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Use of a Disposable Modified Carbon Paste Electrode for Liquid Chromatography-amperometric Detection of Theophylline and Three Metabolites in Human Serum

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Chemically modified electrodes, constructed by incorporating potassium hexachloroplatinate K_2PtCl_6 into the graphite power/paraffin oil matrix used to fabricate conventional carbon paste electrodes, were shown to catalyze the electrooxidation of purine compounds including theophylline, 3-methylxanthine, 1,3-dimethyluric acid and 1-methyluric acid; the modifier electrodes display electrocatalytic activity for the oxidation of purines at 170 mV (*vs* Ag/AgCl) when used as sensing electrodes in amperometric detection following liquid chromatography. The K_2PtCl_6 electrodes permitted detection of the purines at similar values of applied potential. The limit of detection (LOD) of theophylline, 1-MUA, 3-MXT and 1,3-DMUA were 1.1 ng mL^{-1}, 3.2 ng mL^{-1}, 1.0 ng mL^{-1} and 11 ng mL^{-1}, respectively. The calibration graphs were linear for theophylline and metabolites over the range of concentration used (50-3200 ng mL^{-1}). Theophylline and its metabolites can easily be separated and analyzed in one run using electrochemical sensor chromatography with a methanol – phosphate buffer containing 20 mM di-potassium hydrogen phosphate (pH 5.50). Serum samples were collected after the oral administration of black tea from human volunteers. Quantitative data for the determination of a micromolar amount of theophylline and its metabolites in serum samples are in agreement with data obtained by HPLC-UV.

Keywords: Electrochemical sensor; Disposable modified carbon paste electrode; Theophylline and three metabolites.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) is produced as primary caffeine (1,3,7-trimethylxanthine) metabolite by N-demethylation. Theophylline is metabolized in man by 1-N-demethylation to 3-methylxanthine and 1-methylxanthine (xanthine oxidase to 1-methyluric acid) and by 8-hydroxylation to 1,3-dimethylic acid. A number of studies have been published during the past 15 years, which have been directed toward the characterization of the cytochrome p 450 (cyp) isoforms involved in the different pathways. Reported analytical procedures for theophylline metabolites in body fluids have been based on the methods of liquid chromatography with ultraviolet,[1-9] electrochemical,[10-12] immunoassay[13-14] and capillary electrophoresis detection.[15-16] In a previous investigation the advantage of using a solvent program[1,2,6,8] and ternary solvent[4,5,7,9] for LC-UV determination of theophylline and its metabolites in serum and urine samples was established. However, the separation time needs more than 45 min. The coupling of LC with electrochemical detection (ECD) or flow-injection analysis with ECD at a glassy carbon electrode was employed for the determination of theophylline. The main advantage of immunoassay is that it makes the assay faster because no separation step in needed and no time is lost in incubation. Immunoassay for theophylline, in which it was labeled with fluorescein isothiocyanate or drug-enzyme conjugate was added, competed for the antibody. Capillary electrophoresis with micellar systems, in the presence of sodium dodecyl sulphate, has been employed for analysis of theophylline. Theophylline in tea and drug at a Nafion®/lead-ruthenium oxide pyrochlore modified glassy carbon

Dedicated to the memory of the late Professor Ho Tong-Ing.

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electrode has been studied by J-M Zen et al.[17] Electrochemical oxidation of theophylline was investigated by means of cyclic voltammetry and UV-vis spectroscopy at multi-wall carbon nanotube modified glassy carbon electrodes.[18] The electrochemical oxidation of biological compounds such as 7-methyluric acid and 5,6-dihydroxytryptamine in aqueous solutions at glassy carbon and pyrolytic graphite electrodes has been studied.[19] Electrochemical and peroxidase-catalysed oxidation of 1-MUX was investigated at a pyrolytic graphite electrode (PGE) in the pH range 2.1-10.0,[20] 1,3-DMUA in phosphate buffers of pH 2.3-10.3 at PGC[21] and voltammetry of 7,9-dimethyluric acid in aqueous solution at a PGE.[22] Nevertheless, no study has been attempted to simultaneously measure concentrations of theophylline and three of its metabolites. The goal of our study was to determine the serum metabolites of theophylline in humans after oral administration of black tea. Therefore, we designed a low-cost sensor for the determination of purines. In this work, we were able to apply a disposable carbon paste electrode for the measurement of theophylline and its metabolites, which has the advantages of being selective and sensitive in serum.

EXPERIMENTAL

Apparatus and Materials

The high-performance liquid chromatography system used consisted of a Shimadzu Model LC-10 AD_{vp} pump with a Rheody 7125 injection valve with a 20-μL sample loop coupled with a DECADE II (Antec Leyden B.V., Netherlands) electrochemical detector and an L-7420 (Hitachi, Japan) spectrophotometric detector. The flow cell was designed and the electrodes were as follows: Ag/AgCl/0.1 M KCl reference electrode, platinum auxiliary electrode, and K_2PtCl_6 modified carbon paste (CPE) electrode for the detection of 1-methyluric acid (1-MUA), 3-methylxanthine (3-MXT), 1,3-dimethyluric acid (1,3-DMUA), and theophylline. Differential pulse voltammetric experiments were performed using an EG&G Princeton Applied Research (Princeton, NJ) Model 394 connected to an EG&G325 Faraday cage with Smart stir and KO269 A Faraday cage. All solvents and analytes were filtered through 0.45 μm cellulose acetate and polyvinylidene fluoride syringe (PVDF) membrane filters, respectively. Chromatograms of 1-MUA, 3-MXT, 1,3-DMUA, and theophylline were acquired and peak height calculated by means of an SISC Chromatogram Data Integrator. All other reagents were analytical grade.

Construction of a flow-through voltammetric detector

DC mode electrochemical detection was accomplished at a flow-through electrolysis cell. The detection cell (Fig. 1) was constructed in the laboratory. The modified K_2PtCl_6/CPE electrode was produced by a previous method.[23] The carbon paste (graphite powder: paraffin oil: K_2PtC1_6 (4:1:0.1 w/w) was inserted into one end of the Teflon tube (1/32 in. i.d., 1/16 in. o.d.) and sealed with acrylic resin (obtained from Struers). A small copper wire was placed at the other end of the Teflon tube to allow for electrical connection to the carbon paste. The platinum wire which served as counter and the Ag/AgCl that served as reference electrodes were then attached in series with the Teflon tube.

Human volunteers

The volunteer population composed of four healthy subjects (1 male, 3 females, ages 22 ± 1.0 year) who had not

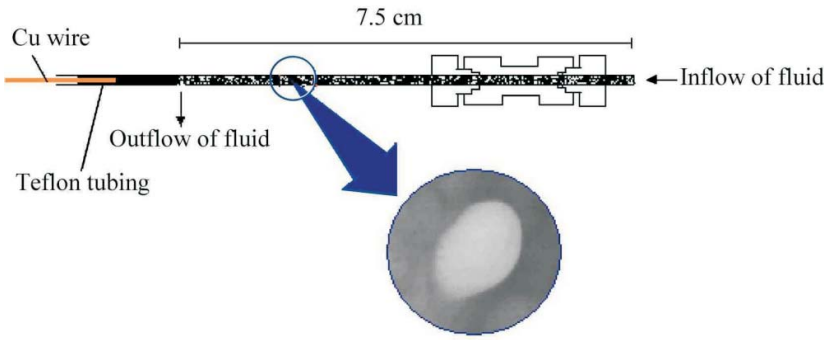

Fig. 1. Diagram of modified K_2PtCl_6/CPE flow-through electrolysis cell.

received any tea during the previous week participated in the experiment. Serum samples were collected prior to various teas (intake time 0) and 1 day after black tea intake. Time 0 values were used as background and subtracted from sample values. The remainder of the samples was kept in high density polyethylene containers and stored in a freezer (-20 °C) for further determination of theophylline and its metabolites by HPLC.

Extraction of theophylline and its metabolites

Samples of 5 mL plasma from human were centrifugated at 6000 g for 30 min. The supernatant serum (2-3 mL) was transferred to another centrifugal tube containing 2 mL ethanol and centrifugated for 30 min to sediment aggregates. The deproteinized samples were then extracted three times with 6 ~ 15 mL ethyl acetate. The organic phase was collected and evaporated under nitrogen at a temperature less than 37 °C. The dried extract was reconstituted with 1 mL of 50% (*v/v*) methanol-water and loaded onto a Sep-Pak® C_{18} Waters cartridge which had been conditioned with 2 mL of methanol and 2 mL water prior to sample loading. An additional 0.5 mL of methanol was used to rinse the sample vial and was also loaded onto the C_{18} cartridge. The sample on the C_{18} cartridge was washed three times with 9 mL of ethyl acetate. These three fractions were combined and dried under nitrogen at 45 °C. The dry extract was reconstituted with 1 mL of deionized water and filtered through 0.45 μm membrane filters before LC analysis.

Determination by liquid chromatography

Stock standard solution was prepared by dissolving 10 mg of 1-MUA, 3-MXT, 1,3-DMUA, and theophylline in 10 mL of methanol, respectively. Working standard solutions were prepared from a stock standard solution in methanol in the range 50-3200 ng mL^{-1}. RP-HPLC was performed on a Phenomenex Luna C_{18} (5u, 250 × 4.6 mm) column eluted methanol - phosphate buffer (20:80, *v/v,* pH 5.50) as the mobile phase at 1 mL min^{-1}. Detection after separation on the Phenomenex Luna C_{18} column was carried out using an ultraviolet detector set at 271 nm. The EC detector was operated at + 1.4 V. By means of the injection valve, 20 μL of the prepared sample solution and standard solution were chromatographed under the operating conditions described above. Quantitation was based on the peak height of the sample.

RESULTS AND DISCUSSION

Choice of analytical method

Fig. 2 shows three pictures of carbon paste electrodes deposited with different K_2PtCl_6 concentrations taken with a scanning electron microscope (SEM, JXA-840, JEOL). For comparision, no platinum particles of zero concentration were detected on the surface of the carbon paste electrode (CPE, Fig. 2a). The particle size of platinum concentration 3.1 μM (Fig. 2b) was less than 7.0 μM (Fig. 2c). The particles were dispersed with very slight aggregation, as seen in Fig. 2b, because of less concentration on the surface CPE. As can be seen from the pictures, the surface has few cavities as the concentration of K_2PtCl_6 increases. The final micrograph, Fig. 2c, shows a thick deposit; this confirms the uniformity of the deposit. To confirm the electroanalytical utility of K_2PtCl_6/CPE composite electrode, we

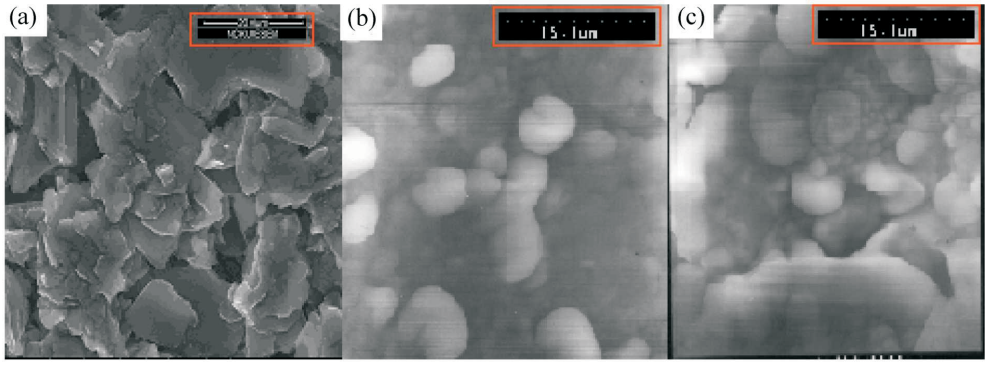

Fig. 2. Scanning electron micrographs of different concentrations of K_2PtCl_6 (a) unmodified CPE (b) modified K_2PtCl_6 (3.1 μM)/CPE (c) modified K_2PtCl_6 (7.0 μM)/ CPE.

studied. 1-MUA and 3-MUX oxidation were performed using several electrodes containing different concentrations of K_2PtCl_6/CPE (in the range from 0 to 40 mg L^{-1}, see Fig. 3). K_2PtCl_6 (30 mg L^{-1}) was used for incorporation of graphite power, because the peak current of 1-MUA and 3-MUX (6.4 mg L^{-1}) was the largest using this procedure. The oxidation of 1-MUA and 3-MXT in phosphate buffer pH 2.08 was studied at the carbon paste electrode (CPE) and the K_2PtC1_6 carbon paste electrode by differential pulse voltammetry (DPV), respectively. The oxidation potentials of 1-MUA and 3-MXT were 0.39 V and 0.97 V at K_2PtC1_6 modified CPE and 0.48 V and 1.14 V at CPE, respectively. Two anodic waves of 1-MUA and 3-MUX show that the K_2PtCl_6/CPE is at a lower potential and higher current than CPE (Fig. 4). The K_2PtCl_6/CPE showed an increase of approximately five times in the activity of electrodes. Therefore, the K_2PtCl_6/CPE was chosen for use in the determination of theophylline and metabolites in serum. Figs. 5A and 5B compare a K_2PtCl_6/CPE and pure CPE as a working electrode carried out a flow-through voltammetric detector. The K_2PtCl_6/CPE has a higher peak height than CPE.

Comparative tests of various pH and supporting electrolytes were taken for 1-MUA, 33-MXT, 1,3-DMUA, and theophylline in a 20 mM phosphate buffer (pH 5.5), 2 mM tetraethylammonium perchlorate (pH 8.3), 50 mM acetate buffer (pH 4.4), and 10 mM sodium perchlorate (pH 6.7). The best results were achieved with the operational electrolyte of 20 mM phosphate buffer (pH 5.5) since the EC response of the theophylline and three metabolites was found to be much higher than in the other supporting electrolytes.

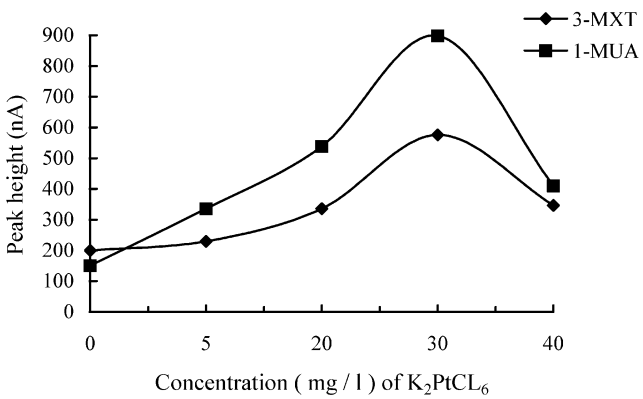

Fig. 3. Effect of the concentration of K_2PtCl_6 on the electrocatalytic oxidation of 1-MUA (6.4 ppm) and 3-MXT (6.4 ppm) on K_2PtCl_6/CPE.

Various methanol-dipotassium hydrogen phosphate ratios (10:90, 20:80, 30:70, *v/v*) were tested on metabolites of theophylline. After various studies of the retention behavior of the theophylline and metabolites, we achieved base-

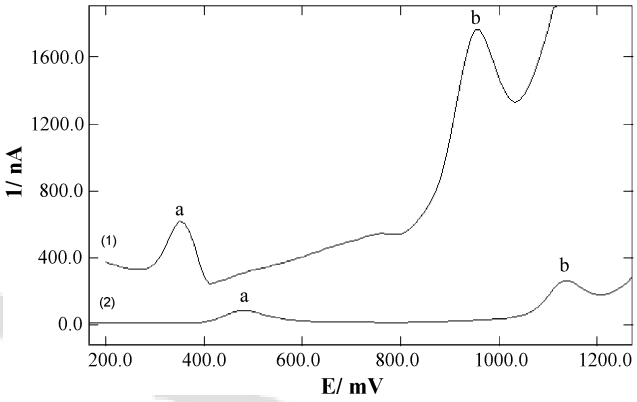

Fig. 4. The DPV recorded to produce analytical curves for (a) 1-MUA and (b) 3-MXT in 0.1 M phosphate buffer (pH 2.08) at the K_2PtCl_6 carbon paste electrode (curve 1); carbon paste electrode (curve 2), respectively. Scan rate, 10 mVs^{-1}; pulse height, 50 mV.

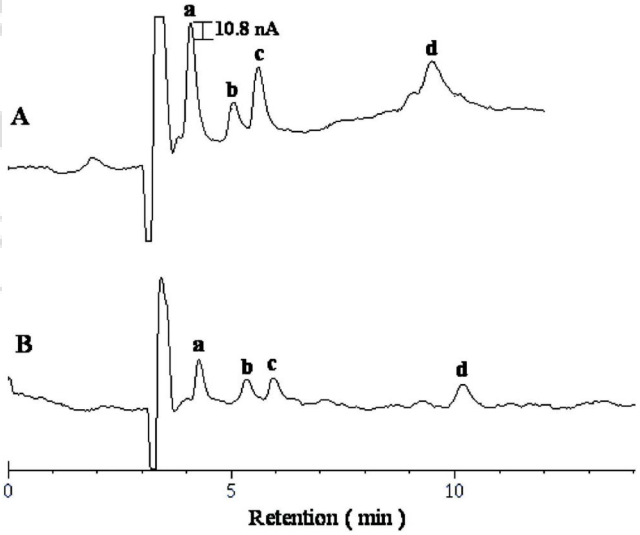

Fig. 5. Chromatograms obtained by ECD for (a) 1-MUA (20 ng); (b) 3-MXT (10 ng); (c) 1,3-DMUA (40 ng); (d) theophylline (20 ng) by use of (A) a K_2PtCl_6 carbon paste electrode and (B) a carbon paste electrode (0.8 mm i.d.). Stationary phase, Phenomenex Luna C_{18} column (particle size 5 μ, 250 mm × 4.6 mm i.d.); Mobile phase, methanol-water (20:80, *v/v*) containing 0.02 M K_2HPO_4 (pH 5.50); flow rate 0.7 mL/min.

line separation. Methanol-phosphate buffer (20:80, *v/v*) was found to be the best condition for a good resolution and the least peak interference in the matrix. In order to determine the optimum applied potential for electrochemical detection, following HPLC, hydrodynamic voltammograms were constructed (Fig. 6) for theophylline and metabolites. The maximum current, measured as peak height, was achieved at a potential of +1.4 V, so this was used. The peak of the height was dependent on the mobile phase flow-rate and varied from 0.3-0.9 mL min^{-1} (Fig. 7).

Linearity and limit of detection

The calibration graphs were linear for theophylline and metabolites over the range of concentration used (50-3200 ng mL^{-1}). The regression equation was y = 34.60 + 3158 x (r = 0.9992), y = 34.59 + 1192 x (r = 0.9999), y = 4.46 + 1432 x (r = 0.9991) and y = 23.45 + 574 x (r = 0.9997) for theophylline, 1-MUA, 3-MXT, and 1,3-DMUA, respectively. The limit of detection (LOD) of theophylline, 1-MUA, 3-MXT and 1,3-DMUA were 1.1 ng mL^{-1}, 3.2 ng mL^{-1}, 1.0 ng mL^{-1} and 11 ng mL^{-1}, respectively.

Recovery and precision

When real samples are analyzed, some other interferences appear in the chromatogram which need to be separated from the metabolites of interest. We examined interference due to the biological sample matrix by standard recovery studies A known amount of theophylline and its metabolites standards (286-1231 ng mL^{-1}) were spiked into serum samples (n = 5) and subjected to the whole procedure. The calibration plots of theophylline, 1-MUA, 3-MXT, and 1,3-DMUA in human serum show good linearity over the range. As shown in Table 1, excellent recoveries and precision were observed (recoveries ranged from 98% to 105%).

Application to human serum

The proposed HPLC method was applied to the determination of theophylline and three of its metabolites in human serum. Representative LC-ECD and LC-UV chromatograms for the theophylline and metabolites in serum before and after continuous oral administration of various teas (2 g/day) are shown Fig. 8. Fig. 8 compares pure theophylline and its metabolites (Fig. 5). Sample constituents with retention characteristics identical to those of theophylline and metabolites were identified and measured. Results for the analysis of human serum after oral administration of various teas is shown in Table 2. A maximum concentration plasma level of theophylline was observed at 3-6 h single post-administration, and plasma drug disappearance half-life was 12 h.[24] Since the blood from the volunteers was collected during 24 h after continuous oral administration of various teas, individual differences appeared. In two volunteers, no theophylline was detected and another volunteer had only a very low level of theophylline that could not be determined by UV detection. However, the concentrations of 1-MUA, 3-MXT, and 1,3-DMUA in volunteers and control serum are in agreement with published literature values of 559 ng mL^{-1}, 709 ng

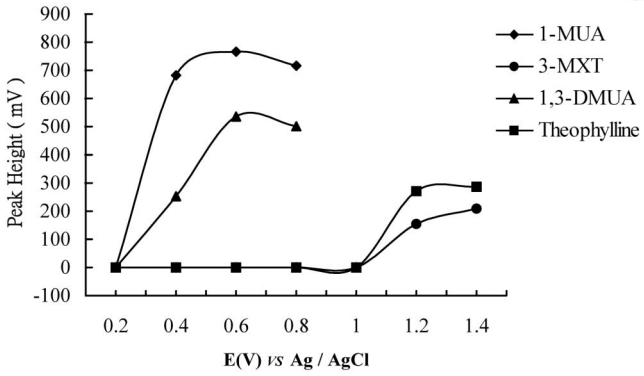

Fig. 6. Hydrodynamic voltammogram obtained for 1-MUA (20 ng); 3-MXT (10 ng); 1,3-DMUA (40 ng); theophylline (20 ng) by use of 85 mm carbon paste electrode detector. Analysis conditions are identical to those listed in Fig. 5.

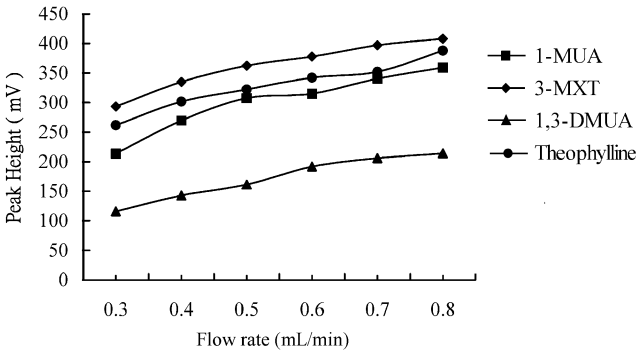

Fig. 7. Dependence of peak height on the flow rate (mL min^{-1}); 1-MUA (20 ng); 3-MXT (10 ng); 1,3-DMUA (40 ng); theophylline (20 ng). Analysis conditions are identical to those listed in Fig. 5.

Table 1. Recovery of 1-methyluric acid (1-MUA), 3-methylxanthine (3-MXT), 1,3-dimethyluric acid (1,3-DMUA), and theophylline from human serum measured by LC-ECD and LC-UV

	LC-ECD			LC-UV		
	Added ($ng\ mL^{-1}$)	Found ($ng\ mL^{-1}$)	Recovery (%)	Added ($ng\ mL^{-1}$)	Found ($ng\ mL^{-1}$)	Recovery (%)
1-MUA	286	296	104	286	278	97.1
	641	670	105	641	593	93.0
	1800	1810	100	1800	1560	86.7
3-MXT	286	285	99.6	286	279	97.7
	641	652	102	641	631	98.4
	1800	1760	97.8	1800	1770	98.3
1,3-DMUA	571	554	97.1	571	556	97.4
	1510	1562	103	1510	1498	99.2
Theophylline	444	440	99.0	444	436	98.2
	615	605	98.3	615	609	99.0

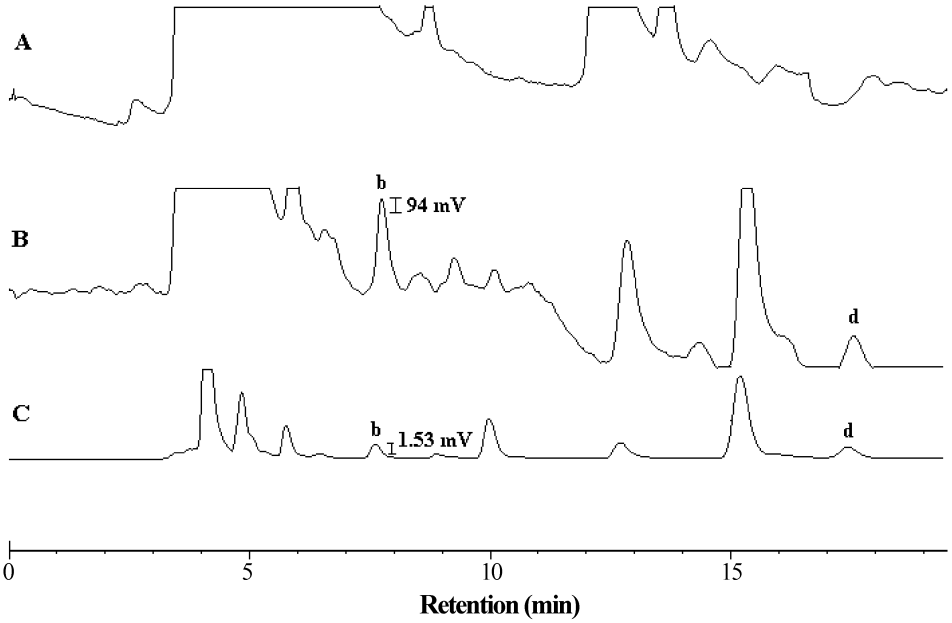

Fig. 8. Chromatograms obtained by LC-ECD (A) and (B); LC-UV (C) from before and after oral administration of black tea (2 g/day) in humans. (A) Untreated black tea serum (B) treated black tea 24 h serum (3-MXT concentration, 0.931 μg/mL; theophylline concentration, 0.882 μg/mL), (C) treated black tea 24 h serum (3-MXT concentration, 0.414 μg/mL; theophylline concentration, 0.712 μg/mL). The peaks are identified as follows: (b) 3-MXT; (d) theophylline. Analysis conditions are identical to those listed in Fig. 5 except the flow rate is 0.5 mL/min.

mL^{-1}, and 920 ng mL^{-1}, respectively.[7]

CONCLUSIONS

A procedure is presented here for a rapid and routine analysis of theophylline and three of its metabolites in serum, based on HPLC separation and subsequent electrochemical detection. The procedure described, DPV, of the experiment is sensitive and selective. The one-step voltammetric detection is a more convenient but less selective assay due to other compounds totally or partially overlapping the analyte signal, and it is not suitable for physiological fluid samples. To avoid background interferences, it is ad-

Table 2. Serum metabolites of theophylline samples from various teas taken orally by human volunteers

	Mean concentration (ng mL^{-1})[a]							
	1-MUA		3-MXT		1,3-DMUA		Theophylline	
	LC-ECD	LC-UV	LC-ECD	LC-UV	LC-ECD	LC-UV	LC-ECD	LC-UV
M1	1384 (0.8%)[b]	1451 (4.3%)	344 (3.3%)	369 (1.1%)	ND[c]	ND	ND	ND
F1	ND	ND	ND	ND	1041 (7.6%)	905 (13.5%)	790 (11.7%)	700 (1.8%)
F2	ND	ND	1407 (6.1%)	1010 (1.7%)	2370 (6.8 %)	1745 (15.5%)	ND	ND
F3	ND	ND	ND	ND	543 (17.3%)	479 (4.9%)	446 (4.3 %)	ND
Control	ND	ND	ND	ND	ND	ND	ND	ND

[a] Number of determinations (n=3).
[b] RSD, relative standard deviation.
[c] ND, not determined.

visable to perform a two-step LC-ECD. This procedure provides a better sensitivity and accuracy but is more time consuming. In comparision with non-disposable electrodes, disposable electrodes, K_2PtCl_6/CPE offer improved simplicity and cheapness.. The detection limit was reduced to 1.0 ng mL^{-1} (S/N = 3) at an operation potential of +1.4 V with attenuation set at 100 nA. An obvious advantage of LC-ECD over LC-UV detection is that it allows a considerable reduction in sample site to be used for analysis.

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