Journal of Chromatography, 233 (1982) 51—60
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1438

REVERSED-PHASE LIQUID CHROMATOGRAPHIC INVESTIGATION OF UV-ABSORBING LOW-MOLECULAR-WEIGHT COMPOUNDS IN SALIVA

KATSUYUKI NAKANO

PL Medical Data Center, 1 Kamiyama-cho, Tondabayashi, Osaka 584 (Japan)

and

SEBASTIAN P. ASSENZA and PHYLLIS R. BROWN*

Department of Chemistry, University of Rhode Island, Kingston, RI 02881 (U.S.A.)

(Received June 8th, 1982)

SUMMARY

The reversed-phase mode of high-performance liquid chromatography was used to determine the intra- and inter-individual levels of UV-absorbing low-molecular-weight compounds in saliva. Many of the compounds known to occur in serum were also found in saliva; however, concentrations in saliva are lower. Both the intra- and inter-individual levels of these compounds vary significantly; in most cases, the inter-individual variance is 2—3 times the intra-individual variance.

Caffeine and its metabolites in saliva are also reported. A greater number of metabolites were found in the saliva of habitual coffee drinkers. After caffeine was administered orally, paraxanthine, theobromine, theophylline, 1-methylxanthine, and 1-methyluric acid were found in the saliva of an individual who did not drink coffee regularly. In this subject, the serum half-life for caffeine was 3.49 h and the saliva half-life was 3.27 h. The half-life of caffeine in an habitual coffee drinker who had refrained from caffeine products for four days was 4.39 h.

INTRODUCTION

Since dosages required for optimal effects can vary widely among patients, drugs with a narrow therapeutic range, such as the methylated xanthines, must be monitored periodically to ensure that an effective and nontoxic dose has been prescribed. However, frequent collection of blood is inconvenient and can add discomfort, especially with pediatric and geriatric patients. Saliva, on the other hand, is convenient; sample collection is noninvasive and can

0378-4347/82/0000—0000/$02.75

be repeated often. Therefore, saliva has been investigated as a matrix to monitor caffeine [1] and theophylline [2, 3].

Numerous methods have been developed to measure the methylated xanthines in biological matrices [1—5]. In recent years, high-performance liquid chromatography (HPLC) has been shown to be ideally suited for the assay of these compounds [6—13]. Many of these methods have been applied directly to saliva analysis [1—3, 9, 12—14]. However, little information has been reported on the metabolites of the methylated xanthines in saliva. Moreover, the UV-absorbing low-molecular-weight endogenous constituents of saliva have not been fully studied; knowledge of these compounds could provide useful clinical data and may aid in the identification of possible interfering substances.

In this paper, the reversed-phase mode of HPLC was used to determine the intra- and inter-individual levels of the UV-absorbing low-molecular-weight endogenous compounds in the saliva of a group of subjects. In addition, caffeine and its metabolites in saliva are reported. The effects of diet and sample preparation on the chromatographic profiles of saliva constituents were also examined.

EXPERIMENTAL

Chromatographic equipment

A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with two M 6000A pumps, M 660 solvent programmer, U6K injector, and M 440 dual-wavelength UV detector was used. A Houston Instrument (Austin, TX, U.S.A.) two-pen recorder was used to trace the 280-nm and 254-nm signals and to calculate peak-height ratios (PH-280/PH-254). A scanning UV—VIS spectrophotometer (SF 770 Spectroflow Monitor) and fluorescence detector (FS 970 L.C. Fluorometer) manufactured by Schoeffel Instrument Division (Kratos, Westwood, NJ, U.S.A.) were used to aid in the identification of the saliva constituents. The output of the ancillary detectors was recorded on a Kratos dual-pen recorder. Peak areas and retention times were obtained with an HP 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Chromatographic conditions

The low-molecular-weight UV-absorbing compounds in saliva were separated on commercially available columns (300 × 3.9 mm I.D.; Waters) packed with 10-μm octadecyl-silica (Waters). A Waters precolumn (25 × 3.9 mm I.D.) packed with 25—37-μm octadecyl-silica (Whatman, Inc., Clifton, NJ, U.S.A.) was used to protect the analytical column.

A screening separation of the endogenous compounds was done with a 5-min isocratic elution with 0.02 *M* potassium dihydrogen phosphate, pH 5.7, followed by a 35-min linear gradient to 24% methanol. Paraxanthine, theophylline, theobromine, caffeine, and 8-chlorotheophylline (as an internal standard) were separated isocratically with 0.01 *M* dibasic ammonium phosphate (pH 4.5)—methanol—acetonitrile (91:6:3, v/v). In addition, the chromatographic conditions developed by Orcutt et al. [9] were used to aid in the identification of the caffeine metabolites. Flow-rates were 1.5 ml/min and column temperature was ambient. All eluents were degassed by sonication and helium purge.

Chemicals and chromatographic standards

The phosphate and acetate buffers were obtained from Fisher Scientific Company (Fair Lawn, NJ, U.S.A.). Glass-distilled methanol and acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were used throughout. Standard compounds and enzymes were from Sigma Chemical Company (St. Louis, MO, U.S.A.) and Vega Biochemicals (Tucson, AZ, U.S.A.). Caffeine tablets (100 mg, Bristol-Meyers Co., New York, NY, U.S.A.) were used in the caffeine metabolism studies. The chemical reagents for qualitative identifications were obtained from Fisher and other sources.

Sample collection

Saliva samples were obtained from donors with no known disease or abnormalities. The subjects were not maintained on strict diets; however, inventories of foods, beverages, and drugs were kept. Samples were taken in the morning after a 12-h fasting period unless otherwise stated. The saliva was collected without the aid of saliva-stimulating agents.

Serum was obtained by subcubital venipuncture drawing of blood into 5-ml tubes and clot formation was allowed for 15—20 min at room temperature, after which the tubes were centrifuged at 1145 relative centrifugal force (RCF) for 5—15 min. The serum was then removed from the packed materials.

Sample preparation

Serum samples were processed according to protocol developed elsewhere [15, 16]. Sera were filtered through membrane cones (CF-25, Amicon Corp., Lexington, MA, U.S.A.) at 640 RCF for 20 min to remove material with molecular weights greater than 25,000.

The effects of handling procedures on the chromatographic profiles of saliva were examined. The techniques investigated were filtration with membrane cones, centrifuging at 1145 RCF for 5—10 min and removal of the upper layer, and direct injection of whole saliva. To test each method, 20 ml of saliva from one subject were collected, vortexed, and divided into six lots; of the six lots, three were used for blank determinations. For the remaining three lots, 100 μl of saliva and 100 μl of 10^{-5} M standard solution were mixed and processed.

Peak identification

The low-molecular-weight UV-absorbing constituents of the saliva matrix were identified from the combined data of retention time, UV spectra, fluorescence, enzyme reactions, and chemical reactions [16—18].

RESULTS

Chromatography

Fig. 1 shows a chromatographic profile of saliva constituents. Based on the combined data from the identification techniques, the endogenous compounds present in the majority of saliva samples from fifteen subjects were identified as creatinine, uric acid, tyrosine, hypoxanthine, uridine, xanthine, kynurenine,

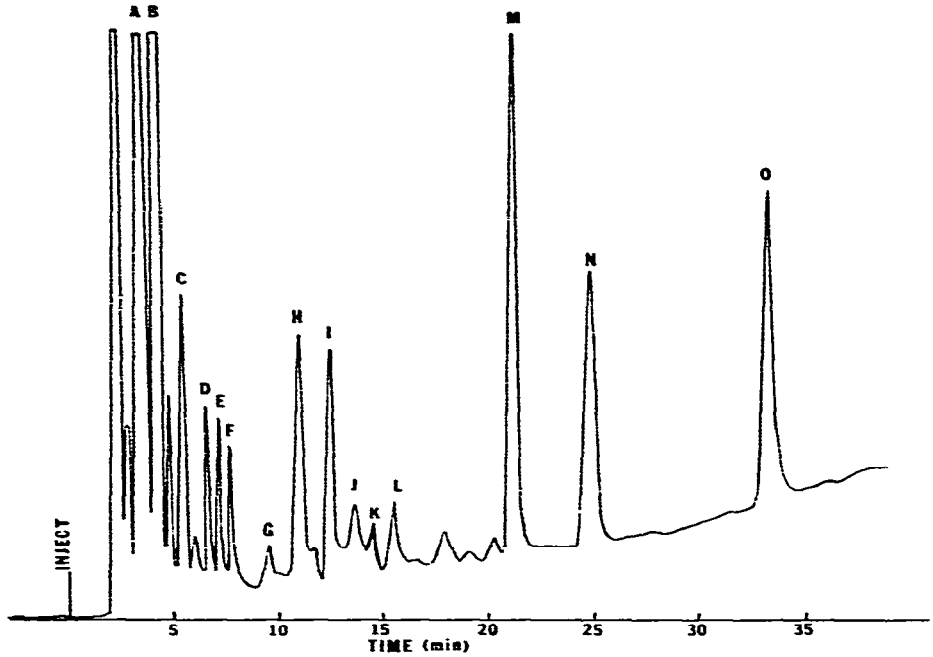

Fig. 1. Chromatogram of a saliva sample (100 μl). Conditions are listed in the text. Peaks: A = creatinine; B = uric acid; C = tyrosine; D = hypoxanthine; E = uridine; F = xanthine; G = kynurenine; H = 5-hydroxytryptophan; I = inosine; J = guanosine; K = hippuric acid; L = tryptophan; M = theobromine; N = paraxanthine/theophylline; O = caffeine.

5-hydroxytryptophan, inosine, guanosine, hippuric acid, and tryptophan. The exogenous compounds theobromine, paraxanthine/theophylline, and caffeine were also found to occur frequently in the saliva profiles.

Sample handling

A study of serum processing techniques prior to chromatography has been reported [15, 16]. Filtration through membrane cones was the preferred method to remove the high-molecular-weight materials in serum [16]. How-

TABLE I

PERCENTAGE RECOVERY OF STANDARD COMPOUNDS

Compound	Direct injection		Ultrafiltration	
	Whole saliva	Saliva	Saliva	Serum
Tyrosine	38.7	35.5	19.8	86.3
Xanthine	72.3	70.6	73.5	92.7
Inosine	52.8	51.6	50.6	96.0
Guanosine	76.5	75.8	79.4	61.5
Tryptophan	34.4	33.9	18.1	11.9
Theobromine	90.9	88.1	86.2	79.5
Paraxanthine	88.7	86.9	89.2	86.5
Caffeine	87.3	84.7	87.6	84.1

ever, filtering the saliva samples is not required since only a small percentage of whole saliva is high-molecular-weight material. In addition, differences in recoveries of the constituents of saliva and serum were noted (Table I). The recovery differences could be due to the pH of the two matrices.

Using the precolumn to trap high-molecular-weight material, we found the direct injection of whole saliva convenient for 40 serial assays; purging with 100% methanol is recommended after the last sample. However, when many samples (> 300) must be analyzed and time is an important factor, centrifuging the whole saliva and injection of the upper layer eliminates the need for frequent purging of the system. The differences between recoveries of these two methods are shown in Table I.

Constituents of saliva and serum

Many of the compounds found in serum [16, 19—21] were also noted in saliva. However, saliva contains lower concentrations of these compounds (Table II).

TABLE II

AVERAGE CONCENTRATIONS OF SALIVA COMPOUNDS

Compound	Saliva (μM; mean ± S.D.)	Serum range (μM)
Creatinine	3.71 ± 2.52	71.0—133
Uric acid	44.8 ± 21.5	155—429
Tyrosine	6.63 ± 2.92	44.0—71.0
Hypoxanthine	1.09 ± 1.00	1.56—12.8
Uridine	0.214 ± 0.231	<0.100—5.39
Xanthine	1.18 ± 0.814	0.542—4.70
Kynurenine	0.331 ± 0.289	55.0—151
5-Hydroxytryptophan	0.272 ± 0.174	—
Inosine	1.13 ± 0.610	<0.100—11.4
Guanosine	0.216 ± 0.168	<0.100—1.98
Hippuric acid	0.159 ± 0.125	<0.100—1.57
Tryptophan	1.55 ± 0.713	9.16—17.0

Reproducibility of saliva constituents

By monitoring five subjects for several months, the intra-individual levels of the saliva constituents were determined; these values are for samples taken after the donors had fasted for 12 h (Table III). Qualitatively, the saliva profiles are reproducible. Each individual appears to maintain a characteristic pattern of peaks in their profile. However, the concentrations of these compounds can vary greatly from day to day. These variations could be linked to foods consumed prior to sampling; however, no direct correlations were determined. Furthermore, the inter-individual levels of the endogenous saliva compounds are distributed over a larger range than the intra-individual concentrations (Table III). The inter-individual variance, in most cases, is 2—3 times that of the intra-individual variance.

TABLE III

VARIATIONS IN SALIVA CONCENTRATIONS OVER THREE MONTHS

Compound	Intra-individual		Inter-individual	
	Range (μM)	C.V. (%)	Range (μM)	C.V. (%)
Creatinine	3.01—4.42	40.3	2.45—5.01	69.4
Uric acid	34.8—56.3	38.1	27.8—61.8	51.7
Tyrosine	6.03—7.23	17.6	3.71—9.55	44.8
Hypoxanthine	0.970—1.21	23.1	0.589—1.59	92.6
Uridine	<0.100—0.325	100	<0.100—0.554	100
Xanthine	1.08—1.28	17.5	0.773—1.59	69.3
Inosine	0.949—1.32	33.0	0.825—1.44	54.2
Tryptophan	1.33—1.75	26.8	1.19—1.91	46.4

Methylated xanthines in saliva

The conditions of Orcutt et al. [9] were used to determine the methylated xanthines and methylated uric acids in saliva. A great number of these compounds were found in the saliva of habitual coffee drinkers. In one subject, 1,3-dimethyluric acid, 1-methyluric acid, 7-methylxanthine, 3-methylxanthine, and 1-methylxanthine were observed; however, the concentrations of these compounds were small compared to the levels of paraxanthine, theophylline, theobromine, and caffeine.

Metabolites of caffeine in saliva

Two habitual coffee drinkers were used to measure the rate of metabolism of caffeine from coffee. The subjects had refrained from consuming caffeine-containing products for 24 h. A cup of coffee (approx. 150 mg of caffeine) was given at the start of the experiment. Saliva samples were collected immediately before and after drinking the coffee, and thereafter at intervals spanning 20 h. The chromatographic conditions for the separation of paraxanthine, theophylline, theobromine, and caffeine were used.

The change in caffeine, paraxanthine, and theobromine concentrations in saliva are shown in Fig. 2. Within the first hour, caffeine concentrations in saliva decrease rapidly. A maximum concentration is reached at 4 h, followed by a steady decrease with a calculated half-life of 5.7 h. These changes in caffeine with time were similar in both subjects. The increase in caffeine in saliva after 4 h is explained by the redistribution of caffeine from blood back to saliva. The increase of paraxanthine and theobromine in saliva immediately after drinking coffee is due to their presence in the coffee. Relatively steady levels of paraxanthine and theobromine are observed from 2 to 9 h. After 9 h, paraxanthine and theobromine decrease at approximately the same rate. Theophylline levels followed those of paraxanthine.

To follow the metabolism of caffeine from an oral administration, a subject who was not an habitual coffee drinker was given 100 mg of caffeine to hold in the oral cavity. Saliva samples were collected at set intervals over 9 h. None of the metabolites of caffeine are observed in the saliva in the first 4 h. However, when the same subject, several days later, ingested a 100-mg tablet, paraxanthine is seen after 20 min and reaches a maximum concentration at 5 h (Fig. 3);

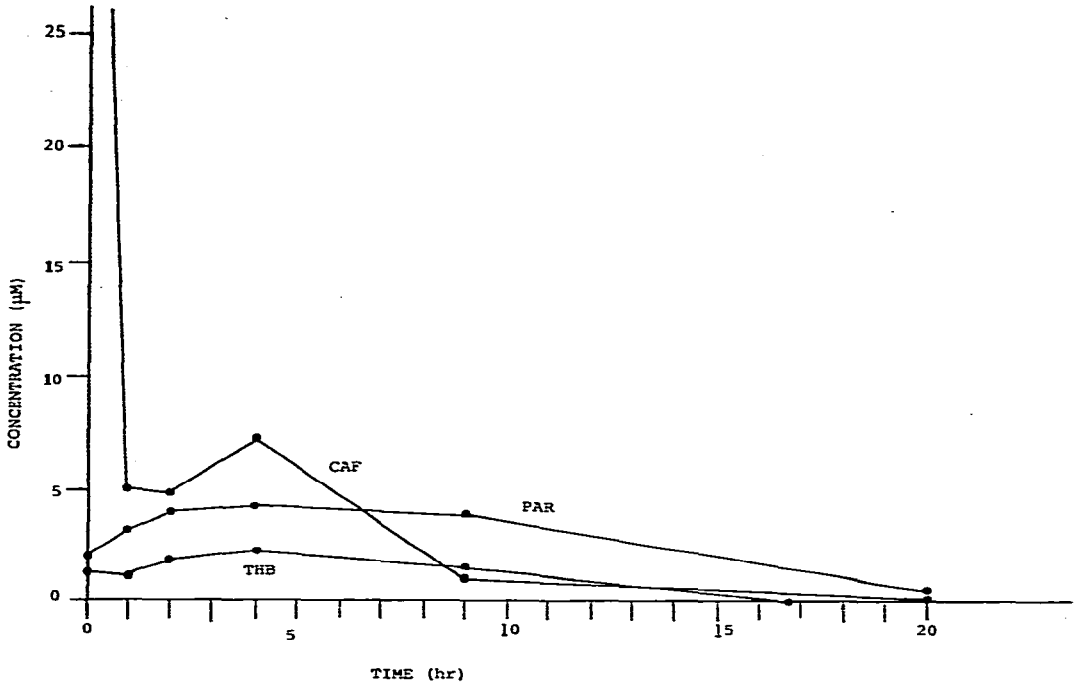

Fig. 2. Time curves for caffeine (CAF), paraxanthine (PAR) and theobromine (THB) in the saliva of an habitual coffee drinker.

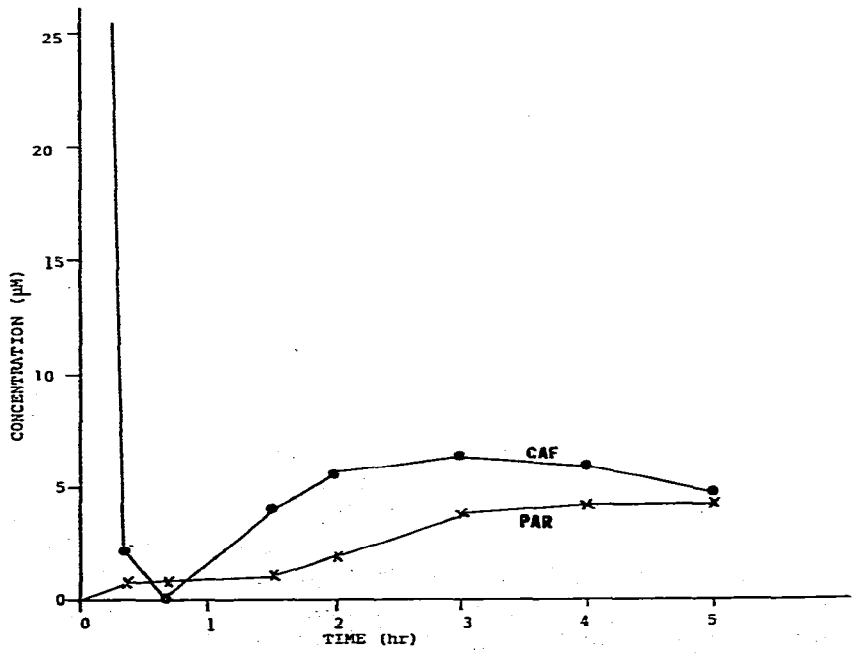

Fig. 3. Time curves for caffeine (CAF) and paraxanthine (PAR) in saliva after an oral administration of caffeine.

caffeine levels peak at 3 h and the drug has a half-life of 4.39 h. Theobromine concentrations reach maximum levels 5 h after caffeine administration. Maximum theophylline concentrations occur at approximately 3 h; the levels of theophylline are less than those of theobromine and paraxanthine. The caffeine metabolites 1-methyluric acid and 1-methylxanthine were also observed.

Caffeine in saliva and serum

Fig. 4 illustrates the changes with time in concentrations of caffeine and paraxanthine in saliva and serum of an habitual coffee drinker. The subject had avoided all products containing caffeine for four days prior to testing. Both the saliva and serum profiles obtained before the administration of 100 mg of caffeine showed no caffeine or metabolites.

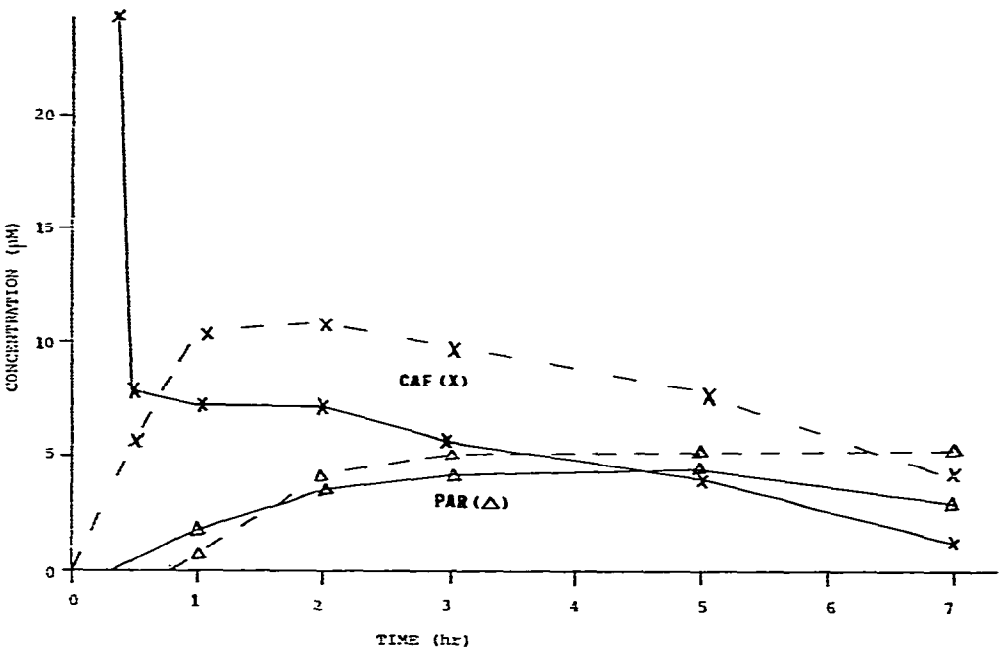

Fig. 4. Time curves for caffeine (CAF) and paraxanthine (PAR) in saliva (——) and serum (— — —) after an oral administration of caffeine to a decaffeinated habitual coffee drinker.

Between 1 and 2 h after administration of caffeine, a maximum in caffeine concentration is observed in saliva and serum. The rate of appearance of caffeine in serum is much slower than the initial disappearance of caffeine from saliva. However, the time curves for caffeine in saliva (half-life = 3.27 h) and serum (half-life = 3.49 h) are very similar after 2 h. For paraxanthine, the rate of appearance in serum follows the rate in saliva. After 50 min paraxanthine is observed in both saliva and serum; it is believed that caffeine is converted to paraxanthine in the liver [22, 23]. Comparing the data of Figs. 3 and 4, the kinetics of caffeine differ in individuals who drink coffee habitually and those who do not; Christensen and Whitsett [24] previously reported on these differences and also noted the influence of smoking on the rate of caffeine metabolism.

DISCUSSION

The saliva profiles of low-molecular-weight UV-absorbing compounds are similar to serum profiles. The major difference, however, is that saliva contains much lower concentrations of these compounds than serum. Furthermore, there appears to be a much greater variation in the intra-individual profiles of saliva than serum [13]. In addition, the tremendous variation in the inter-individual saliva profiles warrants great care in drug monitoring and metabolic studies.

The major metabolites of caffeine found in urine were shown to be 1-methyluric acid and 1-methylxanthine [22]. It was also suggested that paraxanthine, which was found in small amounts in urine, may be the intermediate in the formation of 1-methylxanthine [22]. After an oral dose of caffeine, the major metabolites of caffeine in saliva were found to be paraxanthine, theobromine, theophylline, 1-methylxanthine, and 1-methyluric acid. The other possible metabolites [22, 23] were not readily observed.

The decay of caffeine in saliva was found to consist of two different rates (Fig. 4). The first rate of disappearance of caffeine in saliva is very rapid, as seen within the first 30 min, and the second rate is much slower. The exponential rates of caffeine in saliva and serum can be expressed as

$$-dX(\mathrm{L})/dt = A(\mathrm{L1})X(\mathrm{L}) \quad (1)$$

$$-dX(\mathrm{L})/dt = A(\mathrm{L2})X(\mathrm{L}) \quad (2)$$

$$-dX(\mathrm{R})/dt = A(\mathrm{R})X(\mathrm{R}) \quad (3)$$

where $X(\mathrm{L})$ and $X(\mathrm{R})$ represent the caffeine concentrations in saliva and serum, respectively. $A(\mathrm{L1})$ and $A(\mathrm{L2})$ are the first and second decay constants in saliva and $A(\mathrm{R})$ is the rate of decay in serum. When the redistribution rate constant is denoted by $A(\mathrm{D})$ the following expressions between the rates could be expected

$$\text{if } A(\mathrm{D}) >> A(\mathrm{L1}) \text{ then } A(\mathrm{L2}) \approx A(\mathrm{R}) \quad (4)$$

$$\text{if } A(\mathrm{D}) << A(\mathrm{L1}) \text{ then } A(\mathrm{L2}) \approx A(\mathrm{L1}) \quad (5)$$

From Fig. 4, the rate constant $A(\mathrm{L2})$ approximates $A(\mathrm{R})$ and not $A(\mathrm{L1})$. Applying the least-squares method to the observed kinetics of caffeine in Fig. 4, we find $A(\mathrm{L1}) = 8.5$, $A(\mathrm{L2}) = 0.24$, and $A(\mathrm{R}) = 0.15$; thus, $A(\mathrm{L1}) >> A(\mathrm{L2}) > A(\mathrm{R})$. Therefore, caffeine disappearance in saliva proceeds according to the conditions expressed in relationship 4. However, the exact quantitative solution would require compartment analysis of the two matrices.

The rates of caffeine metabolism differ in individuals who are habitual coffee drinkers from those who are not. Caffeine in saliva peaks at 3 h and has a 4.4-h half-life in persons with no coffee habit. For individuals who drink coffee regularly, a 3.3-h half-life is found after a four-day fast from caffeine and a 5.7-h half-life when caffeine is removed from the diet for only 24 h. The 4.4-h half-life in saliva (and serum) is in good agreement with the 4-h half-life reported by Cook et al. [1] and the 4.7-h half-life reported by Christensen and Whitsett [24]; in addition, it compares well with the 4.3-h half-life Tse and Szeto [11] found for caffeine in the male beagle dog.

The rates and levels of caffeine are similar in saliva and serum; a simple relationship is evident between the two matrices from the log—linear decline of caffeine in saliva and serum. This would suggest, as reported elsewhere [25], that wherever such relationships exist, saliva can be a useful matrix to monitor. However, in monitoring drugs such as theophylline, the presence of possible interfering substances such as paraxanthine should be noted; especially when the interfering substance has a long half-life and its distribution correlates between the two matrices.

ACKNOWLEDGEMENTS

The authors acknowledge Dr. T. Yasaka (PL Medical Data Center), Dr. R.P. Panzica, Dr. H.A. Scoble and M. McKeag (University of Rhode Island) for helpful comments and technical assistance.

This study was supported by a grant to K.N. from Oshieoya T. Miki and the "Perfect Liberty" Organization.

REFERENCES

1 C.E. Cook, C.R. Tallent, E.W. Amerson, M.W. Meyers, J.A. Kepler, G.F. Taylor and H.D. Christensen, J. Pharmacol. Exp. Ther., 199 (1976) 679.
2 G. Levy, E.F. Ellis and R. Koysooko, Pediatrics, 53 (1974) 873.
3 R. Koysooko, E.F. Ellis and G. Levy, Clin. Pharmacol. Ther., 15 (1974) 454.
4 H. Milon and J.A. Antonioli, J. Chromatogr., 162 (1979) 223.
5 J.A. Schack and S.H. Waxler, J. Pharmacol. Exp. Ther., 97 (1949) 283.
6 M. Weinberger and C. Chidsey, Clin. Chem., 21 (1975) 834.
7 A.H. van Gennip, J. Grift and E.J. van Bree-Blom, J. Chromatogr., 163 (1979) 351.
8 H.F. Walton, G.A. Eiceman and J.L. Otto, J. Chromatogr., 180 (1979) 145.
9 J.J. Orcutt, P.P. Kozak, S.A. Gillman and L.H. Cummins, Clin. Chem., 23 (1977) 599.
10 A.J. Quattrone and R.S. Putman, Clin. Chem., 27 (1981) 129.
11 F.L.S. Tse and D.W. Szeto, J. Chromatogr., 226 (1981) 231.
12 G.W. Peng, V. Smith, A. Peng and W.L. Chiou, Res. Commun. Chem. Pathol. Pharmacol., 15 (1976) 341.
13 J.R. Miksic and B. Hodes, J. Pharm. Sci., 68 (1979) 1200.
14 G.F. Johnson, W.A. Dechtiaruk and H.A. Solomon, Clin. Chem., 21 (1975) 144.
15 R.A. Hartwick, D. Van Haverbeke, M. McKeag and P.R. Brown, J. Liquid Chromatogr., 2 (1979) 725.
16 R.A. Hartwick, A.M. Krstulovic and P.R. Brown, J. Chromatogr., 186 (1979) 659.
17 A.M. Krstulovic, R.A. Hartwick, P.R. Brown and K. Lohse, J. Chromatogr., 158 (1978) 365.
18 S.P. Assenza and P.R. Brown, Anal. Chim. Acta, 123 (1981) 33.
19 W.E. Wung and S.B. Howell, Clin. Chem., 26 (1980) 1704.
20 S.P. Assenza and P.R. Brown, J. Chromatogr., 181 (1980) 169.
21 E.H. Pfadenhauer and S.-D. Tong, J. Chromatogr., 162 (1979) 585.
22 H.H. Cornish and A.A. Christman, J. Biol. Chem., 228 (1957) 315.
23 M. Bonati, R. Latini, E. Marzi, R. Cantoni and G. Belvedere, Toxicol. Lett., 7 (1980) 1.
24 H.D. Christensen and T.L. Whitsett, in G.L. Hawk (Editor), Biological/Biochemical Applications of Liquid Chromatography, Marcel Dekker, New York, 1977, p. 507.
25 J.C. Mucklow, M.R. Benoling, G.C. Kahn and D.T. Dollery, Clin. Pharmacol. Ther., 24 (1978) 563.