H. DIX CHRISTENSEN: Caffeine in plasma and saliva by a radioimmunoassay procedure. J. Pharmacol. Exp. Ther. **199:** 679–686, 1976.

Caffeine was analyzed in human plasma and saliva by a simple, rapid, and sensitive radioimmunoassay procedure. Immunization of rabbits with an antigen prepared by coupling 7-(5-carboxypentyl)-1,3-dimethylxanthine to bovine serum albumin resulted in the formation of antibodies selective for caffeine as opposed to various mono- and dimethylxanthines, mono-, di-, and trimethyluric acids and a variety of common drugs. The radioligand used for competitive binding studies was 7-(2,3-$^3H_2$-propyl)-1,3-dimethylxanthine. The procedure permits direct analysis of caffeine in plasma or saliva without extraction. Comparison with a high pressure liquid chromatography method for the analysis of caffeine gave satisfactory results and showed no evidence for interference by metabolites. A caffeine half-life of 4.0 hours determined by the radioimmunoassay was in agreement with previous work. Comparison of human plasma and saliva levels by the radioimmunoassay procedure indicated approximately equal concentrations in the two fluids.

Caffeine (**Ia,** fig. 1) is undoubtedly the most widely ingested alkaloid. In 1972, the average consumption of coffee in the United States was over two cups per day for persons 10 years old or older (Pan American Coffee Bureau, 1973). This would imply, conservatively, a consumption of 10 million kilograms of caffeine from coffee alone. In addition to its further consumption in tea and other drinks, caffeine is also present in a large number of over-the-counter and prescription medicines.

Long known for its diuretic and central nervous system-stimulating abilities, caffeine has also been reported to exhibit a variety of other physiological effects. Peters (1967) has reviewed the factors affecting caffeine toxicity. Among its other properties, caffeine has been reported to cause mutations in human cells in culture (Osterstag *et al.*, 1965) and appears to pass into fetal and gonadal tissues (Goldstein and Warren, 1962). Caffeine treatment of ejaculated human sperm increases motility without any decrease in longevity, possibly by inhibition of cyclic nucleotide phosphodiesterases (Schoenfeld *et al.*, 1975).

Axelrod and Reichenthal (1953) described a method for the determination of caffeine in

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biological material which involved extraction with an organic solvent, re-extraction into aqueous acid and measurement of the ultraviolet (UV) absorbance. This method, with various modifications (Routh *et al.*, 1969), has been used until fairly recently, when other techniques such as gas liquid chromatography (Gurtoo and Phillips, 1973) have been applied to the determination of caffeine. The advantages (in particular, selectivity and sensitivity) of radioimmunoassay (RIA) for analysis of drugs in plasma led us to believe that development of a RIA procedure for caffeine would be a useful contribution to the methodology available for analysis of this widely used drug and would aid in further studies of its pharmacological activity. The expected sensitivity of an RIA method should also permit determination of saliva levels of the drug. If a simple relationship existed between levels in the two fluids, saliva analysis would be of interest as a noninvasive means for following levels of caffeine. Such a technique has been shown to offer promise in the case of the related drug theophylline (Koysooko *et al.*, 1974).

Methods

Chemicals were reagent grade from commercial sources. Sources of relatively uncommon xanthines are given in the footnotes to table 1.

7-(5-Carboxypentyl)-1,3-dimethylxanthine (Ic). Theophylline (**Ib**, fig. 1, 3.96 g, 22 mmol) was stirred and heated for 2.25 hours at 80°C with the lithium salt of ω-bromohexanoic acid (4.41 g, 22 mmol, prepared by dissolving molar equivalents of lithium hydroxide and ω-bromohexanoic acid in water and lyophilizing the solution) and potassium carbonate (3.31 g, 24 mmol) in dry N,N-dimethylformamide (66 ml). Water (80 ml) was added; the solution was adjusted to a pH of 8 and extracted with three portions of 50 ml of methylene chloride to afford 0.85 g of unreacted theophylline. Acidifying the aqueous solution to a pH of 1 with cautious addition of concentrated hydrochloric acid and extraction with methylene chloride as before afforded a semicrystalline residue (4.72 g). One gram of this residue was triturated with 7 ml of CH_2Cl_2 to give a further 135 mg of theophylline and 865 mg of material soluble in CH_2Cl_2. This soluble material was separated by chromatography [monitored by thin-layer chromatography (TLC) (methanol/chloroform, 1:9 on Silica Gel HF_{254})] on 115 g of TLC grade Silica Gel HF (Brinkmann Instruments, Inc., Westbury, N.Y.) in a 2.5 × 57 cm column utilizing a gradient of chloroform to 10% methanol in chloroform (2000 ml) at 80 psi (Chromatronix CMP-3 pump) into four substances. The first substance eluted off the column was a trace of ω-bromohexanoic acid [$\upsilon_{max}^{CH_2Cl_2}$ 1710 cm^{-1} (COOH) and M^+ at *m/e* 194 ($C_6H_{11}O_2Br$ requires 194)]. The second substance eluted was shown to be 7-[5-(5-carboxypentyloxycarbonylpentyl)] - 1,3 - dimethylxanthine **(Id)** formed by alkylation of **Ic** with another molecule of lithium ω-bromohexanoate. It exhibited nuclear magnetic resonance (NMR) [$CDCl_3$, tetramethylsilane (TMS)] peaks at δ 3.4 (3H, singlet, N—CH_3), 3.58 (3H, singlet, N—CH_3), 4.31 (2H, triplet, N—CH_2), 7.64 (1H, singlet, 8—H), 2.31 (2H, triplet, —CH_2·CO—), 9.26 (1H, broad singlet, COOH), 4.08 (2H, triplet, OCH_2); $\upsilon_{max}^{CH_2Cl_2}$ 3300 to 2500 cm^{-1} (COOH), 1708 cm^{-1} (COOH), 1725 cm^{-1} (—COOR); ms M^+ at *m/e* 408.2004 ($C_{19}H_{28}N_4O_6$ requires 408.2009).

The third substance eluted was theophylline and the fourth was the desired 7-(5-carboxypentyl)-1,3-dimethylxanthine (**Ic**). It was recrystallized from acetone (2 ml/g) to yield an analytical sample, m.p. 128–131.5°C; λ_{max}^{MeOH} 272.5 (ε 8740); $\upsilon_{max}^{CH_2Cl_2}$ 2500 to 3500 cm^{-1} (COOH); 1708 cm^{-1} (C=O), 1660 cm^{-1} (C=O); NMR peaks at δ 2.36 (2H, triplet, —CH_2·CO—), 3.4 (3H, singlet, N—CH_3), 3.58 (3H, singlet, N—CH_3), 4.31 (2H, triplet, N—CH_2—), 7.64 (1H, singlet, 8—H), 9.64 (1H, broad singlet, COOH); and M^+ at *m/e* 294 ($C_{13}H_{18}N_4O_4$ requires 294).

Analysis. Calculated for $C_{13}H_{18}N_4O_4$: C, 53.05; H, 6.16; N, 19.04. Found: C, 52.94, H, 6.15; N, 19.00 (Micro-Tech Laboratories, Skokie, Ill.).

Relative amounts of products were determined by integration of the 3 to 4.5 ppm region of an NMR spectrum of the methylene chloride-soluble fraction placed on the column. This calculation was possible because of the separation of the O—CH_2— resonance of the ester from the N—CH_2— resonances and the fact that the theophylline methyl resonances were displaced slightly from those in the products. It indicated a total yield of 2.28 g (35%) of desired monoalkylated product (**Ic**) from the reaction.

Formation of hapten-protein conjugate. Acid **Ic** was allowed to react with bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, Mo.; recrystallized) by a modification (Cook *et al.*, 1974) of the mixed anhydride method (Erlanger *et al.*, 1957). The

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FIG. 1. Structures of compounds discussed. Substituents are as follows: **Ia**, R = —CH_3; **Ib**, R = —H; **Ic**, R = —$(CH_2)_5COOH$; **Id**, R = —$(CH_2)_5$·COO—$(CH_2)_5COOH$; **Ie**, R = —$(CH_2)_5CONH$—(BSA)$_{1/n}$; **If**, R = —$CH_2CH{=}CH_2$; **Ig**, R = —$CH_2CH_2CH_3$; **Ih**, R = —CH_2—CHTCH_2T.

product (**Ie**) was chromatographed on Bio-Gel P-6 to separate unreacted **Ic** (Cook *et al.*, 1974). Incorporation of the caffeine moiety into protein was measured by differential UV (Erlanger *et al.*, 1957). Covalent bonding was demonstrated by unsuccessful attempts to extract UV-absorbing substances into methylene chloride.

Preparation of 7-(2,3-$^3H_2$-propyl)-dimethylxanthine. Theophylline was alkylated with allyl bromide as described above to yield 7-allyl-1,3-dimethylxanthine (**If**), m.p. 110–111.5°C which on reduction (H_2, 10% Pd/C) gave 7-propyl-1,3-dimethylxanthine (**Ig**), m.p. 102.5–103.5°C. The tritiated compound (**Ih**, 62.6 Ci/mmol) was obtained by use of tritium gas in the reduction. Radiochemical purity was greater than 98% and the compound has been radiochemically stable for over a year.

Antibody formation and characterization. One milligram of the antigen was emulsified in 3 ml of equal volume 0.9% saline and Freund's complete adjuvant and administered intradermally in 30 sites to each of three New Zealand White rabbits (Vaitukaitis *et al.*, 1971). At two separate sites 0.3 ml of crude *Bordetella pertussis* vaccine (Eli Lilly & Co., Indianapolis, Ind.) was administered s.c. A one milligram booster per animal was administered s.c. at four sites after 4 weeks and at subsequent 4-week intervals. Serum (separated from blood obtained from the marginal ear vein) was collected monthly (10–14 days after each booster). The antiserum was characterized by determination of: 1) the titer, which was the dilution of antiserum which bound 30% of approximately 50 pg of tritiated 1,3-dimethyl-7-propylxanthine; 2) the affinity constant, which was measured as the reciprocal of the unbound radioligand concentration at half-saturation of the antibody-binding sites; and 3) the cross-reaction with various compounds, which was calculated at 50% displacement of the radioligand, with caffeine being taken as 100% cross-reaction. In all characterization studies, the volume of incubation was 0.7 ml and the time was 2.5 hours.

Assay. The basic assay consisted of the addition of radiolabeled propyl analog **Ih** (10,000 cpm, 50 pg) and antiserum (sufficient for 30% initial binding) to the caffeine-containing sample in a 0.7 ml final volume of buffer [5.38 g/l of $NaH_2PO_4 \cdot H_2O$, 16.35 g/l of $Na_2HPO_4 \cdot 7H_2O$, 9.0 g/l NaCl, 1.0 g/l of NaN_3, 1.0 g/l of gelatin, pH 6.8]. A displacement curve was generated by the addition of 0.1, 0.3, 1, 3 and 10 ng of caffeine (from stock solutions containing 10 and 100 ng of caffeine per ml of buffer) to the labeled analog and antiserum. After a 2.5-hour incubation interval at 4°C, 0.5 ml of a dextran-coated charcoal suspension (0.5% Norit A and 0.05% Dextran T-70 in buffer), which adsorbed 98% of the tritiated propyl analog in the absence of antibody, was added to separate the bound and unbound radioligand. The supernatant (bound fraction) was decanted into a counting vial and radioactivity was measured by liquid scintillation counting in a medium containing 0.6% Omnifluor (New England Nuclear Corp., Boston, Mass.) dissolved in toluene–Triton X-100 (2:1). Plasma and saliva samples were normally diluted 1:100 with buffer and 0.1- to 0.2-ml aliquots were analyzed in triplicate. Thus only 1 to 2 μl of biological fluid were present in each assay tube. The measured amount of caffeine was determined by a logit-log method (Rodbard *et al.*, 1969).

High performance liquid chromatography (HPLC) analysis of caffeine. The instrument used was a Waters model M-600 equipped with a 4.8 mm inside diameter × 25 cm column of Micro-Bondapak C-18 and a Varian 635 UV detector. Solvent (acetonitrile–water, 30:70, v/v) was passed through the column at a flow rate of 2.0 ml/min. To 2.0 ml of plasma were added 50 μl (1.5 μg) of a solution of internal standard (7-propyl-1,3-dimethylxanthine) prepared in double distilled water. After a 30-minute equilibration period, the plasma was extracted with 5 ml of $CHCl_3$. The residue from evaporation of the $CHCl_3$ was dissolved in 20 to 40 μl of acetonitrile and aliquots (7–10 μl) were injected into the chromatograph. Standard curves were prepared starting with known amounts of caffeine in sheep serum. (Sheep serum was used because of the difficulty of obtaining quantities of caffeine-free human plasma.) Peak heights (H) and retention times (R) were measured and the standard curve was expressed by linear regression in the form

$$\log (W_C) = a \log [(H_C \times R_C)/(H_S \times R_S)] + b,$$

where b includes the logarithm of the constant weight (1500 ng) of internal standard. (W = weight in total sample in nanograms, subscript C denotes caffeine and subscript S internal standard.)

Human studies. Six informed volunteers were requested to refrain from consuming caffeine for 72 hours before taking 100 mg of caffeine in tablet form (NoDoz, Bristol Myers, Syracuse, N.Y.). Heparin-treated blood samples were taken just before (zero) and at time periods 1, 3 and 6 hours after administration of the drug. (Heparin did not interfere with the assay.) Plasma was separated by centrifuging. Whole saliva was collected by spitting into glass vials. To provide additional samples for comparison assays, equal portions of the 0- and 6-hour plasma samples were combined for each subject, as were samples from the 1 and 3 and the 3 and 6 hour times. The plasma samples were analyzed in duplicate by both RIA and HPLC. Plasma samples were also obtained from seven subjects administered 65 mg of caffeine mixed with aspirin, phenacetin and codeine.

Results

Antigen synthesis. A 35% yield of acid **Ic** was obtained. Reaction of this compound with

BSA gave an incorporation of 24 caffeine residues per molecule of BSA (**Ie**, n = 24).

Antisera characteristics. In one of three rabbits, a good antibody titer (final dilution of 1:35,000 bound 30% of about 50 pg of radioligand) was obtained by 3rd month after inoculation. The affinity constant was 4.5×10^9 liters/mol for the propyl analog (**Ih**) of caffeine. Logit transformation resulted in a linear response to caffeine from 0.2 to 10 ng with a correlation coefficient of −0.99. Cross-reactions with a variety of xanthine analogs are shown in table 1. Several miscellaneous compounds including oral contraceptives, salicylic acid, acetylsalicylic acid, phenylbutazone, propoxyphene, codeine, phenobarbital, pentobarbital, diphenylhydantoin and primidone at 10 μg did not compete with **Ih** for binding sites. Phenacetin had a displacement curve closely parallel to that of caffeine, but the cross-reaction was only 0.004%.

The applicability of the assay to a wide range of plasma or serum levels was demonstrated by analyzing spiked samples of human plasma (100 ng/ml–100 μg/ml) and sheep serum (0.2 ng/ml–100 μg/ml) (fig. 2). The average error over all these levels was 9%. The regression line (r = 0.99) for the combined set of values was

$$\log y = 0.985 \log x + 0.027$$

Analysis of plasma samples in the usual range (>25 ng/ml) required the presence of 20 μl or less of plasma in the assay tubes and these amounts caused no interference in the assay. However, if very low levels are to be measured (*i.e.*, if 0.1 ml or more of plasma is added to the assay), nonspecific binding by plasma proteins may become significant. In such a case, it may be necessary to generate a standard curve by using blank serum from the subject. For the analyses in figure 2 below 10 ng/ml, correction for binding was made by subtracting the nonspecific binding of serum-containing control tubes.

Comparison of HPLC and RIA. The retention times for caffeine and internal standard were 1.97 and 3.07 minutes, respectively, under the conditions used. Blank plasma gave no interference and it was shown that metabolites such as paraxanthine (retention time 1.65 minutes) and trimethyluric acid (retention time 0.98 minutes) did not interfere. The standard curve for HPLC analysis was applicable down to at least 0.38 μg of caffeine per ml. Constants for the equation

$$\log (W_C) = a \log [(H_C \times R_C)/(H_S \times R_S)] + b$$

were a = 0.752 and b = 3.248 with a correlation coefficient of 0.994.

Analysis of the same plasma samples by

TABLE 1

Caffeine antiserum cross-reaction

	% Cross-Reaction[a, b]
Caffeine (1,3,7-trimethylxanthine)	100
1,3-Dimethyl-7-propylxanthine	460
Theophylline (1,3-dimethylxanthine)	7
Theobromine (3,7-dimethylxanthine)	0.6
Paraxanthine (1,7-dimethylxanthine)[c]	0.3
1-Methylxanthine[d]	0.03
3-Methylxanthine[d]	0.3
7-Methylxanthine[d]	0.003
Xanthine[e]	0.001
1,3,7-Trimethyluric acid[c]	0.9
1,3-Dimethyluric acid[c]	0.05
3-Methyluric acid[c]	0.003
Uric acid	<0.001
Urea	<0.001

[a] Molar basis.

[b] At radioligand bound/free ratio of 0.18.

[c] Obtained from Adams Chemical Company, Round Lake, Ill.

[d] Obtained from Chemalog, Chemical Dynamics Corporation, South Plainsville, N.J.

[e] Obtained from Sigma Chemical Company, St. Louis, Mo.

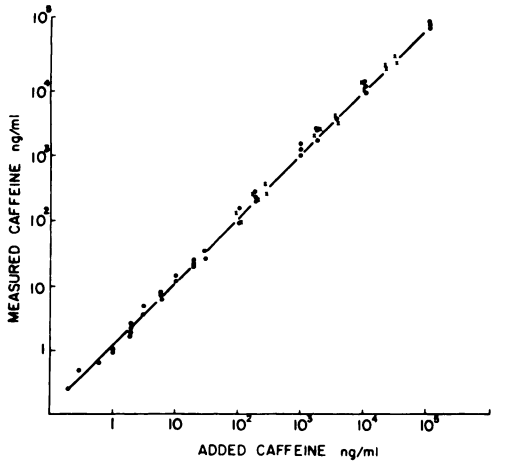

FIG. 2. Regression line for the analysis of plasma spiked with caffeine.

HPLC and RIA showed good agreement (table 2). The linear regression equation was

$$C_{HPLC} = (1.02 \pm 0.08)\ C_{RIA} + (0.04 \pm 0.13)$$

with $r = 0.916$ ($n = 31$) over a concentration range of 0.4 to 3.1 μg/ml. The percent difference between RIA and HPLC did not increase with increasing time, ($r = 0.0$, $n = 15$); thus metabolites do not interfere at the later time periods.

Determination of human plasma levels. Three of the six subjects in the study avoided all caffeine-containing products for 72 hours before the study. Plasma levels of these subjects before administration of caffeine were <0.1 μg/ml. (We did not attempt to measure lower levels.) Illustrative of the ubiquitous nature of caffeine was the failure of the other three to avoid it completely, in spite of reasonably careful instructions. Caffeine levels at 1 hour after ingestion of 100 mg of the drug averaged 2.2 ± 0.6 μg/ml.

TABLE 2

Comparison of HPLC and RIA results for plasma samples from human subjects

	RIA	HPLC
	μg/ml	μg/ml
	2.45	2.25
	1.91	1.89
	1.04	1.03
	0.63	0.68
	2.16	1.98
	1.39	1.52
	1.54	1.56
	1.54	1.57
	0.46	0.65
	1.43	1.65
	1.20	1.02
	1.71	1.63
	0.75	0.72
	0.50	0.71
	1.60	1.79
	3.86	3.85
	1.43	2.10
	1.00	1.40
	0.36	0.45
	3.00	2.73
	2.01	1.90
	1.13	0.75
	0.82	0.55
	2.61	3.23
	1.55	1.57
	2.21	1.56
	1.49	1.15
	1.05	0.76
	0.52	0.44
	1.77	1.33
	1.19	1.00
Mean ± S.D.	1.47 ± 0.72	1.46 ± 0.80

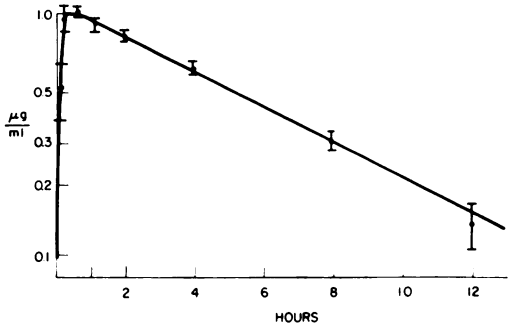

FIG. 3 Average (±S.D.) caffeine levels in seven human subjects administered a combination of caffeine, phenacetin, aspirin and codeine.

In a separate study, when 65 mg of caffeine were ingested with phenacetin, codeine and aspirin by seven subjects, the levels increased to an average peak of 1.14 μg/ml and then declined with an excretion half-life of 4.0 hours (fig. 3). Levels were still measurable (about 150 ng/ml) 12 hours after administration.

Comparison of saliva and plasma levels. Figure 4 shows the correlation between the two fluids. When fitted on a logarithmic basis, the data fit the following equation ($r = 0.96$, $n = 19$);

$$\log C_{Saliva} = (0.965 \pm 0.069)\ \log C_{Plasma} + (0.0067 \pm 0.0224)$$

The constants in the above equation indicate a linear relationship with an equal distribution between plasma and saliva (saliva/plasma ratio = 1.02).

Discussion

The molecular size of the caffeine molecule necessitates conjugation to a large molecule to achieve a substance capable of eliciting antibody formation. Antibodies to a given hapten-protein conjugate will have greatest selectivity[3] for those portions of the hapten molecule not located at the point of attachment to the

[3] We are using "selectivity" instead of the more commonly used "specificity," since the latter has an absolute connotation. Antisera may be highly (and usefully) *selective* in their preference for binding to a given compound, but are not generally *specific* to the point of exclusion of all other compounds.

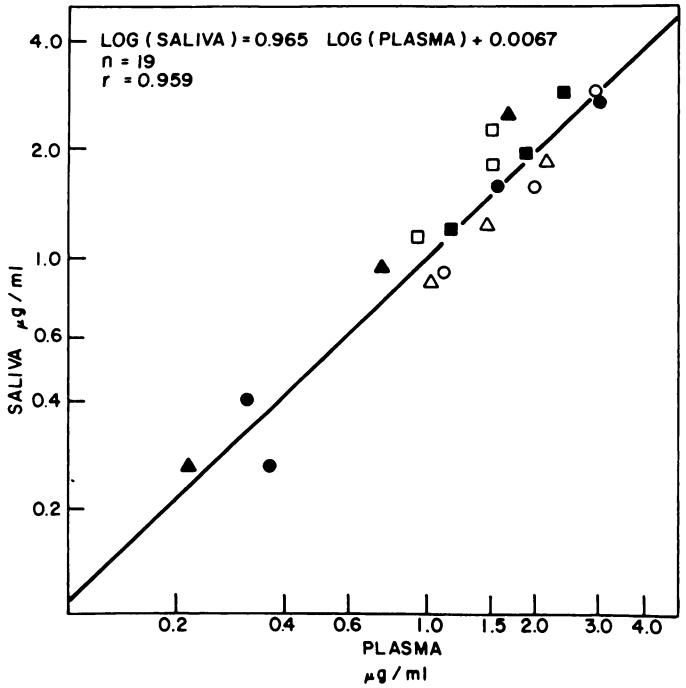

FIG. 4. Comparison of plasma and saliva caffeine levels in human subjects. Each symbol (○, ●, △, ▲, □, ■) refers to a different subject.

protein (Landsteiner, 1962). Thus, to choose the best point of attachment in design of the conjugate of a drug, one must consider the metabolism of the parent compound in order to achieve greatest selectivity of the antibodies for parent drug *vs.* its metabolites.

Caffeine undergoes metabolism by N-demethylation and oxidation as well as eventual ring cleavage. N-demethylation occurs principally in the pyrimidine ring with a major metabolite in man reported (Cornish and Christman, 1957) to be 1,7-dimethylxanthine (**II**). Theophylline (1,3-dimethylxanthine, **Ib**), formed by N-7 demethylation, is a minor caffeine metabolite in man (Sved *et al.*, 1976) and forms a minor percentage of the total metabolites in other species (Khanna *et al.*, 1972). In the rat, 8,9-dihydro-1,3,7-trimethyluric acid has been reported to be a significant urinary metabolite (Rao *et al.*, 1973).

Linkage of the caffeine moiety to a protein *via* a 7-alkyl substituent should result in an antigen which would stimulate formation of antibodies capable of distinguishing between caffeine and its N-1 and N-3 demethylated metabolites. We also expected such antibodies to be relatively insensitive to uric acid derivatives. It seemed reasonable that a moderately long alkyl chain at position 7 would lead to antibodies which had a selectivity for 7-alkyl substituents over compounds possessing a hydrogen at position 7. Thus, it might be possible to also obtain some selectivity between caffeine and theophylline.

Alkylation of theophylline with either ω-bromohexanoic acid or allyl bromide occurred in position 7 to give caffeine analogs. This was demonstrated by the UV spectra of the two compounds which exhibited a single maximum in the 270-nm region in addition to low wavelength absorption. It has been shown that isoxanthine derivatives such as isocaffeine (1,3,9-trimethyl substitution) exhibit a second UV maximum around 240 nm (Golovchinskaya and Chaman, 1960).

The use of tritiated 7-propyl-1,3-dimethylxanthine as the radioligand for competitive binding studies proved convenient, since the 7-allyl analog could be readily made and catalytically reduced with tritium gas to give material with a high specific activity. The fact that the propyl analog binds more readily to the antibody than caffeine suggests that the antibody recognizes a significant portion of the alkyl side chain of the hapten.

The selectivity[4] of the antiserum for caffeine was quite good. Since theophylline reaches levels only 5% those of caffeine after caffeine administration (Sved *et al.*, 1976), in most biological situations there will be no interference from theophylline. A relatively large volume (up to 0.1 ml) of serum/plasma can be measured directly in the assay without correction. Thus, the assay is extremely simple as the procedure involves only the addition of antiserum and label to a serum/plasma aliquot. The assay limit is then about 2 ng/ml, well below the probable caffeine serum levels of most persons. Correction for nonspecific binding can reduce the limit by a factor of 10.

The correlation of results between the RIA and HPLC methods was quite good. The HPLC work was carried out principally to act as a check with respect to possible metabolite interference and thus was not necessarily optimized. The propyl analog of caffeine was useful as an internal standard since its extraction and chromatographic properties closely resembled those of caffeine. Although the HPLC technique was convenient, the direct assay of plasma by RIA was even more convenient and efficient. Its sensitivity has been utilized in studies dealing with rats (Parsons and Aldridge, 1976) and premature infants (Aranda *et al.*, 1975).

The human studies confirm the applicability of the procedure. The plasma levels and the half-life determined here are consistent with those found in earlier studies. Axelrod and Reichenthal (1953) reported a half-life averaging 3.5 hour and plasma concentrations of 0.7 to 2.7 μg/ml 1 hour after ingestion of two cups of coffee. In contrast to theophylline (Koysooko *et al.*, 1974), in which case the ratio of saliva/plasma has been reported to be about 0.52, caffeine appeared equally distributed between plasma and saliva. This is presumably due to the lesser protein binding of caffeine, which is also reflected in the insensitivity of the RIA to a large proportion of added plasma. Axelrod and Reichenthal (1953) found only 15% binding to plasma at 37°C and showed that the drug was distributed in tissues according to water content. The data in figure 3 indicate some intersubject variability in the saliva/plasma ratio, but this appears to be relatively low.

In conclusion, the analytical methodology described in this paper has proven to be sensitive, simple and selective for the analysis of caffeine in either plasma or saliva. Saliva levels in the subjects studied directly reflected plasma levels. Although more extensive studies are required, it appears that caffeine may join the growing list of pharmacological agents for which saliva analysis may be used to reflect plasma concentration. As in the case of phenytoin (Cook *et al.*, 1975) the sensitivity and selectivity of RIA procedures make them attractive for saliva determinations.

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[4] Selectivity was determined in this instance by comparison of the concentrations of various xanthine derivatives required to displace 50% of the initially bound radioligand. Ekins and Rodbard have pointed out (see Rodbard and Lewald, 1970) and reiterated (*inter alia*, Ekins, 1975) that in the case of a homogeneous antibody this type of calculation does not reflect the exact ratio of binding constants of the two compounds, that it varies with the bound/free ratio (R) of radioligand in the assay system and that the response curves for two different compounds can never be exactly parallel. An adaptation of Rodbard's equation vii (Appendix III, Rodbard and Lewald, 1970) is shown below in which K is the affinity constant for binding, C is the molar concentration at a given value of R (ratio of bound to free radioligand) and subscripts M, D, and * refer to metabolite, drug and radioligand, respectively.

$$\% \text{ Cross-reaction} = 100 \left[\frac{1 + \left(\frac{K_D}{K^*}\right)R}{1 + \left(\frac{K_M}{K_D}\right)R} \right] \frac{K_M}{K_D}$$

$$\left(\frac{C_D}{C_M}\right)$$

In a large number of cases in RIA of drugs, the conditions are such that $K_M < K_D \approx K_*$. If, as is usual, the initial bound fraction of radioligand is 0.3 to 0.5 and R at 50% displacement is thus 0.18 to 0.33, calculations based on the above equation indicate no severe errors will result from comparison of 50% inhibition concentrations. Thus in the case where $K_M = 0.1\ K_D = 0.1\ K_*$, the calculated cross-reaction could vary from 13.9 to 10.5%, based on the initial bound fraction (0.3–0.5) and the percent displacement (10–90%) chosen for comparison. This is slightly higher than the "true" cross-reaction (based on K values) of 10%, but the variation is not very significant from a practical RIA standpoint. If, as in the present case, $K_M < K_D < K_*$, the differences are even smaller. We conclude that when $K_M < K_D \leq K_*$ and $R < 0.5$, comparison of 50% displacement values is a reasonable means for estimating the selectivity of an antiserum for a drug *vs.* potential cross-reactants. Selective antisera can then be subjected to more intensive study and the problems of antibody heterogeneity (not taken into account in the above discussion) can be considered.

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