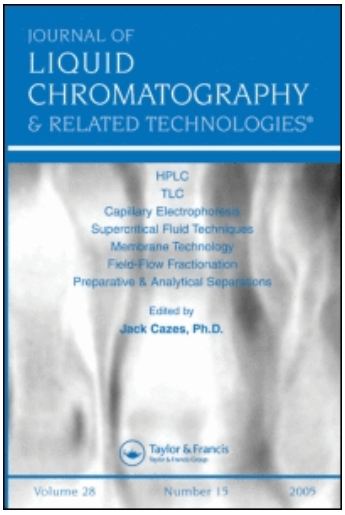

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Rapid Simultaneous Determination of Three Methylxanthines in Human Plasma by Capillary Electrophoresis

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ABSTRACT

A simple capillary electrophoresis method is described for simultaneous determination of three methylxanthines, theophylline, dyphylline, and caffeine in human plasma. Plasma proteins are precipitated by acetonitrile and the supernatant was performed in Tris buffer (20 mM; pH 9) with sodium dodecyl sulfate (SDS) (150 mM) as an anionic surfactant. Several parameters affecting the separation of the drugs were studied, including the pH and concentrations of the Tris buffer and SDS. Application of the proposed method to the determination of theophylline and dyphylline in human plasma proved to be feasible. The practicability of the proposed method is demonstrated on a healthy volunteer.

Key Words: Capillary electrophoresis; Human plasma; Methylxanthines; CZE; Validation; MEKC-CE.

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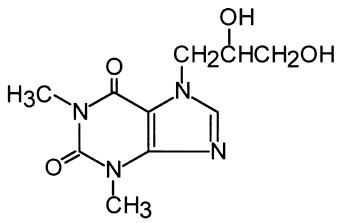
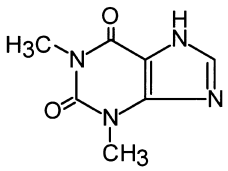
INTRODUCTION

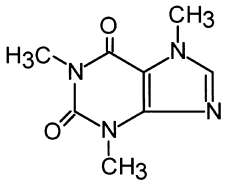
Theophylline and caffeine are structurally closely related alkaloids occuring in plants. Dyphylline(dihydroxypropyl theophylline) is a derivative of theophylline and not metabolized to theophylline in vivo. They are all methylxanthines and have similar chemical structures (Fig. 1). Theophylline and dyphylline are widely employed to relax bronchial smooth muscle in treatment of asthma and chronic obstructive pulmonary disease. The therapeutic range of theophylline is 5–20 $\mu g\,mL^{-1}$. The minimal effective therapeutic concentration of dyphylline is 12 $\mu g\,mL^{-1}$. Methylxanthines of CNS stimulant properties are often encountered as side effects, including nausea, vomiting, insomnia, and tremors. Seizures have sometimes occurred on patients which the blood concentrations of theophylline are only about 50% above the top of the accepted therapeutic range.[1] As a consequence of the variation of pharmacokinetics between patients, it is necessary to monitor concentration of drugs in individual patients to ensure the maximum clinical response and to avoid undesirable side effects. Because of shorter half-life of dyphylline, it has a lower incidence of side effects than does theophylline in clinical use. There is some evidence that dyphylline combined with theophylline may exhibit less



dyphylline



theophylline



caffeine

Figure 1. Chemical structures of theophylline, dyphylline, and caffeine.

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frequent adverse side effects than an equivalent dose of theophylline alone.[2] Therefore, the monitoring of dyphylline and theophylline levels in plasma is required for therapeutic use and toxic control.

For the analysis of theophylline, dyphylline, and caffeine in plasma samples, spectrophotometry,[3,4] immunoassays,[5–7] chromatographic methods,[8–14] and capillary electrophoresis (CE)[15–21] have been introduced. The immunological and spectrophotometric methods would be inappropriate for determination of the combination of different methylxanthines. Only chromatographic and CE methods can be applied to differentiate and measure these drugs, simultaneously. But HPLC methods produced a lot of solvent waste and complicated the pretreatment of biological samples needed. The advantage of small amounts of sample size and running buffer needed for CE methods have become the major analysis tool to determine chemical concentration in serum. Theophylline alone or theophylline with other drugs or with its metabolites in plasma has been determined by CE. So far, there is not a CE method for simultaneous determination of widely clinical used of theophylline and dyphylline in human plasma has been reported.

In this work, a simple plasma pretreatment and CE method is developed for the simultaneous determination of theophylline, dyphylline, and caffeine in human plasma. The proposed method also can be applied to pharmacokinetic researches and clinical monitoring of these methylxanthines. The proposed CE method was applied for the determination of analytes extracted from human plasma taken from a volunteer dosing a 300 mg theophylline, and 100 mg dyphylline tablets, simultaneously.

EXPERIMENTAL

Capillary Electrophoresis Conditions

A Beckman P/ACE MDQ system (Fullerton, CA) equipped with a filter UV detector and a liquid-cooling device were used. Samples were performed in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ) of 40 cm (effective length 30 cm) × 75 μm I.D. and injected by pressure (0.3 psi) for 3 s and the applied voltage was 12 kV. Separations were performed at about 25°C in Tris buffer (20 mM; pH 9.0) with SDS (150 mM). Detection was carried out by the on-column measurement of UV absorption at 214 nm (cathode at the detection side). Capillary conditioning before startup is methanol for 10 min, 1 N HCl aqueous solution 10 min, deionized water 2 min, 1 N NaOH aqueous solution 10 min, and deionized water 2 min. The conditioning between runs was effected by rinsing with 0.1 N NaOH (3 min), deionized water (2 min), and running buffer (3 min), under positive pressure

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applied at the injection end. A Beckman P/ACE MDQ software system was used for data processing.

Chemicals and Reagents

Theophylline and caffeine were from Sigma (St. Louis MO). Imidazole (internal standard, IS), sodium hydroxide (NaOH), *tris*(hydroxymethyl)-aminomethane (Tris), sodium dodecyl sulfate (SDS), and phosphoric acid (H_3PO_4, 85%) were supplied by E. Merck (Darmstadt, Germany). Dyphylline and theobromine were from Acrós (Geel West Zone, Belgium). Acetonitrile (ACN) and other reagents were of analytical-reagent grade. Blank plasma from healthy donors was obtained from Department of Transfusion Medicine, University of Kaohsiung Medicine. Milli-Q (Millipore, Bedford, MA) treated water was used for the preparation of buffer and related drugs. Solutions of various Tris buffers at different pH were prepared by neutralizing the related Tris solution with H_3PO_4. Solutions of SDS at various levels were obtained by dissolving different amounts of SDS in water then diluted with Tris buffer.

Sample Preparation

A 200 μL aliquot of human plasma was pipeted into a 1.5-mL eppendorf vial, and 50 μL mixed solution containing theophylline, dyphylline, caffeine, and imidazole (IS) was added. Each vial of the theophylline, dyphylline, and caffeine final concentrations in plasma sample is over the range of 20–200 μM as studied samples. The vials were mixed for 10 s. Then 300 μL of ACN was added and mixed. The vials were centrifuged (35,000*g*) for 5 min. A 0.2-mL supernatant was transferred to a 0.2-mL mini-vial that could be placed into the autosampler for CE analyses.

Method Validation

Calibration curves were prepared by simultaneously adding theophylline, dyphylline, and caffeine at five different concentrations and fixed concentration of IS in drug-free plasma to make the final concentrations of theophylline, dyphylline, and caffeine in plasma are 20, 50, 100, 150, and 200 μM. The calibration graphs were established with the peak area ratio of theophylline, dyphylline, and caffeine to IS as an ordinate (y) vs. the concentration of these drugs in μM as abscissa (x). Intra-day precision was based on six replicate analyses for three concentration levels at 20, 100, and 200 μM for theophylline, dyphylline, and caffeine. Inter-day precision was calculated from six consecutive days for these three concentrations of theophylline, dyphylline, and caffeine. All relative recoveries of theophylline and dyphylline from the

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plasma were tested by spiking with three different concentrations of theophylline, dyphylline, and caffeine (30, 90, and 180 μM), and treated as Section Sample Preparation.

Application

We investigated the application this developed CE method to analysis one healthy male took theophylline, and dyphylline tablets. The volunteer was treated with an oral sustained release theophylline 300 mg tablet at 9:00 a.m., then took dyphylline 100 mg tablet at 12:00 p.m. Venous blood sample was collected in heparinized tube at 2:00 p.m. The plasma collected before dosing was employed as a blank. Both blood samples were centrifuged and analyzed immediately.

RESULTS AND DISCUSSION

Preliminary test of theophylline, dyphylline, and caffeine standards by capillary zone electrophoresis (CZE) was briefly studied at 12 kV with 20 mM of Tris, borate, or phosphate buffers at pH 9.0 in the absence of SDS. Dyphylline and caffeine did not separate in these background electrolytes. This indicates the differences of charge to mass ratio of dyphylline and caffeine is not enough to differentiate. Therefore, Micellar electrokinetic capillary chromatography (MEKC), with SDS as a micellar source, was tried to separate the analytes. The SDS in 20 mM of Tris, borate, or phosphate buffer was studied; MEKC of the standard methylxanthines at 30 mM SDS give resolved peaks, but a lot of plasma proteins interfere with the analysis of the test drugs. At the higher concentrations of SDS, the lesser interference of electropherograms was obtained. Tris or borate buffer with SDS concentration beyond 150 mM as running buffer can get good background and-resolution for determination of these methylxanthines in human plasma matrix. The separation efficiency under Tris buffer is better than borate buffer. The SDS micelle may play two roles in this separation: provide the chromatographic pseudostationary phase to differentiate the methylxanthines, and retain a lot of plasma proteins in SDS micelle. After each run, the capillary must be washed for 3 min with 0.1 N NaOH aqueous solutions. Because of the high concentration of SDS, much plasma protein may be retained and may adsorb onto the capillary wall and cause noise during consecutive analysis. Rinsing with NaOH between each injection can make the maximal electroosmotic flow (EOF) wash out the protein-SDS micelle. In order to determine the optimal separation conditions, parameters affecting the theophylline, dyphylline, and

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caffeine separations were studied. Different voltages were tested, 12 kV can provide suitable separation and better efficiency.

Concentration and pH of Tris Buffer

The retention behavior of theophylline, dyphylline, and caffeine in Tris buffer (pH 9.0) at a concentration range of 10–50 mM with 150 mM SDS was studied. The migration times of theophylline, dyphylline, and caffeine in pH 9 Tris buffer over the range of 10–50 mM with 150 mM SDS, did not change remarkably. From the electropherograms it is indicated that baseline resolution of dyphylline standard from plasma blank is unattainable at 10 mM Tris buffer and the peaks are broader. The baseline resolution of the drugs from plasma blank is obtainable at Tris buffer concentrations >20 mM. At high concentrations, the Joule heat increases. The optimal Tris buffer concentration is set at 20 mM. Then 20 mM Tris buffer with SDS (150 mM) at different pH (7, 7.5, 8, 8.5, and 9) are studied. Some of the interferences from plasma affected the accuracy of quantification in pH 7.5–8.5 of 20 mM tris buffer with 150 mM SDS. The complete drugs baseline resolution from plasma matrix and the shortest time of the run were obtained at pH 9.0. The effect of pH on the migration time was shown as Fig. 2.

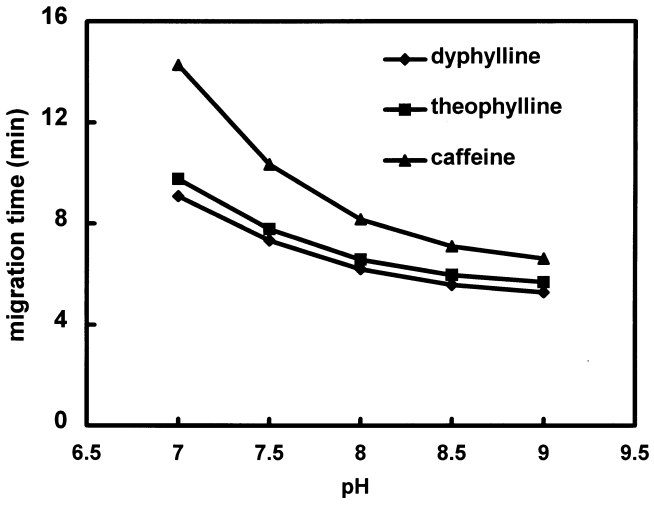

Figure 2. Effect of pH of 20 mM Tris buffer with 150 mM SDS on the migration of theophylline, dyphylline, and caffeine. Capillary electrophoresis conditions: applied voltage, 12 kV (detector at cathode side); uncoated fused-silica capillary, 30 cm × 75 μm I.D.; sample size, 0.3 psi, 3 s; wavelength, 214 nm.

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Concentration of Sodium Dodecyl Sulfate

The effects of SDS at a concentration range of 0–150 mM in Tris buffer (20 mM; pH 9.0) on the separation are showed in Fig. 3. The results indicate that electrophoresis of the drugs in the absence of SDS result in no resolution of dyphylline and caffeine. Micellar electrokinetic capillary chromatography of the standard methylxanthines at 30 mM SDS give resolved peaks, but a lot of plasma proteins interfere in the analysis of the test drugs. At the higher concentrations of SDS, the lesser interference of electropherograms was obtained. When the concentration of SDS at 150 mM in Tris buffer (20 mM, pH 9), the best efficient resolution from plasma matrix was achieved. The results of SDS and the buffer effects on the MEKC mode for analysis of these methylxanthines with optimization of the CE conditions, was 20 mM Tris buffer (pH 9.0) with SDS (150 mM) at 214 nm. Figure 4(A) and (B) present the typical electropherograms of the MEKC separation of human plasma blank and methylxanthines spiked in plasma, respectively. Peaks 1, 2, and 3 represent dyphylline, theophylline, and caffeine, respectively. Owing to these methylxanthines are neutral species in running buffer at pH 9; the migration velocity of the analyte depends on the distribution coefficient between the micellar and the nonmicellar (aqueous) phase. Dyphylline is a theophylline derivative containing a polar functional group, 2,3-dihydroxypropyl at 7-position. So dyphylline retains in SDS micelles less than

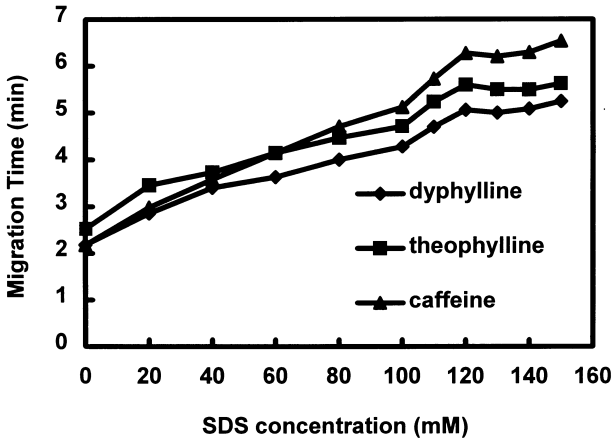

Figure 3. Effect of SDS concentrations (0–150 mM) on the migration of theophylline, dyphylline, and caffeine in Tris buffer (20 mM, pH 9.0). See Fig. 2 for other CE conditions.

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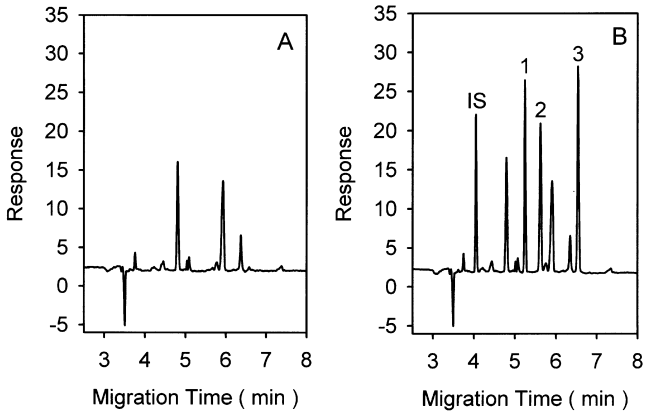

Figure 4. Electropherograms of (A) plasma blank and (B) theophylline, dyphylline, and caffeine spiked in plasma. Peaks: IS, 1, 2, and 3 for imidazole, dyphylline, theophylline, and caffeine, respectively. Capillary electrophoresis conditions: 20 mM Tris buffer with 150 mM SDS, pH 9.0; applied voltage, 12 kV (detector at cathode side); uncoated fused-silica capillary, 30 cm × 75 μm I.D.; sample size, 0.3 psi, 3 s; wavelength, 214 nm.

theophylline. On the other hand, caffeine is more lipophilic than theophylline owing to a methyl group substitute in 7-position. It can be seen that caffeine has the longest migration time in this running buffer. To identify that the observed peaks were the analytes, we changed the UV detector to a photodiode array detector, and three-dimensional electropherogram which represents the absorbance vs. migration time vs. wavelength relationship was obtained. By comparing the three-dimensional electropherogram of the analyte standards and that which was spiked in plasma for identification, we note that the same absorption spectra were obtained.

Validation of Methylxanthines Spiked in Plasma

For evaluating the quantitative applicability of the method, five different concentrations of theophylline, dyphylline, and caffeine, in the range 20–200 μM, were analyzed using imidazole (1 mM) as an IS. The linear regression equations are listed in Table 1. The data indicate high linearity of this method for the intra- and inter-day assays. The precision (relative standard deviation, RSD) and accuracy (relative error, RE) of the proposed method at 20, 100, and 200 μM of these methylxanthines spiked samples was studied. The results (Table 2) show that the intra-day RSD ($n = 6$) and RE of the

Table 1. Regression analysis for determination of theophylline, dyphylline, and caffeine in human plasma.

Concentration range, μM	Regression equation	Correlation coefficient (r)
Intra-day[a] (20–200)		
Theophylline	$y = (0.0066 \pm 0.0001)x + (0.037 \pm 0.004)$	0.999
Dyphylline	$y = (0.0066 \pm 0.0001)x - (0.009 \pm 0.007)$	0.999
Caffeine	$y = (0.0067 \pm 0.0001)x - (0.009 \pm 0.006)$	0.999
Inter-day[b] (20–200)		
Theophylline	$y = (0.0066 \pm 0.0001)x + (0.039 \pm 0.010)$	0.999
Dyphylline	$y = (0.0065 \pm 0.0001)x - (0.005 \pm 0.009)$	0.999
Caffeine	$y = (0.0066 \pm 0.0001)x - (0.008 \pm 0.006)$	0.999

[a]Regression equations on intra-day analysis were calculated from the assay values of prepared standards on a single day ($n = 6$).
[b]Regression equations on inter-day analysis were calculated from the assay values of prepared standards on six different days ($n = 6$).

analytes at three concentrations were all below 3.8 and 2.8%, respectively; in parallel, the inter-day RSD ($n = 6$) and RE were all below 6.5 and 3.1%, respectively. The relative recoveries of the proposed method were also evaluated at 30, 90, and 180 μM of these methylxanthines in spiked plasma, from three different subjects. The relative recoveries are all greater than 90%. The limit of detection (LOD) was defined as the analyte concentration giving a signal exceeding that of the blank by three times. The LOD of theophylline, dyphylline, and caffeine in plasma (S/N = 3, 0.3 psi, 3 s) were found to be 4, 2, and 2 μM, respectively.

The selectivity of the proposed method was briefly tested on the separation of a standard mixture of methylxanthines and others may be combined in clinical use. Under present MEKC conditions, a complete separation of methylxanthines and other drugs, including theobromine, erythromycin, prednisolone, procaterol HCl, zafirlukast, ketotifen, dextromethorphan, codeine phosphate, carbetapentane citrate, ambroxol HCl, terbutaline sulfate, fenoterol HBr, ipratropium, metronidazole, vancomycin, oxacillin sodium, meropenem, amphotericin B, hydrocortisone sodium succinate, dobutamine, cephalexin,

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Table 2. Precision and accuracy for determination of theophylline, dyphylline, and caffeine in human plasma.

	Concentration known, μM	Concentration found, μM	RSD, %	RE, %
Intra-day ($n = 6$)				
Theophylline	20.00	19.60 ± 0.66	3.4	−2.0
	100.00	102.81 ± 2.49	2.4	2.8
	200.00	197.98 ± 1.49	0.8	−1.0
Dyphylline	20.00	19.81 ± 0.76	3.8	−1.0
	100.00	99.21 ± 1.01	1.0	−0.8
	200.00	200.68 ± 2.20	1.1	0.3
Caffeine	20.00	19.67 ± 0.56	2.8	−1.6
	100.00	99.78 ± 0.98	0.9	−0.2
	200.00	200.35 ± 1.24	0.6	0.1
Inter-day[a] ($n = 6$)				
Theophylline	20.00	20.50 ± 1.34	6.5	2.5
	100.00	103.05 ± 2.28	2.2	3.1
	200.00	200.21 ± 2.03	1.0	0.1
Dyphylline	20.00	20.27 ± 0.68	3.4	1.4
	100.00	99.15 ± 1.53	1.5	−0.9
	200.00	200.42 ± 2.54	1.3	0.2
Caffeine	20.00	20.19 ± 0.96	4.8	0.95
	100.00	102.18 ± 0.98	0.9	2.1
	200.00	202.38 ± 1.13	0.6	1.2

[a]Intra-day data were based on six replicate analyses and inter-day were from six consecutive days.

cefadroxil, cefuroxime, cefoxitin, cefazolin, cefotaxime, cefamandole, amikacin, tobramycin, gentamicin, and kanamycin A was obtained. None of these commonly used drugs were found to interfere in the procedures developed for theophylline, dyphylline, and caffeine determination in plasma.

Application

The volunteer ingested a sustained release 300 mg tablet of theophylline. After three hours, he took a dyphylline 100 mg tablet. Then, we measured his plasma concentration of theophylline and dyphylline two hours later. The electropherograms of plasma blank (before dosing) and after ingesting the drugs are shown in Fig. 5 (A) and (B), respectively. The concentration of

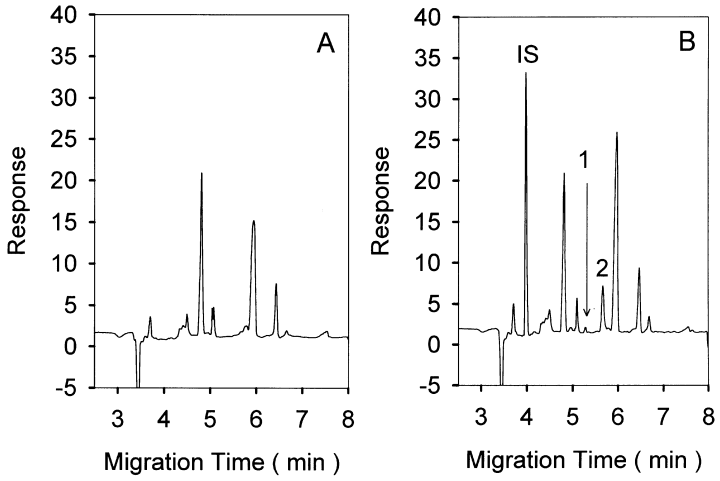

Figure 5. Electropherograms of (A) plasma before dosing and (B) the plasma of a volunteer dosing theophylline and dyphylline tablets. Peaks: IS, 1 and 2 for imidazole, dyphylline, and theophylline, respectively. Capillary electrophoresis conditions: see Fig. 4.

theophylline found was $6.9\,\mu g\,mL^{-1}$ ($38.3\,\mu M$), which is in the therapeutic range ($5–20\,\mu g\,mL^{-1}$). Dosing of dyphylline is low and is rapidly metabolited. This proposed method can detect the presence of dyphylline, but cannot quantitatively provide the concentration of dyphylline in plasma because its concentration did not fall in the linear quantitative range of this proposed method. It had been reported that volunteers took 300 mg dyphylline, and after two hours the serum concentration of dyphylline was about $4\,\mu g\,mL^{-1}$.[12] Therefore, the application of this proposed method is feasible for clinical studies.

CONCLUSION

A simple, rapid, and selective MEKC method has been developed for the quantitative of theophylline, dyphylline, and caffeine in human plasma. The optimized conditions for analysis of theophylline, dyphylline, and caffeine in plasma are described. The method was validated for quantification in terms of selectivity, accuracy, precision, recovery, and linearity. In addition, the minimal amount of sample required, simple sample pretreatment, and short analysis times required was observed in this proposed method. The method can be applied to theophylline and dyphylline in clinical drug monitoring. Other commonly used

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drugs and the same kind of xanthines (caffeine and theobromine) did not interfere in this method. In summary, the proposed method is an effective method for determination of these methylxanthines in human plasma.

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