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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR MONITORING CAFFEINE AND ITS METABOLITES IN BIOLOGICAL FLUIDS OF MONKEYS CONSUMING CAFFEINE

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SUMMARY

A recently reported high-performance liquid chromatographic procedure, using a 5 μm C_{18} reversed-phase column to separate and quantitate caffeine and seven of its metabolites was modified for use with an automatic sampler to allow the continuous analysis of a large number of samples of various biological fluids obtained from monkeys consuming caffeine. The sensitivity for most metabolites was in the range of 0.1—0.3 μg/ml from a 0.1 ml sample. The repeatability of the method regarding within-day variations was excellent and the absolute retention time for eight standards differed by less than ± 0.03 min. Excellent repeatability in the day-to-day assay, with almost quantitative recoveries, was found for most of the analyzed compounds in various biological fluids. The standard deviation for the quantitation of all standards was in a range of 0.41—2.01 μg/ml, with the standard error less than 0.02. Using this method an analytical chemist could process between 40 and 60 samples of biological fluids in 24 h.

The main metabolite of caffeine in the plasma of the monkey was theophylline, while theophylline and 1,3-dimethyluric acid were the major metabolites in urine. A close correlation was observed for the pattern of metabolites found in plasma and milk.

INTRODUCTION

Coffee is certainly one of the most frequently consumed beverages not only

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in North America but all over the world [1]. Coffee drinking has been associated with a number of pharmacological and physiological effects [2]. Several epidemiological studies have associated coffee consumption with an increased risk for cancers of the bladder, pancreas, prostate, and esophagus or for renal carcinoma. However, most of these reports are controversial and it appears that the association is not causal [3]. The more obvious effects of coffee consumption are physiological in that it causes cardiovascular and psychological disorders [4].

Caffeine is considered to be the most physiologically active component in coffee [5], contributing to pronounced behavioral effects [6]. In addition, caffeine is present in tea, soft drinks, cocoa, chocolate and some medications. Caffeine and its metabolites readily cross the human placenta [7]. The biotransformation of caffeine in the newborn infant is different relative to the adult [8] and the elimination pattern of caffeine and its metabolites during pregnancy is also changed [9]. Due to the fact that the embryo is subjected to the influence of caffeine and its metabolites, it is important to determine the effects of caffeine on the developing nervous system. To study such effects it was proposed that the behavioral consequences of in utero exposure to caffeine be determined in the cynomolgus monkey (*Macaca fascicularis*), both immediately following birth and during the animal's first year of life. In addition, it is important to assess whether pregnant monkeys handle caffeine in a way similar to humans during their pregnancy.

For a long-term study with a large number of samples obtained from monkeys, a simple, uniformly applicable, fast and precise method for the determination of caffeine and its metabolites in biological fluids is required. However, the determination of caffeine metabolites in biological fluids poses analytical problems not encountered in other matrices [10]. There are several combined reasons for this difficulty: first, low dilute concentration and a limitation in the available sample size; secondly, extractions from matrices containing a variety of chemically different compounds; thirdly, the presence of structurally similar metabolites; and fourthly, individual variability in the metabolic pattern. Therefore, a method with high selectivity as well as sensitivity, employing a small sample size is required. Our previously developed method, employing a reversed-phase 5 μm C_{18} column for the high-performance liquid chromatographic (HPLC) separation of caffeine and its metabolites [11], was adapted for use with an automatic sampler for this purpose. This report describes a simple, fast, reliable and accurate method for monitoring caffeine and metabolites in different biological fluids of monkeys, using an automatic sampler, which allows continuous injections of large numbers of samples onto the HPLC column.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph Series 2/2 (Perkin-Elmer, Norwalk, CT, U.S.A.) provided with a Perkin-Elmer high speed 5 μm C_{18} column (15 cm × 4.6 mm I.D.) was used. The ultraviolet (UV) detector (Perkin-Elmer LC-55) connected to a Perkin-Elmer Deuterium Supply (Model

22320) was set at 276 nm. The flow cell had a volume of 8 μl. Samples were injected into the HPLC system using the Perkin-Elmer Intelligent Automatic Sampling System (ISS-100). The automatic sampler can handle up to 100 samples for continuous analysis. The sampler can be pre-programmed for a number of different parameters, such as volumes to be injected, repeated analysis of the same sample, and time between injections.

Data were recorded using a Perkin-Elmer Sigma 10 Data Processing system. The results (the values for the concentrations of the metabolites in μg/ml) were entered into a Data General NOVA computer (Westboro, MA, U.S.A.) for storage, retrieval and statistical manipulation.

All instruments and the column were operated at ambient laboratory temperature (approximately 22° C), although occasional fluctuations within the room (between 18° C and 28° C) were observed.

Chemicals

Sources and the abbreviations for the reference chemicals are: caffeine (1,3,7-trimethylxanthine or 1,3,7-TMX), theobromine (3,7-dimethylxanthine or 3,7-DMX) and theophylline (1,3-dimethylxanthine or 1,3-DMX) from Eastman Kodak, Rochester, NY, U.S.A.; 3,7-dimethyluric acid (3,7-DMU) from Sigma, St. Louis, MO, U.S.A.; paraxanthine (1,7-dimethylxanthine or 1,7-DMX) and 3-methylxanthine (3-MX) from Tridom/Fluka, Toronto, Canada; 1,3-dimethyluric acid (1,3-DMU) from Adams, Round Lake, IL, U.S.A. 8-Chlorotheophylline (8-CT) was purchased from ICN Pharmaceutical. All the standard stock solutions (10 μg/ml) and the internal standard, 8-CT (20 μg/ml) were prepared in 0.9% sodium chloride solution.

For the quality control, a mixture of 100 ml of all standards, at a concentration of 10 μg/ml in 0.9% sodium chloride was prepared. Aliquots of the stock standard solution were placed in several appropriate sample containers (scintillation vials or reagent bottles with glass stopper) and stored in a freezer. Every month (or if required more frequently), a fresh bottle of the standard solution was used and the repeatability for the retention time and peak heights of the "new" and "old" standards was compared.

All solvents used for extractions and for the chromatography were either of HPLC purity or were glass-distilled. All chemicals were analytical grade.

The extracting solvent was chloroform—isopropanol (85:15). The mobile phase consisted of a daily prepared mixture of water—isopropanol—acetonitrile—acetic acid (91:4:4:1), degassed for 2 min under partial vacuum in an ultrasonic bath (Branson Cleaning Equipment, Shelton, CN, U.S.A.).

Biological fluids

Biological fluids (urine, plasma, milk and saliva) for these analyses were obtained from the female monkeys (*Macaca fascicularis*), either from non-treated controls or from animals treated with different concentrations of caffeine in drinking water. Blood was collected from the femoral vein or artery in unheparinized vacutainers containing EDTA (Becton-Dickinson, Rutherford, NJ, U.S.A.), and centrifuged to obtain plasma. Urine was collected over a 24 h period in special metabolism cages. Milk was collected by hand expressing milk from a lactating monkey that did not have an infant.

Saliva was collected as follows: a plastic suction tube, normally used by dentists to remove saliva from a patient's mouth, was inserted into the monkey's mouth. The other end was connected to a small filtering test tube with side arm, which was further connected to a water pump partial vacuum. By applying a low suction pressure, a small sample of saliva (about 200 μl) collected in the plastic tube, was sufficient for the assay, although not every monkey cooperated readily for this type of collection.

Treatment of the monkeys

The monkeys were exposed to caffeine in doses of 0, 0.15 and 0.35 mg/ml in drinking water, seven days a week. Monkeys were given a fixed amount (ten cubes) of Purina monkey chow daily.

Sample preparation

One hundred microliters of the sample to be analyzed (urine, blood, serum or plasma, milk, saliva, or standards) was added to the test tube containing 1.2 g of ammonium bicarbonate and 100 μl of internal standard solution. After adding 8.0 ml of the extraction solvent, the mixture was well agitated on a Vortex mixer for 30 sec. After settling, the clear extracting solvent was filtered through Ottawa sand placed (approx. 1—2 mm depth) on the bottom of a polypropylene disposable mini-column 10 cm long (Alltech Associates, Deerfield, IL, U.S.A.). The extraction was repeated once more, and the pooled extracts were evaporated to dryness using a stream of nitrogen in a water bath (temperature not exceeding 50° C). After adding approximately 500 μl of the mobile solvent, the test tubes were briefly (15—20 sec) warmed (50° C) and sonicated for 10 sec to complete the dissolution. The samples were transferred into automatic HPLC sample vials for the analysis. The vials were not capped. The automatic sampler was set for 80 μl of sample per injection.

Setting the automatic sampler

Vials containing an extracted standard solution were incorporated into the tray of the automatic sampler: the first vial into position No. 1, and then one vial after every ten samples, i.e. in the positions numbered 12, 23, 34, etc.

A reasonable "wash-out" time was allowed between the injections of individual samples. This was important to allow complete elution of some slowly eluting endogenous compounds, which were found in urine. Therefore, urine samples needed an additional 20 min of wash-out time after the elution of the internal standard. There were no visible interference (peaks) with the extracts from plasma, saliva or milk. The wash-out time for the extracts from these samples was set at 5 min. Therefore, when using an automatic sampler it is preferable to group sets of identical fluids in consecutive order and to select the proper "elution" time for each set.

Quality control for the recoveries, repeatability and accuracy

Tests for accuracy were performed regularly using a mixture of standards (10 μg/ml of each) as a means of quality control.

For the purpose of controlling the repeatability of the methodology (extractability, interference) and the performance of the instruments, in addition

to the regular analysis of analytical standards, blank samples of biological fluids were periodically spiked with a mixture of analytical standards, and then analyzed. These tests, indicating the recoveries for different metabolites, were performed by adding 1.0 ml of the mixture of standards (containing 10 μg of each standard) to blood, urine, milk or saliva obtained from control, non-treated monkeys, prior to the analysis.

Additional tests for intra- and interday variability were performed using samples of urine or pooled plasma from animals on test. Samples of plasma were combined in two separate pools: (1) plasma of monkeys treated with low dose of caffeine and (2) plasma from animal treated with high dose of caffeine. These analyses, performed in nine or ten replicates per day, were repeated three times within twelve days. Samples of urine were analyzed in nine or ten replicates each day, and the analysis was repeated three times within one week.

Statistical evaluation

The analytical standards from all runs were pooled and the mean, median, standard deviation and standard errors calculated for each standard.

RESULTS

Very little variation was observed in the elution time for the standards within the day. The results from six injections performed during one working day are presented in Table I. If the room temperature remained constant the differences for the actual retention time differed less than ± 0.03 min for the internal standard, which was the last eluting compound from the column, i.e. peak 9 in Table I. Other metabolites showed even smaller differences. Also very little variation was found in the quantitation (peak heights or area) for the individual standards, when this mixture was repeatedly injected during the same working day.

TABLE I

ACTUAL ELUTION (RETENTION) TIME FROM SIX INDIVIDUAL INJECTIONS OF STANDARDS DURING ONE WORKING DAY

Retention times are given in minutes.

Injection number	Compound/peak number*							
	2	3	4	5	6	7	8	9
1	1.95	2.10	2.41	2.82	3.82	4.13	6.82	8.77
2	1.95	2.09	2.40	2.81	3.80	4.11	6.78	8.73
3	1.94	2.09	2.40	2.81	3.80	4.11	6.75	8.69
4	1.94	2.08	2.39	2.81	3.80	4.12	6.80	8.74
5	1.96	2.10	2.42	2.83	3.82	4.13	6.81	8.76
6	1.96	2.11	2.42	2.84	3.83	4.15	6.82	8.77
Average	1.95	2.095	2.41	2.82	3.81	4.125	6.81	8.74
S.D.	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.03

*For identification of the compound/peak numbers see Fig. 1.

Generally, the results for accuracy and precision in the day-to-day operations were also very good. Table II evaluates the results obtained from 124 injections of the mixture of standards using the same column. The concentration of each added standard was 10 μg/ml. These analyses were performed on 59 different days over an eight-month period. Excellent repeatability with almost quantitative recovery was regularly obtained for most of the analyzed compounds. The compound with the highest variability was 1,3-DMU, with a standard deviation of 2.01 μg/ml, and with a standard error of 0.02. The ranges, standard deviation and standard error values for all other compounds were considerably lower.

The intraday variability for urine samples is presented in the Table III. Similar results (mean, median, standard deviation and standard error) were obtained for the other two sets of analyses performed on different days. The standard error for all metabolites in the interday variability tests was in a range between 0.01 (for 1,7-DMX and 3-MX) and 0.11 (for theophylline).

There were little variations in tests for the intra- and interday variability either for plasma of low or high caffeine treated monkeys. The combined results of the interday variability for serum samples is presented in the Table

TABLE II

REPEATABILITY FOR QUANTITATION OF STANDARDS

Number of analyzed samples of processed mixed standards only = 124. Number of days the analysis was performed = 59. Time period = 8 months.

	Added (μg/ml)	Found (μg/ml)		S.D.	S.E.	Coefficient of variation
		Mean	Median			
Caffeine	10	9.96	10.00	0.84	0.01	0.084
Theophylline	10	10.18	10.15	0.58	0.00	0.057
Paraxanthine	10	10.11	10.10	0.41	0.00	0.041
Theobromine	10	9.96	10.00	0.48	0.00	0.048
1,3-DMU	10	9.66	9.30	2.01	0.02	0.208
3-MX	10	9.91	9.90	1.28	0.01	0.129

TABLE III

INTRADAY VARIABILITY FOR CAFFEINE AND METABOLITES FOUND IN URINE OF TREATED MONKEY

Number of replicates = 9.

	Found (μg/ml)		S.D.	S.E.
	Mean	Median		
Caffeine	9.13	9.30	0.54	0.07
Theophylline	68.49	68.90	1.76	0.22
Paraxanthine	2.01	2.00	0.33	0.04
Theobromine	6.94	6.80	0.59	0.07
1,3-DMU	26.54	26.00	2.23	0.28
3-MX	4.33	4.30	0.28	0.03

TABLE IV

INTERDAY VARIABILITY FOR CAFFEINE AND METABOLITES FOUND IN POOLED PLASMA OF TREATED MONKEY

	Found (μg/ml)		S.D.	S.E.
	Mean	Median		
Low dose of caffeine*				
Caffeine	2.87	2.95	0.31	0.01
Theophylline	8.01	8.00	0.79	0.03
Paraxanthine	0.51	0.50	0.16	0.01
Theobromine	0.60	0.60	0.10	0.00
1,3-DMU	0.17	0.10	0.18	0.01
3-MX	0.09	0.10	0.08	0.00
High dose of caffeine**				
Caffeine	7.57	7.60	0.58	0.02
Theophylline	14.46	14.20	1.09	0.04
Paraxanthine	0.69	0.70	0.20	0.01
Theobromine	1.01	1.00	0.12	0.00
1,3-DIMU	0.51	0.50	0.14	0.01
3-MX	0.18	0.20	0.06	0.00

*Total number of replicates = 27. Tests repeated three times, nine samples/day.
**Total number of replicates = 28. Tests repeated three times, nine or ten samples/day.

IV. The maximum standard error of 0.04 was found for theophylline in the high dose treated samples.

The results of the recovery studies of the mixture of standards added to various biological fluids were the same as previously described [11].

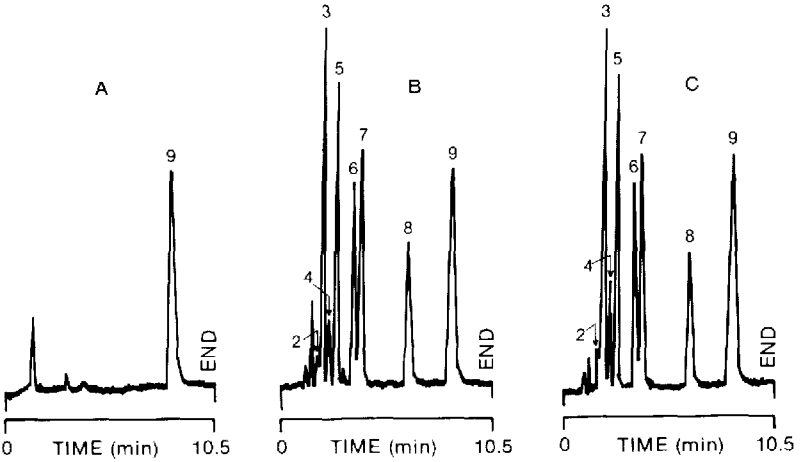

Fig. 1. Recovery profiles of standards (10 μg of each) added to the plasma of a non-treated monkey, before processing for HPLC analysis. (A) non-spiked plasma; (B) spiked plasma; (C) chromatograms of standards (10 μg of each) processed in the same way before the HPLC analysis. Identification of peaks: 2 = 3,7-dimethyluric acid; 3 = 3-methylxanthine; 4 = 1,3-dimethyluric acid; 5 = 3,7-dimethylxanthine; 6 = 1,7-dimethylxanthine; 7 = 1,3-dimethylxanthine; 8 = 1,3,7-trimethylxanthine; and 9 = 8-chlorotheophylline (8-chloro-1,3-dimethylxanthine).

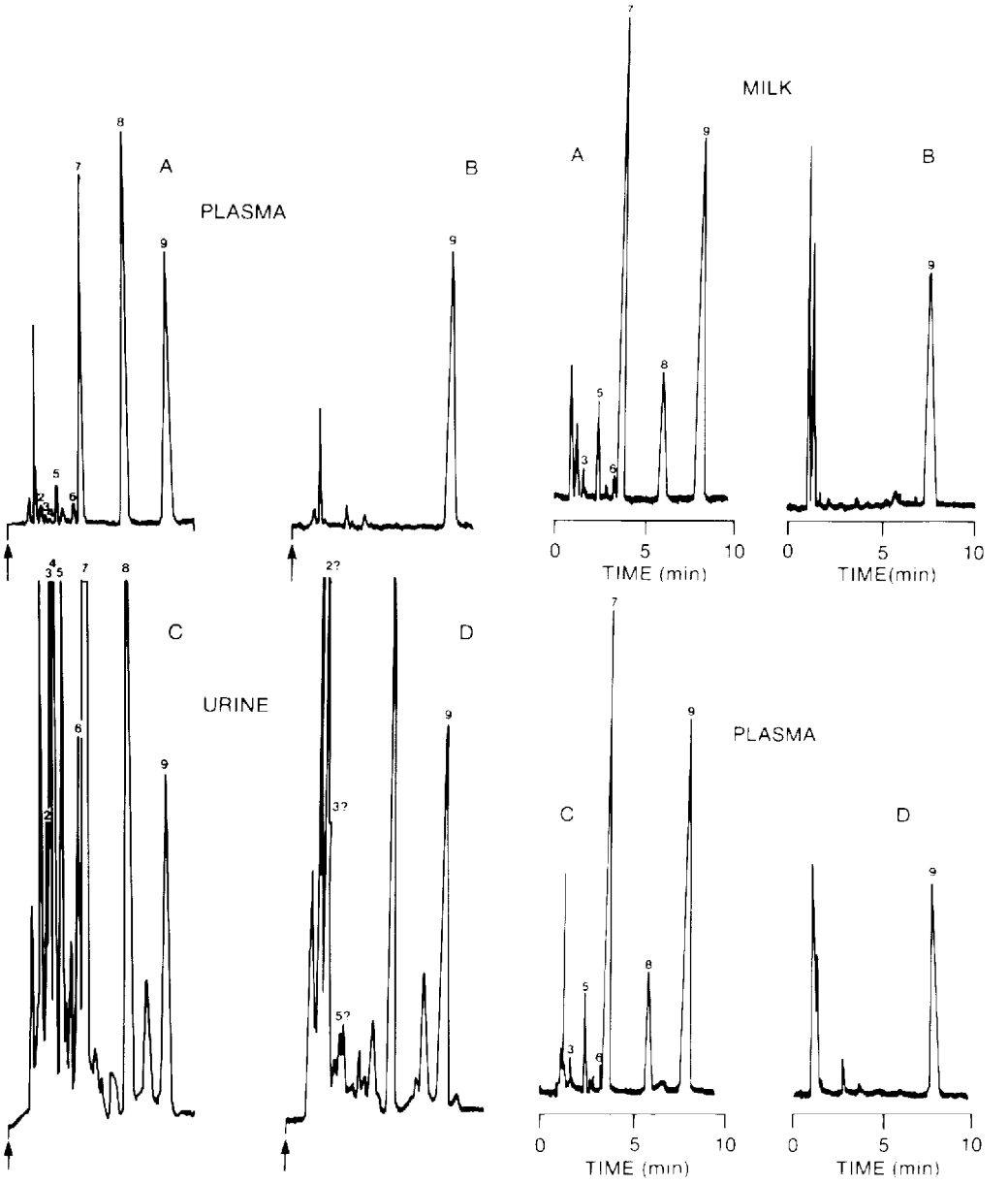

Fig. 2. HPLC profiles of caffeine metabolites in plasma (A) and urine (C) of a monkey consuming caffeine (9 mg/kg) for six months. B and D are comparable chromatograms for plasma and urine from a non-treated control monkey. For the identification of peaks see Fig. 1.

Fig. 3. The correlation between caffeine metabolites in milk (A) and plasma (C) from identical monkey treated with caffeine. B and D are corresponding controls taken from another, non-treated monkey. For peak identification see Fig. 1.

TABLE V

THE AMOUNT OF DIFFERENT METABOLITES FOUND IN PLASMA AND URINE OF A MONKEY RECEIVING 0.35 mg/ml CAFFEINE IN DRINKING WATER, COMPARED WITH A CONTROL

Values are expressed in μg/ml.

Peak number*	Compound	Plasma		Urine	
		A** Treated	B** Control	C** Treated	D** Control
3	3-MX	0.1	0.0	21.6	0.6
4	1,3-DMU	0.5	0.3	67.0	0.0
5	3,7-DMX	0.9	0.0	17.0	1.6
6	1,7-DMX	0.6	0.0	12.6	0.0
7	1,3-DMX	11.4	0.0	128.1	0.0
8	1,3,7-TMX	22.5	0.0	46.5	0.0
9	8-CT	20.0	20.0	20.0	20.0

*For the identification of the peak number see Fig. 1.
**For the chromatograms see Fig. 2.

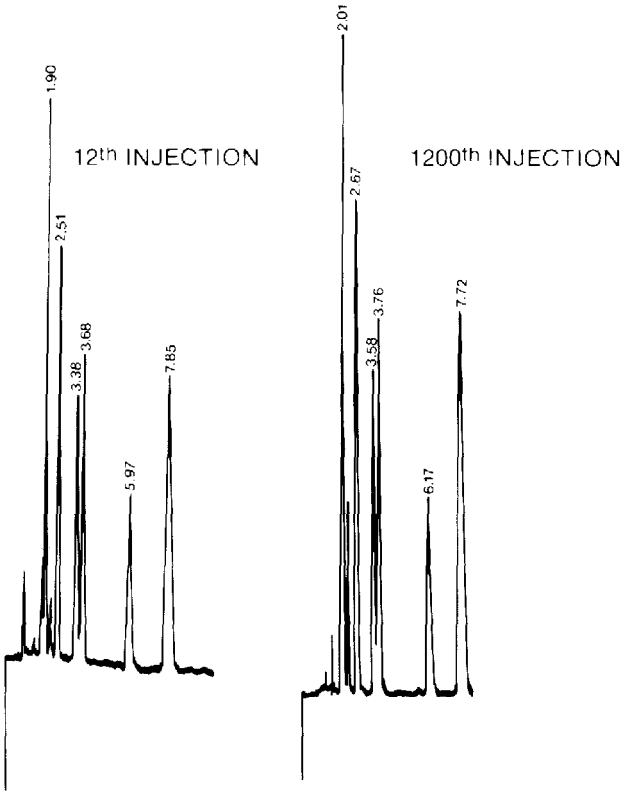

Fig. 4. The HPLC profiles of standards of caffeine metabolites from identical column routinely used for analysis of caffeine metabolites in urine and plasma for five months. Very little differences were observed between the 12th and 1200th injection.

Fig. 1 illustrates the extractability and recovery of metabolites (standards) added to plasma of a non-treated monkey. A is a chromatogram of processed plasma before spiking with standards, B is processed plasma of the same blood after spiking with 10 μg of each standard, and C is processed mixture of 10 μg of each standard. Similarly, very good recoveries for most metabolites, especially for the dimethylxanthines and caffeine, were found for other biological fluids, confirming our previous findings [11].

Typical HPLC profiles of caffeine metabolites in plasma (A) and urine (C) from one monkey consuming approximately 9 mg of caffeine in drinking water daily for six months, are presented in Fig. 2. Chromatogram B and D (Fig. 2) are comparable chromatograms from plasma and urine from another, non-treated control monkey. The concentrations (μg/ml of fluids) of metabolites found in this test are presented in Table V.

Caffeine and its metabolites were found in the milk of a treated monkey. The profiles of these metabolites are presented in Fig. 3A. Fig. 3B is the HPLC profile of milk from a control monkey. Fig. 3C is a chromatogram of metabolites in plasma of the same monkey from whom milk was also analyzed and presented in Fig. 3A.

Fig. 4 gives the HPLC profiles of seven standards using an identical column for 1200 injections of samples from different biological fluids. Very little variation, deterioration or differences were observed in the retention times and separation of peaks with time and usage of the column, when the 12th and the 1200th injection are compared. These two analyses were also incorporated in the above-mentioned evaluation of the repeatability for quantitation of standards, as presented in Table II.

DISCUSSION

The present method has advantages in analysis of caffeine metabolites over some other recently published procedures [12—14] in several aspects. This method avoids the use of elution programming, a procedure [13] that requires more time because of the time necessary for the re-equilibration of the column between runs. The time required for the separation of the caffeine metabolites in our method is less than 10 min, while others [14] need about 30 min. The procedure is equally applicable not only to urine [13, 14] but also to other biological fluids [11]. Using this method we were able to separate and quantitate several metabolites, as theophylline and paraxanthine or caffeine, the separation of which was not possible in some other procedures [12, 14].

From Tables I and II, it is clear that the repeatability, accuracy and the precision when using an automatic sampler was consistently very good. The relative retention time (RRT) for the standards within the day differs less than 0.02. Occasionally, some day-to-day variation in retention time was observed. The absolute retention time was found to fluctuate, up to 1 min, mainly due to changes of room temperature. During some weekends the room temperature was recorded as low as 18° C or as high as 28° C. Using the RRT these variations were easily recognized and corrected by adjusting the acceptable RRT stored in the data system. The difference in the RRT in the day-to-day assay for the standards, analyzed over a period of five months, was less than 0.039 (see Fig. 4).

Intra- and interday variability for the complete procedure, including the extraction from the biological fluids, gave very little variations. Tests with plasma were more consistent than tests with urine (Table III and IV).

The separation/quantitation of 1,7-DMX from 1,3-DMX as well as several other metabolites of caffeine, which was previously described using a Perkin-Elmer high speed 5 μm C_{18} column [11], has been confirmed with two other columns of the same type obtained from the same manufacturer. There were no visible differences in the performance (retention time, response factors) between these individual columns.

The durability of this column (maximum number of injections of samples) has not been reported so far. In our experience, the durability of our first column was excellent. After many months of regular injections of extracts from serum and urine, the separation of peaks, especially those of the 1,7- and 1,3-isomers of DMX was still very good.

The elution pattern of the analyzed caffeine metabolites suffered very little or no interference from certain normal constituents in blood, milk or saliva. However, there were some urine samples (see Fig. 2D) where some normal urinary constituents, such as metabolites from dietary purines (i.e. urates, xanthines), could interfere with the early eluting metabolites. No interferences were found with the major metabolites of caffine, which are eluted later.

The main objective of the feeding study was to find a possible correlation between the levels of caffeine, theophylline, paraxanthine and theobromine in the blood of monkeys during their pregnancy, with a possible neuro-behavioral effect on the infant monkey. For that purpose the method described herein was accurate enough to establish the levels of these metabolites in the blood of individual monkeys before, during and after their pregnancies. In addition, very accurate data have been obtained for the caffeine metabolites in monkey's milk and saliva and, in addition, experimental evidence has been acquired regarding the excretion of the major caffeine metabolites in urine. While this project is still in progress, the experimental data for the metabolism of caffeine during pregnancy will be published elsewhere [15].

Although some expected variabilities in metabolic pattern between individual monkeys were observed, this variation was of a more quantitative than qualitative nature. There were, however, some differences in metabolites found in blood and those excreted in urine. The most significant difference was in the absence (Fig. 3C) or traces only (as in Fig. 2A) of 1,3-DMU in blood (plasma), while there was always a considerable amount of this metabolite in the urine (Fig. 2C). Contrary to this, a very close correlation was regularly observed for the pattern of metabolites found in plasma and in milk (Fig. 3). Similar close resemblances in the content of metabolites was observed between saliva and plasma, which has been also reported for humans [16], and confirmed in our laboratories (results not shown).

Finally, the main difference in the metabolism of caffeine in humans and monkeys seems to be in the reverse ratio of two isomers of dimethylxanthines: while 1,7-DMX is the main metabolite in humans [17] with very little of the 1,3-isomer, the main metabolite in the blood of our monkeys was 1,3-DMX with considerably smaller amounts of the 1,7-isomer (Figs. 2A and 3C).

Once the procedure and the HPLC methodology have been developed, an

automated injection system combined with data system for peak area integration can be used for quantitation of caffeine and its metabolites in biological fluids. This instrumentation increases the analytical precision with additional savings in time, labor and operating cost, with very little supervision from the analyst. Using this method an analytical chemist can handle 40—60 samples of biological fluids in 24 h. The number of samples is determined by the time required for the elution of the other slower moving components between injections, which are found in some biological fluid samples (especially urine). This assay is also applicable to the analysis of caffeine metabolites in biological fluids from other species including humans.

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