The Simultaneous Analysis of Theophylline, Caffeine and Their Metabolites in Human Plasma by HPLC

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Summary: Theoplline, caffeine and their important metabolites were extracted from human plasma using newly developed solid support liquid/liquid extraction tubes. These substances were separated on a 3 µm ODS (250 x 4.6 mm i.d) column with a mobile phase gradient and detected with a UV diode array detector set at two wave lengths of 270 and 290 nm.
The normal ranges for the plasma drug/metabolite molar ratios have been determined.

Introduction

Caffiene and its demethylated metabolites are ubiquitous in human plasma due to the dietary intake of tea, coffee and many soft drinks [1]. Theophylline and coffeine are also known asrespiratory stimulants in premature new-born infants [2].

Theophylline is as well frequently prescribed for the treatment of asthma and chronic obstructive airways diseases [3,4]. Many of the metabolites of theophylline which are formed in plasma are pharmacologically active and effects additive to those of the parent drugs [5].

Besides, many co-administered drugs inhibit the hepatic cytohcrome P450 system which may cause theophylline toxicity [6]. Hence, the quantitation of the plasma levels of methylxanthine metabolites together with the parent drugs is a very vital analysis of these compounds to enable information concerning the nature of cytochrome P450 mediated metabolism.

In this study, we have developed an high-performance liquid chromatographic (HPLC) method of analysis with the detection procedure based on diode-array detection kept at 270 and 285 nm to qualtitate these metabolites along with the parent drugs in one chromatographic system.

Results and Discussion

Fig. 1 shows a chromatogram of standard mixture and Fig. 2 shows the chromatogram of plasma samples.

The precision test conducted on the within- runs which showed the data obtained after 10 chromatographic runs giving coefficient of variations (CV) of 3 and 5 at the concentrations of 50 mmol and 5 mmol respectively.

For patients with normal hepatic cytochrome P450 and renal function, the plasma levels of methyl-

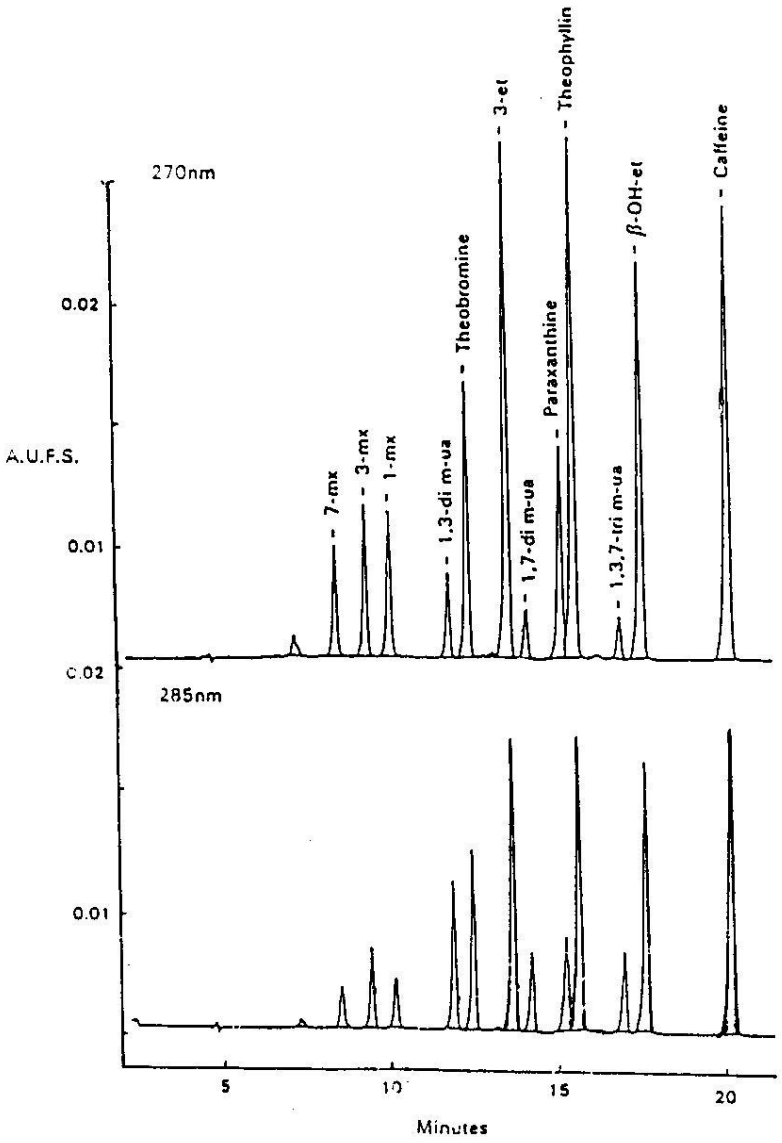

Fig. 1: Chromatogram of current standard calibration plasma. In retention order, the concentration are: 7-methylxanthine (7- mx)=1.3 mmol/1, 3-methylxanthine (3-mx)=15.9 mmol/1(1,1-methyxanthine (1-mx) 17.1 mol/1, 1-3-dimethyluric acid (1,3-dimua=21.3 mmol/1, theobromine=27.5 mmol/1. 1,7-dimethyluric acid (1,7 di mua)=10.2 mmol/1, paraxanthine=24.5 mmol/1, theophylline=57.7 mmol/1, 1,3,7-trimethyluric acid (1,3,7-tri mua)=9.3 mmol/1 and caffeine=61.4 mmol/1.

xanthine metabolites are known to depend upon the concentration of the parent drugs. Therefore, molar ratios were selected which are the most obvious and the convention drug/metabolite was chosen because the whole numbers normally obtained are more convenient to assess than the reciprocal values.

Some metabolites may be formed from both compounds i.e. theophylline and caffeine as are shown in Fig. 3. For example 3-methylxanthine is a dimethylated product of both theobromine and theophylline and therefore a ratio between theophylline and 3-methylxanthine in presence of theobromine may not accurately reflect the demethylation of theophylline or the clearance of 3-methylxanthine [7,8]. However, this difficulty could be overcome to some extent by inclusion of both possible sources of metabolites which in this case would be given as (theobromine + theophylline)/3-methylxanthine. This solution can only hold provided the demethylation of both compounds remains even.

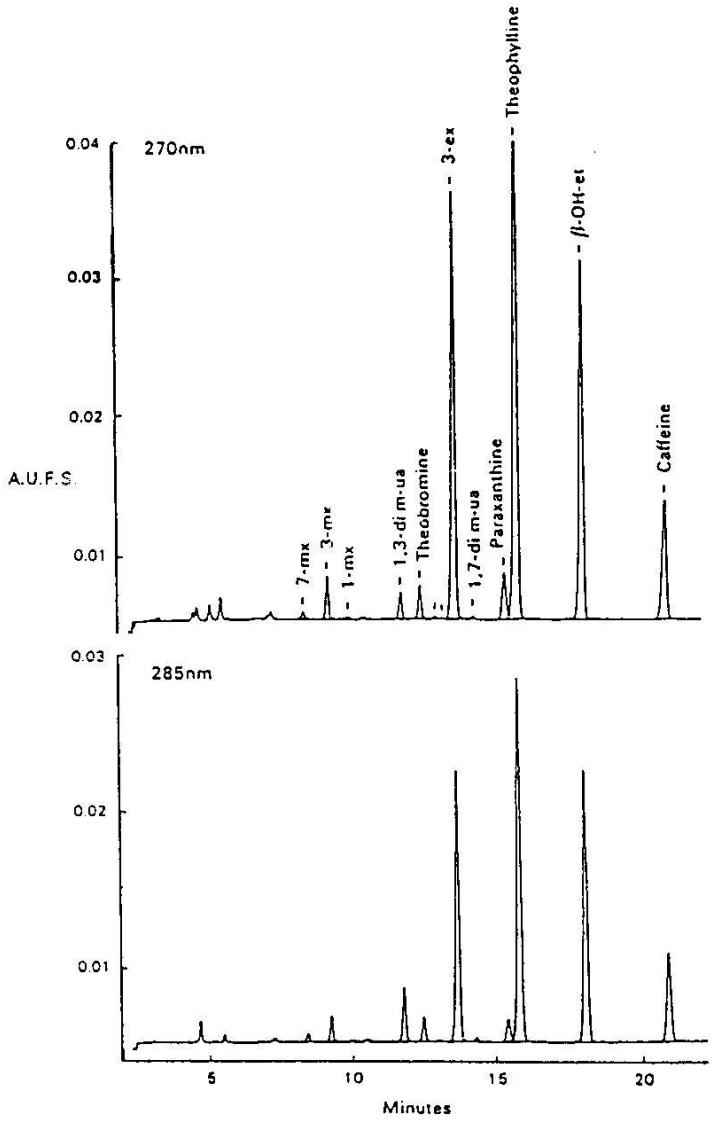

Fig. 2: Chromatographic profile of a normal subject who has taken theophylline within the recommended therapeutic range and moderate coffeine intake.

Table 1 shows the preliminary results on the drug/metabolite ratios since the majority of the samples used in our study were apparently taken at random with respect to dose and time. Previously a study [9] has shown that these ratios very depending on the dose to sample interval and thus, better documentation is necessary to establish the normal ranges with more precision.

In conclusion, we have shown that with our HPLC assay, it is possible to quantitate caffeine, theobromine, theophylline and most of their N-demethylated and C8-oxidized metabolites in plasma. The method of analysis has a potential and could be used to identify abnormal metabolites in several cases of unexpected theophylline toxicity. The use of drug to metabolites ratios compensates for large differen-

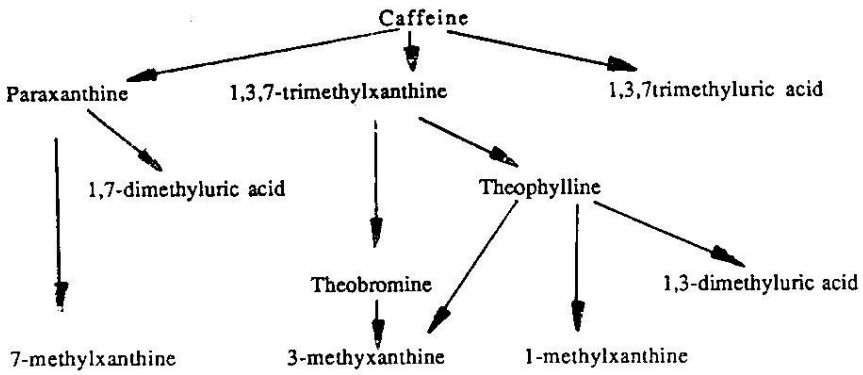

Fig. 3: The metabolism of caffeine and its interrelationship with methylxanthine and methyluricacid in plasma.

Table 1: Group 1: Subjects treated with coffeine (n=16)

Ratio range	Mean	SD	Suggested
Caffeine/Paraxanthine	57.4	21.63	5-79
Caffeine/Theobromine	52.4	21.5	31-74
Caffeine/Theophylline	39.6	13.7	26-53
Group 2: Subjects treated with theophylline (n=19)			
Theophylline/Caffeine	3.7	2.7	1.0-6.4
Theophylline/1,3-dimethyluric acid	22.2	9.01	3.0-31.5

SD=Standard deviation

ces in concentrations and facilitates the distinction between toxicity due to the failures of metabolites and toxicity due to overdose. Therefore, this assay could elucidate impaired hepatic cytochrome P450 mediated clearance of methylxanthine caused either by any disease or by co-administed drugs.

Experimental

Apparatus

The HPLC system consisted of a gradient pump (series 2.2), an automatic sampling system (1SS-100 model), an UV diode array detector (LC-135 model) - all these units supplied by Perkin-Elmer Ltd., New York, USA. An HPLC column block-heater and chromatography work station (Maxima 1M) were obtained from Jones Chromatography Ltd., London, U.K. For the solid-liquid extraction purpose, Celute-Mx tubes and the analytical column 3 µm - ODS Apex I (250 x 4.6 mm i.d) were obtained also from Jones Chromatography Ltd., London U.K. Polyster (0.4 um) filter membranes were obtained from Nucleopore Sterilin Ltd., London, U.K.

Mobile phase

Water used for preparation of mobile phase and standards solutions was polished with Barnstead Water System I (obtained from Gallen Kamp Ltd., London, U.K). This removed contaminants that accumulate in water during its storage and which was responsible for elevated back pressures in the HPLC system.

Stock sodium acetate (20 mmole/l) was adjusted to pH 4.0 with concentrated acetic acid and this buffer was vacuum filtered by passing through 0.4 um polyster membrance. This buffer solution was diluted with the equal amount of distilled water.

Mobile phase A consisted of 25% acetonitrile and 2% tetrahydrofuran and 73% of 10 mmol/l acetate buffer (v/v).

Mobile phase B consisted of 0.02% tetrahydrofuran in 10 mmol/l acetate buffer. Both these solutions were vacuum filtered through 0.4 um polyester membrane before use. Fresh mobile phases were made every day.

The analytical column was maintained at 50°C and the flow arte of 0.8 ml/min was maintained throughout the chromatographic run.

The gradient was started at 100% B increasing linearly with mobile phase A at 2% /min for 40 min

before maintaining at the initial condition for the 10 min prior to the next injection. Channels A and B of the UV detector were set at 270 nm and 285 nm respectively both having a band- width of 15 nm.

Standard

All standards of the reference substances with 100 umol/l concentrations were made in acetate buffer. 3-ethylxanthine was used as the internal standard.

Sample Preparation Procedure:

The systematic scheme is shown below:

Plasma (100 µl) + internal standard (35 µl)

(Mix)

↓

Coelute-Mx

(Dedicated solid support sample preparation tube)

↓

elute

(after 10 min. 10% isopropanol in dichloromethane)

↓

Evaporate

(ambient temp., under oxygen-free nitrogen

↓

Reconstitute

(100 µl Mobile phase B)

↓

Micro-vials

↓

Centrifuge

↓

Inject

(25 µl)

The mixture containing 10,20,30,50 and 100 umol/l concentrations of the reference substances were used to construct the linear relationship between the concentration and UV signal.

All chemicals used were obtained in the puriest quality from Sigma Chemicals Co., St. Louis, USA.

Plasma samples

19 and 16 healthy subjects each were given tablet containing 2 mg coffeine and theophylline respectively. After 2 hrs., 10 ml of blood sample from each individual was collected in heparin containing tubes Plasma samples were separated after centrifuging the blood samples at 2500 x g for 15 mines. These samples were processed as given under sample preparation procedure.

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