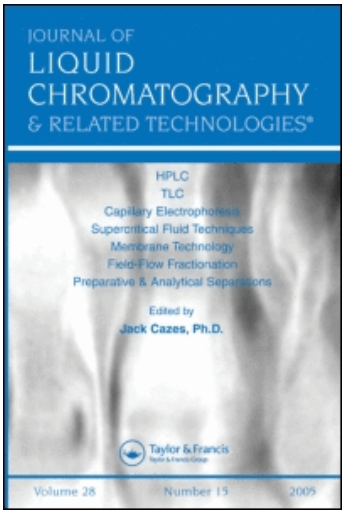

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HPLC SEPARATION OF THEOPHYLLINE, PARAXANTHINE, THEOBROMINE, CAFFEINE AND OTHER CAFFEINE METABOLITES IN BIOLOGICAL FLUIDS

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ABSTRACT

An HPLC method is described for rapid analysis of caffeine and seven of its metabolites in plasma, urine, milk and saliva in a single operation using a 5 μ C_{18} reverse phase column. The metabolites are extracted with chloroform - iso-propanol (85:15) from 100 μL samples added to NH_4HCO_3. No interference from normal blood, urine, milk or saliva constituents was observed. The method is accurate and precise and separates 1,7-dimethylxanthine (paraxanthine) from 1,3-dimethylxanthine (theophylline). Sensitivity for most metabolites is in the range of 0.1 to 0.3 μg/mL and the detectability is at the nanogram level.

INTRODUCTION

A variety of different analytical approaches have been reported for the analysis of caffeine or some of its metabolites. Some GLC procedures involve either multiple extraction steps, derivatization steps, or use of gas chromatography-mass spectrometry (1-3). Several spectrophotometric

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methods are either time-consuming (about 60 min per analysis) (4), need a large volume of material (2 ml of serum or urine) (5) or are specific for determination of caffeine only (4-6). Recently, however, HPLC seems to be the most frequently used approach (7-15). Many of these methods have been developed for analysis of caffeine or other methylated xanthines in special matrices, or to separate and quantitate only some of the metabolites (6,9,11,12). Normally these methods require a specific preparation of the sample and considerable time (23-45 minutes) for the elution from the column (8,13,14). Besides, some of the reported methods can not separate mixtures of some metabolites in a reasonable time period (8,14,15).

One frequent problem encountered by many authors was the separation of paraxanthine from theophylline, as these major caffeine metabolites have identical retention times under most solvent conditions using HPLC (8,10,14,15). One laboratory described an estimation of the relative concentration of these two metabolites by employing the ratio of two different, simultaneously monitored UV wavelengths (8), but this procedure requires special and expensive instrumentation.

Another separation of theophylline from paraxanthine was achieved with radioactive material using two dimensional thin layer chromatography and autoradiography (16), which seems to be not applicable for a routine analysis. One GLC-MS method for determination of theophylline and caffeine using derivatization with ethyl iodide, could not separate ethyl derivatives of theobromine from paraxanthine in their GC column (17).

Some authors reported a separation of paraxanthine from theophylline using normal phase HPLC, but their methods are only evaluated for plasma and only for some caffeine metabolites (7,12). When the work described in this report was already developed, a paper by Tse and Szeto (18) was published, in which a separation of three major caffeine metabolites was achieved using a reverse phase HPLC. Although their procedure separates N-demethylated caffeine metabolites in (dog) plasma, its application for other biological fluids (specifically urine) and other oxidative metabolites has not been investigated.

We studied the paraxanthine-theophylline separation using different columns and/or a variety of elution solvents with blood, urine, milk and

saliva as an analytical substrate. In this report we describe a successful procedure, which is an improved upon version of several previously reported methods (7,14,19).

EXPERIMENTAL

Materials

Standards. Sources of caffeine and its potential metabolites were: 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): Eastman Kodak Co., Rochester, N.Y.; xanthine (X) and 3,7-dimethyluric acid (3,7-DMU): Sigma Chemical Co., St. Louis, MO.; paraxanthine (1,7-dimethylxanthine or 1,7-DMX), 7-methyluric acid (7-MU), 3-methylxanthine (3-MX), 7-methylxanthine (7-MX) and 1-methylxanthine (1-MX): Tridom/Fluka, Toronto, Ont.; 1,3-dimethyluric acid (1,3-DMU): Adams Chem. Co., Round Lake, ILL. 8-Chlorotheophylline was purchased from ICN Pharmaceutical Ltd.

All the standard stock solutions (10 µg/ml) and the internal standard, 8-chlorotheophylline (20 µg/ml) were prepared in 0.9% NaCl solution.

Instrumentation and Operating Procedure

A Perkin Elmer High Performance Liquid Chromatograph, Series 2/2 with a Rheodyne 7125 injector (50 µL loop) was used. Column was a Perkin-Elmer/HS-5 C 18, 5 µm (15 cm; 4.6 mm I.D.) (Cat. Number 0258-1001). The column was at ambient temperature (approximately 22°C) and the flow rate set at 1.5 mL/min (1800 psi). Sample volume of 20 µL were injected (Hamilton 100 µL syringe) per analysis. Detector was a Perkin-Elmer LC-55 instrument with 8 µL flow cell. The UV wavelength was set at 276 µm. A Perkin Elmer Sigma 10 Data Processing system with an input of 1 v was used for recording and manipulation of data.

The mobile phase was prepared daily. It consisted of 10 mL acetic acid, 40 mL acetonitrile (HPLC purity), and 40 mL iso-propanol (glass-distilled) in 910 mL of twice distilled water. It was degassed for 2 minutes using a Branson ultrasonic cleaner (Branson Cleaning Equipment Co., Shelton, Conn.) and water pump vacuum.

Sample Preparation

Pipette (Eppendorff-pipetter) 100 µL of the standard solution or sample and add it to a prepared 16 x 150 mm test tube containing 1.2 g NH_4HCO_3 and 100 µl of the internal standard. Add 8.0 mL of the extracting solvent chloroform-isopropanol (85:15) and agitate test tube using a Vortex mixer for 30 seconds. Allow NH_4HCO_3 particulates to settle for approximately 2 minutes. Filter the supernant to remove undissolved NH_4HCO_3 (e.g. through a polypropylene 10 cm long disposable column with Ottawa sand covered bottom, approx. 1 mm depth). Repeat the extraction and pool the filtrates in a test tube. Evaporate the filtered extract to dryness at 50° under a stream of nitrogen. Add 200 µL of the HPLC eluting solvent, mix well, insert the test tube for 15-20 seconds in a warm (50°) water bath, and then sonicate for 10 seconds to ensure complete dissolution.

RESULTS

Fig. 1 shows the separation of the mixtures of caffeine and its metabolites obtained with the described HPLC procedure. Table I gives the actual elution times of individual metabolites and their relative retention times. The variation in the elution time in the day-to-day analysis was less than $\pm$ 5% . Also little variation was observed in the peak area of metabolites in the mixtures of standards within a day or day-to-day operations.

There was no observed interference from normal blood, milk, or saliva constituents. Fig. 2 shows the profile of caffeine metabolites and

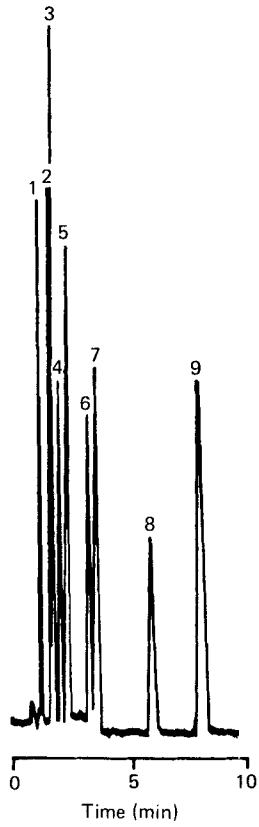

FIGURE 1: The HPLC separation of caffeine and its metabolites.
1 3-MU; **2** 3,7-DMU; **3** 3-MX; **4** 1,3-DMU; **5** 3,7-DMX; **6** 1,7-DMX; **7** 1,3-DMX;**8** 1,3,7-TMX; **9** 8-chlorotheophylline (int. stand). The actual amounts: **1** to **8** 0.2 µg/peak; **9** (int. stand.) 0.4 µg/peak.
The column: Perkin-Elmer/HS C18, 5 µm. Mobile phase: 1% acetic acid, 4% acetonitrile and 4% iso-propanol in water. Other experimental conditions are described in the text.

unchanged caffeine found in plasma of monkey consuming caffeine in drinking water. In some urine samples some additional peaks were obtained, but most were well separated from the major caffeine metabolites. However, there is a possibility of interference from some normal urinary constituents, such as the dietary purine metabolites (urates, xanthines), which elute close to the solvent front.

TABLE 1

Peak Number	Standard	Elution Time in minutes*	RRT**
1	3-MU	1.38	0.165
2	3,7-DMU	1.81	0.216
3	3-MX	1.93	0.230
4	1,3-DMU	2.23	0.266
5	3,7-DMX	2.56	0.306
6	1,7-DMX	3.19	0.417
7	1,3-DMX	3.83	0.458
8	1,3,7-TMX	6.23	0.745
9	Int. Standard***	8.36	1.000

* Actual numbers from a typical HPLC run.

** Relative Retention Time: Ratio between the elution time of the peak vs. that of the internal standard.

*** Internal Standard: 8-Chlorotheophylline.

Fig. 3 shows the profile of caffeine metabolites in the amniotic fluid of a pregnant sheep, when caffeine was introduced i.v. into fetal blood.

DISCUSSION

During the preliminary experiments to develop a procedure to separate 1,7-DMX from 1,3-DMX as well as from other caffeine metabolites, we tested several commercially available HPLC columns with the eluting solvent of 1% acetic acid and 6% acetonitrile in water. [The following columns were used: Bio-Sil ODS-5S Reverse-Phase, 5 µm; Partisil-10 ODS-2, 10 µm C 18; Alltech C 8, 10 µm; Alltech C 8, 5 µm; and Perkin-Elmer/HS-3 C 18 High Speed C_{18} 3µm; the efficiency of

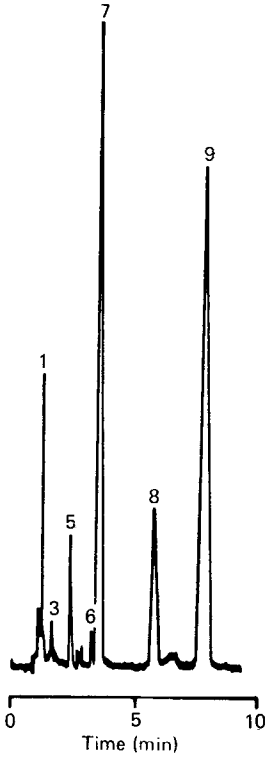

FIGURE 2. The HPLC separation and quantitation (µg/mL in brackets) of caffeine and its metabolites in blood (plasma) of monkey. **1** 3-MU (33.3); **3** 3-MX (1.8); **5** 3,7-DMX (2.1); **6** 1,7-DMX (0.9); **7** 1,3-DMX (15.6); **8** 1,3,7-TMX (5.9); **9** int. standard. The column and experimental condition as in Fig. 1.

these columns were checked according to the manufacturer's specifications.] The separation of 1,7-DMX from 1,3-DMX was successful only with the Alltech 5 µm column and the Perkin-Elmer 5 µm HS column. The separation with the Alltech column was however, short lived. A similar problem of loss of resolution for some caffeine metabolites was recently reported using the Altex 5-µm ODS, 25 cm column (10). Although they did not separate 1,3-DMX from 1,7-DMX, they were able to correct the loss of resolution for other metabolites by over-night flushing of the column with dilute methanol. A very similar treatment in our laboratory

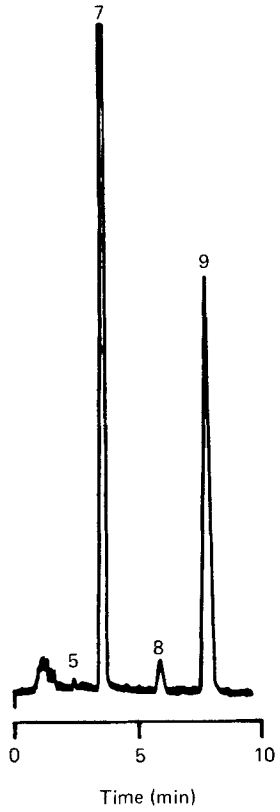

FIGURE 3. The HPLC profile of caffeine and its metabolites in the amniotic fluid from a ewe's fetus treated with a single I.V. dose (30 mg) of caffeine. The ammiotic fluid was collected 53 h after the dosing. **5** 3,7-DMX; **7** 1,3-DMX; **8** 1,3,7-TMX; **9** int. standard.

was unsuccessful in re-generating the initial ability of the Alltech 5 µm column to separate the 1,3-from the 1,7-isomer.

In the second phase of the preliminary tests, different eluting solvents were tried, and the best separation was obtained using the eluting solvent of 1% acetic acid, 4% acetonitrile and 4% iso-propanol in water.

After we achieved a good separation on the 5 µm reverse phase column (Perkin-Elmer/HS-5) with 10,000 efficiency plates, it was expected that using the same eluting solvent system, even better resolution could be obtained in the 3 µm column (Perkin-Elmer/HS-3) with 15,000 plates. But

this was not the case. Difference in the form of the particles of the stationary phase of the column (spherical vs. non-spherical) could be a contributing factor for this observation.

The individual and mixed standard solutions were extracted as described. These extracted standard solutions were used to obtain the relative retention times and relative response factors for each individual standard. These factors were obtained by the Sigma 10 integrator and used for the identification and quantitation of the individual compounds in the samples analysed. Information on recoveries was obtained by adding 1 ml of the standard solution (10 µg/mL) to the blood (plasma), urine, milk, saliva, coffee, or soft drinks before extraction.

The recovery studies of the individual standards or their mixtures added to different biological fluids showed a different, but constant recovery for each metabolite. This is not surprising, since a large difference in physico-chemical properties exists between the methylxanthines and the methyluric acids (13). In our procedure we were concerned with more complete recoveries of the methylxanthines and 1,3-DMU, which were found to be the major caffeine metabolites in monkey (19). The recoveries correlated with the solubility of the particular metabolite in the extraction solvent: 3-MU has a very low solubility, and its recovery was less than 10%. Recoveries for mono- and di-methylxanthines and caffeine from blood or urine were better than 92%, for dimethylxanthines and caffeine usually between 95 to 100%, while the average recovery of the 1,3-DMU was in the 60% range. By calibrating the integrator with a standard solution that has been extracted as described, it is possible to obtain the actual amounts of individual caffeine metabolites initially present.

Usefulness in using a combination of organic solvent - salt for increasing the extraction efficiency has been already reported (12). Instead of using crystalline ammonium sulfate (13) or saturated solution of ammonium sulfate (12,14) we employed crystalline ammonium hydrogen carbonate, which yielded lower backgrounds for blank biological fluids then ammonium sulfate and gave good extractibility for caffeine and its metabolites.

We used 8-chlorotheophylline as internal standard, which is well resolved in our HPLC system from caffeine and other metabolites. It is

not a natural component but has very similar structure to methylxanthines and was used with good results as internal standard by other investigators of caffeine metabolites analysis (8,19).

Some additional caffeine metabolites were also separated in this system. For instance, 1-methyl xanthine was eluted as a separate peak between 1,3-DMU and 3,7-DMX and 1-methyl uric acid eluted between 3-MU and 3,7-DMU. These compounds were not included in our regular standard mixture, primarly because these metabolites were not found in our preliminary study when the whole blood from treated monkey was injected into HPLC. If necessary, additional metabolites could be 'added' into the standard mixture, but for separation, the elution time should then be extended by decreasing the amount of the non-polar solvent in the eluting mobile phase.

The described procedure was found to be equally applicable for the samples of blood, serum or plasma as well as for urine, milk and saliva and it was also successfully used for quantitation of caffeine, theophylline and theobromine in samples of coffees and teas. The results of analysis using this procedure to study the caffeine metabolites in urine, plasma, milk and saliva of monkeys will be published elsewhere (20).

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