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Determination of theophylline and its metabolites in rat liver microsomes and human urine by capillary electrophoresis

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Abstract

A capillary electrophoretic (CE) method has been developed for the determination of theophylline and all of its identified and potential metabolites. The method is rapid, resolves all metabolites to baseline, and requires extraction of only some biological fluids. It has been applied to the analysis of theophylline metabolism by hepatic microsomes from rats treated with a variety of inducing agents for different forms of P450 enzymes which metabolize theophylline, and to human urine spiked with theophylline and its metabolites, and concentrated by solid-phase extraction.

1. Introduction

Theophylline (1,3-DX) is one of the primary caffeine metabolites, and is widely used as a bronchodilating agent in the treatment of chronic obstructive pulmonary disease or asthma. It has long been known that the majority of theophylline (90%) is metabolized in human liver by several forms of P450 (Fig. 1) [1]. The major metabolic route, which accounts for 45–55% of the total clearance, is 8-hydroxylation to 1,3-dimethyluric acid (1,3-DU). 1-Methylxanthine (1-X), which is further metabolized to 1-methyluric acid (1-U) by xanthine oxidase [2], and 3-methylxanthine (3-X) account for 10–13% and 20–25% of theophylline clearance, respectively. Other potential metabolic pathways are the demethylation of 1,3-DU to 1-U or 3-methyluric acid (3-U) and the hydroxylation of 3-X to 3-U [3], but these have not been reported in humans. Based on published results, 3-X, 1-X, and 1,3-DU are probably the only theophylline metabolites formed by P450 catalysis. The P450 enzymes involved in theophylline metabolism have been identified in human liver microsomal incubation assays by inhibition of metabolite formation with specific anti-rat P450 antibodies [4]. The results indicated that the three most likely P450 candidates which participate in the metabolism of theophylline are P4501A2, P4503A3, and P4502E1. Our recent studies on theophylline metabolism in humans confirmed that P4501A2 and P4502E1 are two enzymes catalyzing the majority of theophylline 8-hy-

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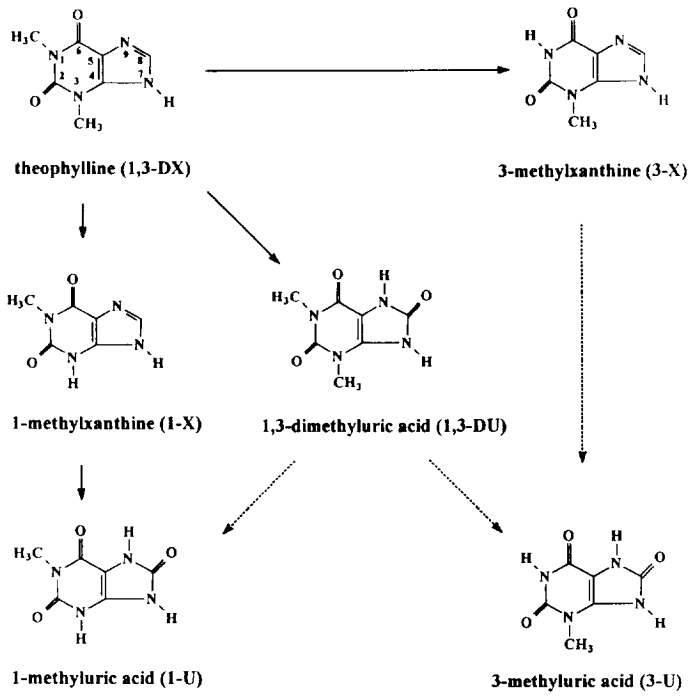

Fig. 1. Theophylline metabolic pathways in humans. → Established pathway, · · · · > potential pathway.

droxylation, while P4502D6 and P4503A4 also have some catalytic activity [5].

HPLC is the most frequently used method for the analysis of theophylline metabolites in biological samples [6–11]. Sarkar and Karnes [11] detected the three major metabolites of theophylline in human liver microsomes, using an HPLC method which had several advantages over previously reported methods in that ion-pair agents or radiolabelled theophylline were not required. Recently, CE has become increasingly popular for the analysis of pharmaceutical compounds and metabolites [12–15]. Thormann et al. [14] separated ten of the caffeine metabolites including theophylline, 1-U, 3-X, and 3-U within 12 min on CE from human serum, saliva, and urine samples. HPLC and CE techniques yielded consistent results in the phenotyping of human N-acetylation by measurement of the ratio of two caffeine metabolites, 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-X, in urine. These results demonstrated the lack of necessity for sample extraction when CE was used because of the occurrence of less interferences [15].

We report here on a novel application of CE to separate all possible theophylline metabolites, including the major metabolite 1,3-DU, which extends the method of Thormann et al. [14]. We have applied the method to monitor these metabolites in incubation media of rat hepatic microsomes containing a variety of specifically induced P450s. Our results demonstrated a diminished interference with the quantitation of 3-X and 1-X, which will obviate the necessity for sample extraction, as well as shorter separation times for theophylline and its metabolites on CE relative to those achieved by HPLC [11]. Furthermore, we successfully applied our method to analyze theophylline and its metabolites in spiked human urine after clean-up by solid-phase extraction. CE has thus been shown to be one of the techniques of choice for the determination of theophylline metabolism both in vitro and in

vivo. We propose to apply the method to a non-invasive test for human P450s using theophylline as a probe.

2. Experimental

2.1. Chemicals

Theophylline, 1-X, 1-U, 3-X, isosafrole (ISO), and sodium dithionite were purchased from Aldrich (Milwaukee, WI, USA); and 3-U, 1,3-DU, 7-(β-hydroxypropyl)theophylline, sodium dodecyl sulfate (SDS), NADPH, Trizma base, and β-naphthoflavone (BNF) from Sigma (St. Louis, MO, USA). Sodium borate was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Sodium dihydrogen phosphate and sodium phenobarbital (PB) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Musk xylene or 2,4,6-trinitro-5-*tert*.-butyl-xylene (MX) was purchased from Penta International (Fairfield, NJ, USA). BCA protein assay reagents were purchased from Pierce (Rockford, IL, USA).

2.2. Theophylline metabolism by rat liver microsomes

Groups of three rats were dosed by i.p. injection with ISO (50 mg/kg/day for 3 days), BNF (40 mg/kg/day for 3 days), MX (50 mg/kg/day for 3 days), or PB (100 mg/kg/day for 2 days). The rats were fasted for 12 h prior to sacrifice by CO_2 asphyxiation 1 day after the last dose. The livers were perfused with physiological saline and microsomes prepared as previously described [16]. The isolated microsomes were stored at −80°C. Microsomal protein concentrations were determined using the BCA protein method [17], while the P450 contents were determined spectrophotometrically using a Beckman DU-70 spectrophotometer [18]. The reaction incubation mixture, in a total volume of 0.50 ml, contained 1.0 mg microsomal protein, 1.0 mg NADPH, 10 mmol theophylline in 0.05 *M* Tris, and 0.015 *M* magnesium chloride. The reaction was initiated by the addition of NADPH solution (20 mg/ml, 50 μl), and incubated with shaking at 37°C for 10 min for rate determinations and for up to 60 min in other experiments. The reaction was terminated by quickly cooling the tubes on ice, and an internal standard solution, 7-(β-hydroxypropyl)theophylline (5 μg, 50 μl), was added. The reaction solution was clarified by centrifugation for 5 min using a Beckman Microfuge E microcentrifuge and the supernatant was filtered through a syringe filter (24 mm, 0.2 μm) into a 0.5-ml CE loading vial.

2.3. Urine clean-up by solid-phase extraction (SPE)

Urine (4 ml) was spiked with 200 μl (20 μg/ml) of standard stock solution of theophylline metabolites and was then acidified with 1 *M* HCl to pH 4.8. The solution was loaded onto a SPE column (Waters C_{18}, 6 ml column volume), which had been conditioned with methanol (5 ml) and water (5 ml) washes. The SPE column was washed with 0.01 *M* ammonium acetate (4 × 5 ml), and then chloroform–isopropanol (19:1, v/v; 3 × 5 ml) was used to elute the metabolites. The eluate was collected and dried in an evaporator at 40°C under a stream of nitrogen. Finally, the residue was dissolved in acetic acid–THF–methanol–water (0.05:0.75:6.5:92.7, v/v) (400 μl).

2.4. Conditions for capillary electrophoresis

The separation buffer consisted of 100 m*M* sodium dihydrogen phosphate in 100 m*M* sodium borate (pH of 8.5), with 200 m*M* SDS solution in a ratio of 7:12 [15]. The pH of the buffer was adjusted to 6.5 by the addition of 3 *M* HCl.

A Waters Quanta 4000 CE system with a Waters SIM Box, Waters uncoated capillary (75 μm I.D. × 60 cm to the detector window, 67 cm total length), and a Millennium version 2.00 software package were used. Sample injection was performed in the hydrostatic mode for 20 s. Prior to injection of an aqueous solution of standards, the capillary was purged with running buffer for 3 min, and for 6 min prior to injection of incubation media and urine samples. The

applied purging vacuum pressure was 2 kPa. The separation potential was 10 kV for microsomal samples and 9 kV for spiked urine samples. The running time was 35 min for all solutions.

3. Results

A typical CE separation of theophylline metabolites (10 μg/ml) is shown in Fig. 2. Resolution of all metabolites was achieved by optimizing the buffer pH at a constant potential, which in this experiment was 10 kV (Fig. 3). At the optimal buffer pH of 6.5, CE resolved all metabolites and the parent theophylline to baseline with 3-X and 1-X being the least well resolved. At higher buffer pH values, pH > 6.5–8.5, these two metabolites were better resolved but the resolution of other components deteriorated. The mobilities of all the metabolites decreased as buffer pH was increased. Precipitation of the buffer reagents at pH values below 6.0 prevented studies at a lower pH. The first peak at approximately 13.5 min was always present in the electropherograms and was due to the difference between the buffer composition of the injected samples and that of the running buffer.

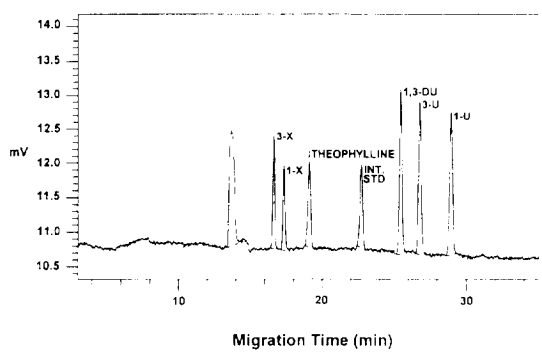

Fig. 2. Electropherogram of an aqueous solution of theophylline, its metabolites 1,3-dimethyluric acid (1,3-DU), 1-methylxanthine (1-X), 3-methylxanthine (3-X), 1-methyluric acid (1-U), and 3-methyluric acid (3-U), and the internal standard, 7-(β-hydroxypropyl)theophylline. A Waters capillary (75 μm I.D. × 60 cm) was used at a potential of 10 kV. The separation buffer was 100 m*M* NaH_2PO_4 in 100 m*M* $Na_2B_4O_7$ (pH 8.5), with 200 m*M* SDS solution in a ratio of 7:12, adjusted to pH 6.5 with 3 *M* HCl. Concentration of each compound is 10 μg/ml.

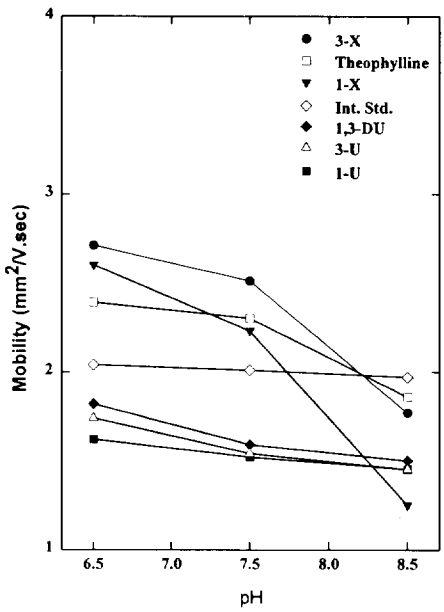

Fig. 3. Effect of buffer pH on the CE mobilities of theophylline and its metabolites. Abbreviations and experimental conditions as in Fig. 2.

The effect of CE running potential on metabolite migration times was also investigated in pH 6.5 buffer (Fig. 4). The migration times for all metabolites as well as their resolution decreased when the potential increased from 10 to 20 kV. Between 20 and 30 kV migration times did not change. Over this range the order of migration of the metabolites did not change. Since migration times are important for identification of the metabolites, their within-day and day-to-day reproducibility was investigated (Table 1). In both cases shifts were small (< 3.6%), with higher day-to-day run shifts probably being a function of small buffer batch-to-batch variability. Calibration curves of theophylline and its metabolites were constructed from serial dilutions of the stock standard solution spiked with a fixed amount of internal standard, 7-(β-hydroxypropyl)theophylline (100 μg/ml). The plots in the range 1–100 μg/ml were all linear with high correlation coefficients ($r^2 > 0.995$) and passed virtually through zero. The detection limits were

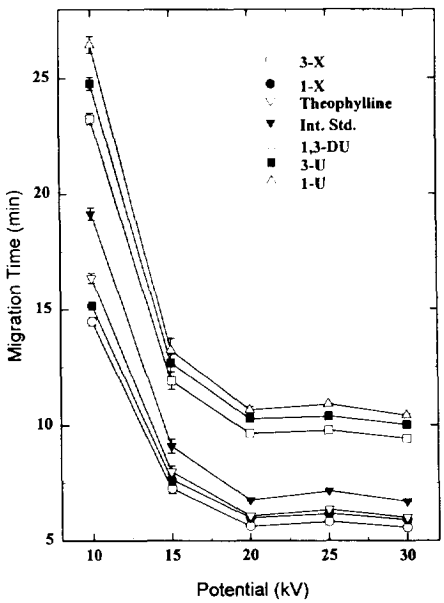

Fig. 4. Effect of CE potential on the migration of theophylline and its metabolites. Each data point represents the mean of four runs. Abbreviations and experimental conditions as in Fig. 2.

about 1 μg/ml for theophylline and its metabolites for a 20-s injection.

An example of an electropherogram of theophylline and its metabolites obtained from theophylline (10 nmol) incubated with liver microsomes from PB-induced rats for 20 min, is shown in Fig. 5. All detectable metabolites were well resolved without interference from other contaminants. The effect of incubation time on the quantities of metabolites formed with hepatic microsomes from PB- and MX-induced rats is shown in Fig. 6. The metabolite formation deviated from linearity with incubation times longer than 20 min and thus a 10-min incubation period was selected for determination of the rates of metabolite formation (Fig. 7). The metabolites 3-X, 1-X and 1,3-DU were all detected in the media of each microsomal prepara-

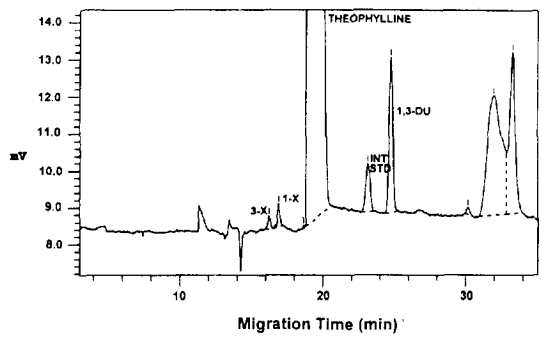

Fig. 5. Electropherogram of theophylline and its metabolic products catalyzed by hepatic microsomes from phenobarbital-induced rats. The incubation medium was injected directly onto the CE capillary without prior extraction. Abbreviations and experimental conditions as in Fig. 2.

Table 1
Within-day and day-to-day run migration time shifts for CE analysis of theophylline and its metabolites

Metabolite	Migration time (min)[a]	
	Within-day[b]	Day-to-day[c]
3-X	13.67 ± 0.12	15.32 ± 0.26
1-X	14.21 ± 0.12	15.88 ± 0.27
Theophylline	15.53 ± 0.15	17.61 ± 0.32
Internal standard	18.23 ± 0.19	21.50 ± 0.48
1,3-DU	20.47 ± 0.19	23.21 ± 0.74
3-U	21.53 ± 0.20	24.60 ± 0.87
1-U	23.30 ± 0.22	27.60 ± 1.00

[a] Values are mean ± S.D.; n = 12 (within-day) and 5 (day-to-day).
[b] CE at 10 kV.
[c] CE at 9 kV.

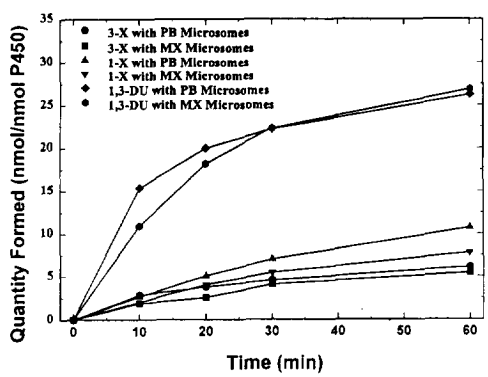

Fig. 6. Time course of theophylline metabolite formation catalyzed by hepatic microsomes from phenobarbital (PB)- and musk xylene (MX)-induced rats. Metabolites were quantified by CE analysis. Abbreviations and CE assay conditions as in Fig. 2.

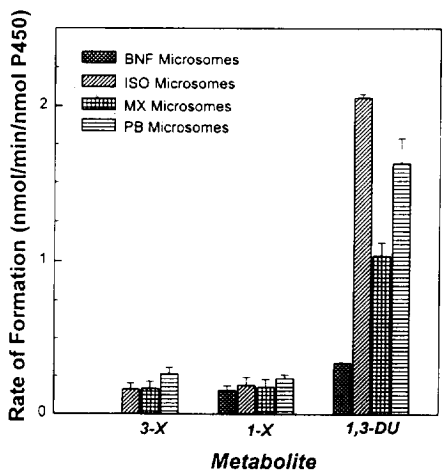

Fig. 7. Rates of formation of metabolites of theophylline catalyzed by hepatic microsomes from β-naphthoflavone (BNF)-, isosafrole (ISO)-, PB-, or MX-induced rats. Metabolites were quantified by CE analysis. Abbreviations are provided in Figs. 2 and 6; CE assay conditions as in Fig. 2.

tion, except for 3-X in microsomes from BNF-induced rats. In all cases the rate of 1,3-DU formation was higher than those for 3-X and 1-X. The former was in the range 0.3–2.1 nmol/min/nmol P450, while the latter were below 0.3 nmol/min/nmol P450. In no case 3-U or 1-U were detected.

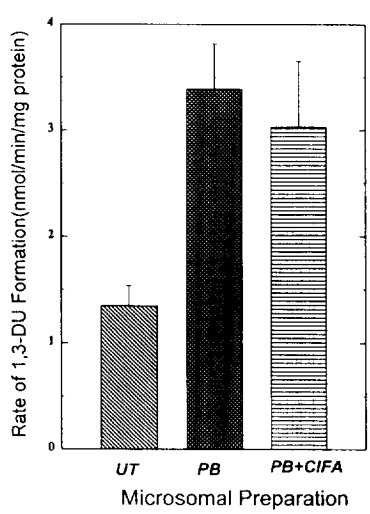

Fig. 8. Rates of 8-hydroxylation of theophylline catalyzed by hepatic microsomes from untreated, PB-induced, or PB-induced and N-(2-*p*-nitrophenethyl)chlorofluoroacetamide (Cl FA)-treated rats. Rates are means ± S.D. with $n = 5$, 13, and 12, respectively. 1,3-DU was quantified by CE analysis. Abbreviations and CE assay conditions are provided in Figs. 2 and 6.

Table 2
Recoveries of theophylline and its metabolites in spiked human urine after solid-phase extraction

Metabolite	Recovery (mean ± S.D., $n = 4$) (%)
3-X	91.0 ± 5.5
1-X	98.0 ± 6.5
1,3-DU	75.8 ± 5.5
Theophylline	91.0 ± 17.2

The rate of 8-hydroxylation of theophylline to 1,3-DU was increased by approximately 2.5-fold when microsomes from PB-induced rats were used instead of microsomes from untreated rats (Fig. 8). However, when microsomes from N-(2-*p*-nitrophenethyl)chlorofluoroacetamide- and PB-treated rats were used, rates of 1,3-DU formation were not significantly decreased.

The recoveries of theophylline and its metabolites when spiked into human urine, as assessed by CE analysis after SPE, are shown in Table 2. The reproducibility of the assays which include the SPE step, can be gauged from the standard deviations from four analyses. A typical electropherogram of theophylline and its metabolites in spiked urine is shown in Fig. 9.

4. Discussion

With the exception of the higher detection limits, our CE assay for theophylline and its metabolites offers several advantages over HPLC methods [9,11]. The metabolites were stable upto high (30 kV) running potentials since no degradation was observed at this potential. The analysis time could thus be shortened to as little as 12 min–which is considerably shorter than that used in HPLC-based analyses where a minimum of 25 min was required [5,9,11]. CE analysis of microsomally-produced theophylline

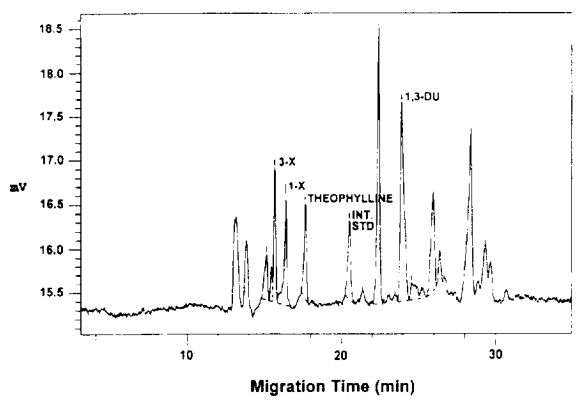

Fig. 9. Electropherogram of theophylline and its metabolites spiked into human urine (0.95 μg/ml). The urine was solid-phase extracted (Waters C_{18}) prior to analysis by CE. Abbreviations and experimental conditions as in Fig. 2.

metabolites was successful without the necessity of extraction of the incubation media, whereas in the HPLC methods a liquid–liquid extraction was essential to obtain clean chromatograms [11]. In particular, resolution of 3-X and 1-X could not be achieved in HPLC without extraction of the incubation medium. Such liquid–liquid extractions, although reported to achieve high recoveries (±80%) [11], introduce additional variabilities in the analysis. The high inter- and intra-run reproducibility reported here for CE analysis of theophylline and its metabolites contrasts with the retention time shifts and poor column-to-column reproducibility that we and others observed with HPLC analysis of these compounds and other xanthines and uric acids [5,19,20].

The relatively high detection limits of the CE method for theophylline and its metabolites are caused by the nanoliter injection volumes used [21] and the limitations of the UV detection system. When extreme sensitivities are required, this can be compensated for by solid- or liquid-phase extraction of the metabolites and reconstitution of the solution at a higher concentration, and by use of more sensitive detection systems. Our studies with theophylline- and metabolite-spiked urine demonstrate how the detection limit can be lowered (Table 2). Since a safe dose of 100 mg theophylline in adult humans gives levels of several micrograms of metabolite per ml in 24-h urine [22,23], which is readily analyzed in our system, we propose to use the CE analysis of such urine samples as a non-invasive probe for the relevant human P450s.

The elevated rates of 1,3-DU formation in hepatic microsomes from ISO- or MX-treated-rats relative to BNF-treated rats (Fig. 7) must reflect the major role of P4501A2 in catalyzing 1,3-DU formation, since P4501A2 is induced by ISO and MX to a much greater extent than by BNF. This role of P4501A2 is consistent with its role in humans [4,10]. The observation that PB treatment of rats increased the rates of 1,3-DU formation by their hepatic microsomes in vitro, is consistent with a previously reported in-vivo observation [24]. P4502B1, the major form of P450 induced in rats by PB treatment was not responsible for catalyzing this metabolism since microsomes from rats treated with PB and N-(2-*p*-nitrophenethyl)chlorofluoroacetamide, a specific P4502B1 inhibitor [25], catalyzed 1,3-DU formation at the same rate as hepatic microsomes from PB-treated rats (Fig. 8). Possible P450 forms, which are inducible by PB, and which could catalyze the metabolism of theophylline, are P4503A1/2, 2D1, 2B2, and 2C6 [26]. The first two forms most likely are responsible for theophylline metabolism since they correspond to human P4503A4 and 2D6, which have been implicated in theophylline metabolism [5].

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