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Simultaneous determination of *p*-aminobenzoic acid and its metabolites in the urine of volunteers, treated with *p*-aminobenzoic acid sunscreen formulation

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Abstract

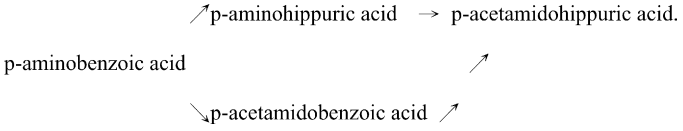
p-Aminobenzoic acid (PABA) and its metabolites (*p*-aminohippuric acid, *p*-acetamidobenzoic acid, and *p*-acetamidohippuric acid) were detected using high-performance liquid chromatography with an electrochemical (carbon paste) detector (HPLC-ECD). For direct current (dc) mode, with the current at a constant potential, and measurements with suitable experimental parameters, a linear concentration from 0.125 to 1.80 μg/ml was found. The detection limit was approximately 2.0 ng/ml. A carbon paste coulometric detector was used to demonstrate that PABA and its metabolites are electrochemically oxidized in acidic media, and to determine, by analyzing human urine, the percutaneous absorption of PABA and its metabolites. Findings using HPLC-ECD and HPLC with an ultraviolet detector (HPLC-UV) were comparable.

Keywords: Percutaneous absorption; High-performance liquid chromatography; Human urine

1. Introduction

Sunscreens are used as a protective measure to prevent sunburn and avoid injury caused by sunlight. Ultraviolet (UV) absorbers are added to many cosmetics to make them effective as anti-ageing and anti-wrinkle creams. *p*-Aminobenzoic acid (PABA), one of the first compounds to be used as a sunscreen agent, is still in use. The use of PABA at 15% is still allowed by the FDA (United States) [1] and at 5% by the EEC (Europe) [2]. During the last few years, researchers have focused on the safety and toxicological and sensitizing properties of PABA and its metabolites, as well as on the effects of PABA on human skin. Nevertheless, few studies have examined its percutaneous absorption [3–6]. Several methods have been proposed to identify PABA and determine its levels in sunscreens and cosmetics: surface-enhanced Raman spectroscopy (SERS) [7], Fourier-transform infrared spectroscopy [8–10], spectrophotometry [11], and liquid chromatography with ultraviolet detection [12,13].

The metabolic processes of PABA in animals have been studied using high performance liquid chromatography with UV or fluorescence detection [14–20]. The percutaneous absorption and metabolism of PABA was determined in human skin [19] and in vitro through hairless guinea pig skin [20] using ^{14}C-labeled PABA. Analytical methodologies employing radioactive PABA cannot be used with live human skin as the substrate because of the hazardous nature of radioactive compounds. PABA is metabolized primarily by acetylation and glycine conjugation in the liver to form *p*-aminohippuric acid (4-AHA), *p*-acetamidobenzoic acid (4-AMB), and *p*-acetamidohippuric acid (4-OCH_3-AHA), as shown in the following scheme [14,18]:



HPLC-UV or fluorometric detection [21–23] and gas chromatography-quadrupole ion trap mass spectrometry (GC-QITMS) [24] detection have been used to measure sunscreen (2-phenylbenzimidazole-5-sulphonic acid, benzophenones, octylsalicylate) and its metabolites in urine or plasma. However, there is only one published report on determining the urinary levels

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of PABA absorbed from sunscreen [3]. No study has attempted to simultaneously measure the concentration of PABA and its metabolites. The goal of this study was to determine the urinary levels of PABA metabolites from dermally absorbed PABA-containing sunscreen. The HPLC-ECD procedure for measuring PABA and its metabolites, which has the advantage of being selective and sensitive in urine, is described.

2. Experimental

2.1. Apparatus and materials

A high-performance liquid chromatograph system (LC-10 AD_{vp}; Shimadzu, Kyoto, Japan) containing a Rheodine 7125 injection valve with a 20-μl sample loop coupled to an electrochemical detector (DECADE II; Antec Leyden B.V., Netherlands) and a spectrophotometric detector (L-7420; Hitachi, Japan) was used. The flow cell was designed with the following electrodes: an Ag/AgCl/0.1 M KCl reference electrode, a stainless steel auxiliary electrode, and a carbon paste electrode to detect *p*-aminobenzoic acid (PABA), *p*-aminohippuric acid, *p*-acetamidobenzoic acid, and *p*-acetamidohippuric acid. All solvents and analytes were filtered through 0.45-μm cellulose acetate and polyvinylidene fluoride (PVDF) syringe membrane filters, respectively. Chromatograms of PABA, 4-AHA, 4-AMB, and 4-OCH_3-AHA were registered and peak height calculated using an SISC Chromatogram Data Integrator. PABA, 4-AHA, and 4-AMB were obtained from Acros Organics (New Jersey, USA) and 4-OCH_3-AHA was synthesized from 4-AHA. All other reagents were locally purchased and of analytical grade.

2.2. Human volunteers

The volunteer population consisted of six healthy adults (three men and three women; mean age, 22 ± 1.0 years) who had not used any sunscreen formulation for 1 month before the study. All the experiments were done using the same sunscreen formula, created for this study, containing 5% PABA, similar to locally available commercial products. The same procedure was used to apply sunscreen solution to all the volunteers. The amount of the mixture involved in the process was determined by weight. The participants were given plastic urine containers for each time period: 0, 1, 2, 4, 8, 16, 24, 36, 42, 48 h, etc.

2.3. Extracting PABA and its metabolites

Solvent extraction efficiency was tested pretreatment, and recoveries were 72, 70, 92, and 99% for chloroform, dichloromethane, ether, and ethyl acetate, respectively. Because ethyl acetate extracts various oxidizable compounds present in urine, such as ascorbic acid, uric acid, and urea, which interfere with determining 4-AMB and 4-OCH_3-AHA, 1-ml human urine samples were centrifuged at $3000 \times g$ for 30 min. The supernatant urine was transferred to another centrifugal tube containing 2 ml of ethanol and centrifuged for 30 min to sediment the aggregates. The deproteinized samples were then extracted three times using 5–15 ml of ethyl acetate. The organic phase was collected and evaporated under nitrogen at a temperature less than 37 °C. The dried extract was reconstituted with 0.5 ml of 50% (v/v) methanol–water and loaded onto a C_{18} cartridge (Sep-Pak; Waters, Massachusetts, USA) that had been conditioned with 2 ml of methanol and 2 ml of water before the samples were loaded. An additional 0.5 ml of methanol was used to rinse the sample vial, and the sample was then loaded onto the C_{18} cartridge. The sample on the C_{18} cartridge was washed three times with 10 ml of dichloromethane. These three fractions were combined and dried under nitrogen at 45 °C. The dry extract was reconstituted with 500 μl of pure methanol and filtered through 0.45 μm membrane filters before HPLC analysis.

2.4. Fabricating a disposable electrode

From Teflon tubing (~7.5 cm long, 1/32 in. internal diameter, and 1/16 in. outside diameter), a coulometric carbon paste electrode, similar to one reported elsewhere [25], was constructed. The carbon paste (graphite powder paraffin oil, 2:1, w/w) was inserted into one end of the tube and sealed with acrylic resin (Struers Ltd., United Kingdom). A small copper wire was placed in the other end of the tube to allow for an electrical connection to the carbon paste (Fig. 1A).

2.5. Constructing the detector

The platinum wire, which served as a counter, and the Ag/AgCl, which functioned as a reference electrode, were then attached in series to the Teflon tube. For stability, the cell compounds were secured with tape to an insulated plastic box (Fig. 1B). The construction was similar to that of a coulometric detector; that is, the eluate was fed to the carbon paste electrode in an overflow vessel containing a counter-electrode and a reference electrode.

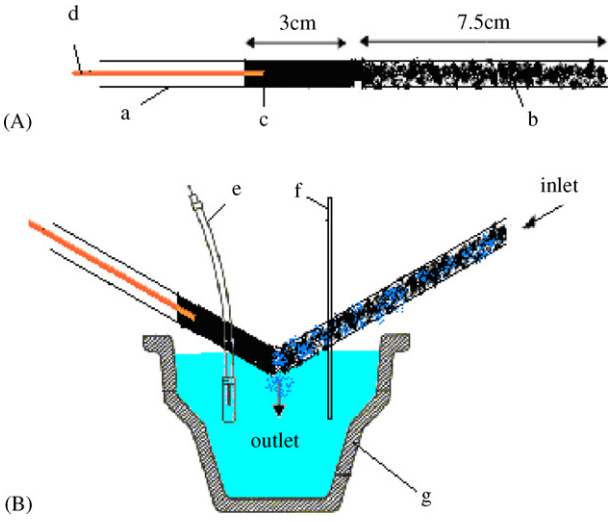


Fig. 1. Schematic of a carbon paste electrode flow-through electrochemical cell. (A) Pattern of a disposable electrode: (a) PTFE tubing, 1/16 in. o.d.; (b) carbon paste; (c) tube-end fitting with carbon powder; (d) copper wire. The flow channel is shown in (B): (e) Ag/AgCl reference electrode; (f) Pt wire auxillary electrode, 0.5 mm; (g) plastic box.

2.6. Determining sample composition using liquid chromatography

A stock standard sample solution was prepared by dissolving 10 mg each of PABA, 4-AHA, 4-AMB, and 4-OCH_3-AHA in 10 ml of methanol. Working standard solutions in the range 125–600 ng/ml were prepared from the stock standard solution. RP-HPLC was done on a Phenomenex Luna C_{18} 5-mm (250 mm × 4.6 mm) column eluted with methanol–phosphate buffer (pH 5.5) (20:80, v/v) as the mobile phase, at a flow rate of 1 ml/min. After the sample components had been separated on the Phenomenex Luna C_{18} column, they were examined using an ultraviolet detector set at 254 nm. The electrochemical detector was operated at +1.4 V. A chromatograph was made of 20 μl of the prepared sample solution and standard solution under the operating conditions described above. Quantitation was based on the peak height of the sample.

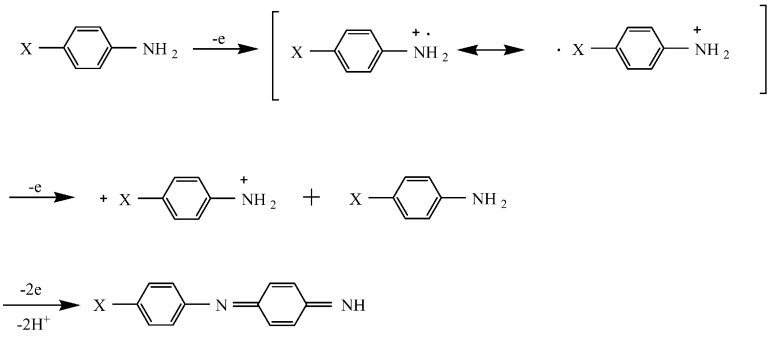
3. Results and discussion

3.1. Optimum conditions for analysis

Radical cations have been obtained using electrochemistry oxidation of monoamine derivatives of primary amines, such as PABA and 4-AHA, and of secondary amines, such as 4-AMB and 4-OCH_3-AHA. The $2e^-$ pathway was proposed for them. They can be oxidized to the corresponding dimer in two steps [26–28]. Therefore, a possible mechanism is given below:

PABA (X = COOH), 4-AHA (X = $CONHCH_2COOH$), 4-AMB (R = OCH_3, X = COOH),

4-OCH_3-AHA ((R = OCH_3, X = $CONHCH_2COOH$)



Various ratios of methanol–dipotassium hydrogen phosphate (90:10, 80:20, 70:30, v/v) were experimented with on metabolites of PABA. After various studies of the retention behavior of the PABA, baseline separation was achieved. Methanol–phosphate buffer (80:20, v/v) was found to be the best mobile phase for a good resolution and for the least peak interference in the matrix. In order to determine the optimum applied potential for electrochemical detection, following HPLC, hydrodynamic voltammograms were constructed for PABA and metabolites (Fig. 2). The maximum current, measured as peak height, was achieved at a potential of +1.4 V. The peak height depends upon the mobile phase flow-rate and varies from 0.2 to 0.6 ml/min (Fig. 3).

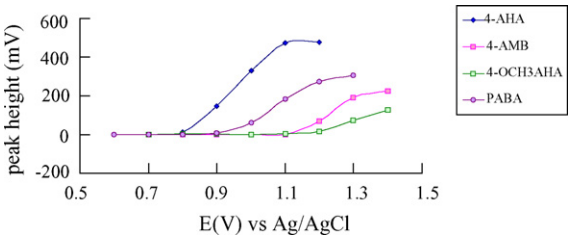


Fig. 2. Hydrodynamic voltammogram obtained for *p*-aminobenzoic acid, PABA (7.5 ng); *p*-aminohippuric acid, 4-AHA (7.5 ng); *p*-acetamidobenzoic acid, 4-AMB (12 ng); *p*-acetamidohippuric acid, 4-OCH_3-AHA (12 ng) by use of 85 mm carbon paste electrode detector. Stationary phase, Phenomenex Luna C_{18} column (particle size 5 μm, 250 mm × 4.6 mm i.d.); mobile phase, methanol–water (20:80, v/v) containing 0.02 M K_2HPO_4 (pH 4.41); flow rate 0.4 ml/min.

3.2. Linearity and limit of detection

The calibration graphs were linear for PABA and metabolites over the range of concentration used (125–1800 ng/ml). The regression equations obtained for PABA, 4-AHA, 4-AMB, and 4-OCH_3-AHA were $y = -14 + 1861x$ ($r = 0.9990$), $y = -48 + 1244x$ ($r = 0.9992$), $y = -8.0 + 1198x$ ($r = 0.9995$), and $y = 5.7 + 597x$ ($r = 0.9999$), respectively. The limit of detection (LOD) was calculated using the equation $LOD = KS_o/S$, where K was a numerical factor chosen according to the confidence level desired, S_o was the standard deviation of the blank measurement ($n = 6$), and S was the sensitivity of the calibration graph. In this study, 3 was the value used for K. The limits of quantification (LOQ) for PABA, 4-AHA, 4-AMB, and 4-OCH_3-AHA were 0.04, 0.04, 0.08, and 0.18 ng, respectively.

3.3. Accuracy and precision

When commercial samples were analyzed, the chromatograms contained some other interference, which needed to be separated from the metabolites of interest. The interference caused by the biological sample matrix was examined using

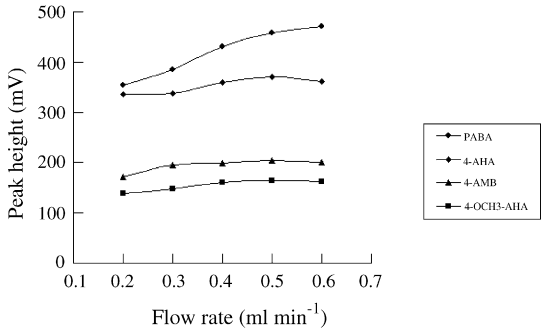


Fig. 3. Dependence of peak height on the flow rate (ml/min); PABA (7.5 ng); 4-AHA (7.5 ng); 4-AMB (12 ng); 4-OCH_3-AHA (12 ng). Analysis conditions are identical to those listed in Fig. 2. Electrode potential was at 1.4 V vs. the Ag/AgCl reference electrode.

standard recovery studies. A known amount of PABA and its metabolite standards (200–1000 ng/ml) were spiked into urine samples ($n = 3$) and subjected to the whole procedure. The calibration plots of PABA, 4-AHA, 4-AMB, and 4-OCH_3-AHA in human urine showed good linearity over the range. The validation of this method was done using different intra- and inter-day concentrations of the four compounds in human urine. Recoveries and precision were excellent using LC-ECD (recoveries and standard deviations ranged from 96 ± 2.0 to $99 \pm 3.8\%$) for intra- and inter-day concentrations ranging from 0.2 to 3.8% and from 1.2 to 3.5%, respectively (Table 1).

3.4. Application to human urine

The proposed HPLC method was used to determine levels of PABA and metabolites in human urine. Fig. 4 is a chromatogram of pure standard and metabolites. Fig. 5 compared with Fig. 4, shows representative LC-EC and LC-UV chromatograms for the PABA and metabolites in urine before and after treatment with PABA-containing sunscreen. Sample constituents with retention characteristics identical to those of PABA and metabolites were identified and measured. Then the urine of the six volunteers, who had used a sunscreen cream preparation containing 5% PABA, was examined and PABA and its metabolites were measured; Fig. 6 shows the time course of their levels in human urine. The major metabolite determined using this approach was 4-AMB, and the minor metabolites were 4-OCH_3AHA and 4-AHA. Acetyl derivatives of PABA were excreted in the urine for

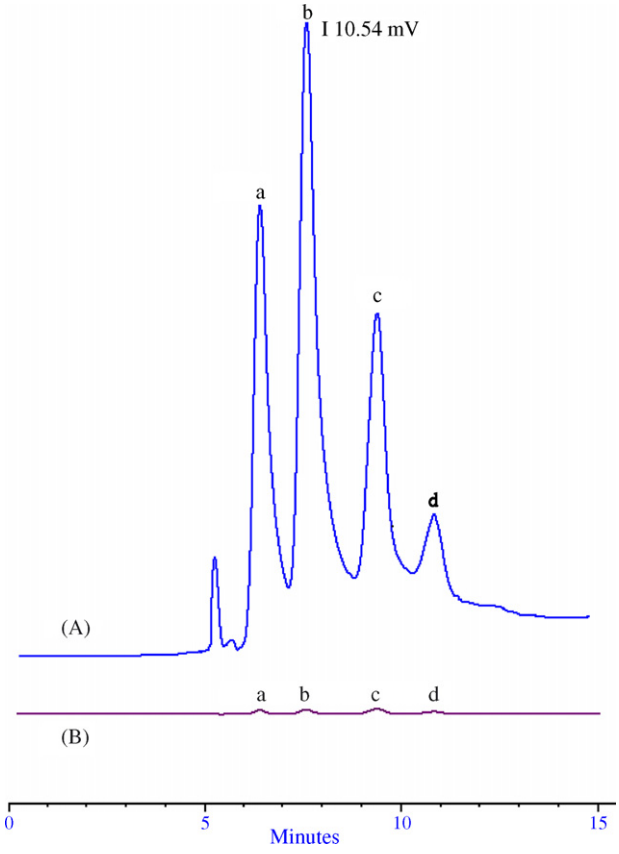


Fig. 4. Chromatograms obtained by (A) LC-ECD and (B) LC-UV; (a) *p*-aminohippuric acid, 4-AHA (7.5 ng); (b) *p*-aminobenzoic acid, PABA (7.5 ng); (c) *p*-acetamidobenzoic acid, 4-AMB (12 ng); (d) *p*-acetamidohippuric acid, 4-OCH_3-AHA (12 ng). Analysis conditions are identical to those listed in Fig. 2. Electrode potential was at 1.4 V vs. the Ag/AgCl reference electrode.

24 h after sunscreen cream had been used. Urine concentration time-profiles for PABA, 4-AMB, 4-OCH_3AHA, and 4-AHA are shown in Fig. 7. Table 2 shows the metabolite concentrations in the urine of the six volunteers using LC-ECD and LC-UV methods to analyze human urine after treatment with PABA-containing sunscreen. ECD is a more specific and sensitive detection method than UV. Metabolite derivatives can be difficult to measure accurately in urine using the UV method. Because the penetration of drugs is affected by anatomical site, age, skin thickness (stratum corneum), and gender, urine specimens from six different participants were used in the metabolism studies.

Table 1
Precision and accuracy in the determination of PABA and its metabolites (4-AHA, 4-AMB, and 4-OCH_3-AHA) in spiked human urine samples by LC-ECD and LC-UV

	LC-ECD					LC-UV				
	Added (ng/ml)	Found (ng/ml)[a] (mean ± S.D.)[b]	Recovery (%)	Variability (CV, %)		Added (ng/ml)	Found (ng/ml)[a] (mean ± S.D.)[b]	Recovery (%)	Variability (CV, %)	
				Intra-day	Inter-day				Intra-day	Inter-day
PABA	200	198 ± 7.50	99	3.8	3.5	900	984 ± 8.36	109	8.5	4.6
4-AHA	1000	961 ± 19.2	96	2.0	3.3	1000	963 ± 25.0	96	2.6	2.5
4-AMB	900	883 ± 1.81	98	0.2	1.2	2000	1840 ± 18.0	92	0.1	1.3
4-OCH_3-AHA	1000	983 ± 4.93	98	0.5	2.0	1000	960 ± 38.4	98	4.0	3.8

[a] Number of determination ($n = 3$).
[b] S.D., standard deviation.

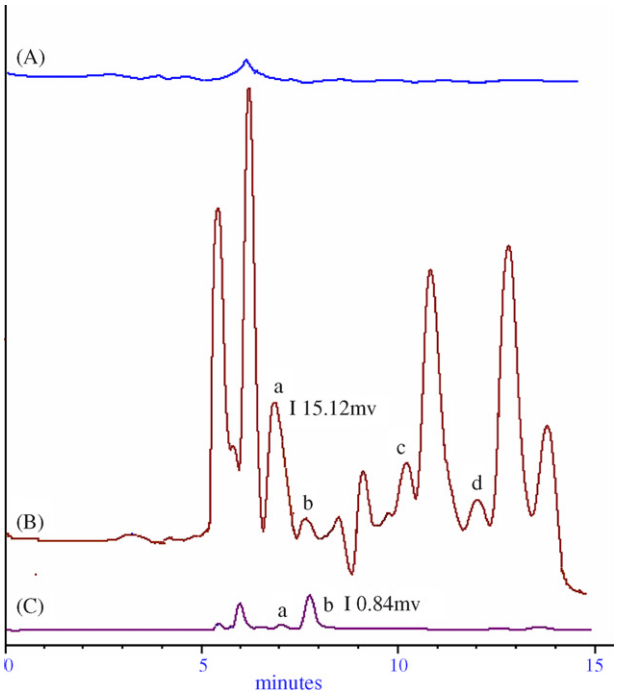


Fig. 5. Chromatograms obtained by LC-ECD (A) blank urine and after 24 h application of a sunscreen cream of 5% PABA to human skin, (B) LC-ECD, and (C) LC-UV.

These results show that dermally absorbed PABA was excreted primarily as the acetyl conjugate 4-AMB. Smaller amounts of 4-AHA and 4-OCH_3-AHA were also found. These quantitative results are similar to those in published reports [18,29] showing serum levels in intravenous studies. The urine samples contained PABA, 4-AHA, 4-AMB, and 4-OCH_3-AHA; PABA, 4-AHA, and 4-OCH_3-AHA were not detected in the urine samples of some participants, because the amounts present were too small. Within the first 24 h post-treatment, the proportions of the dermally absorbed PABA excreted in urine were 0.562 μg/ml for 4-AHA and 967 μg/ml for 4-AMB.

3.5. Sensor stability

The operational stability of the sensors was tested by continuously exposing them to the flow stream, and by monitoring the amperometric response (at +1.4 V versus Ag/AgCl) of methanol–phosphate buffer (pH 5.5) (20:80, v/v) over several hours of repetitive injections. After about 1 h of background stabilization, an average 2.5% decrease of peak height was observed over 15 h.

A number of comprehensive reviews of previous work using liquid chromatography with an ESA Analytical Coulochem II electrochemical detector equipped with a guard cell and an ESA analytical cell have been published [30–35]. These reports

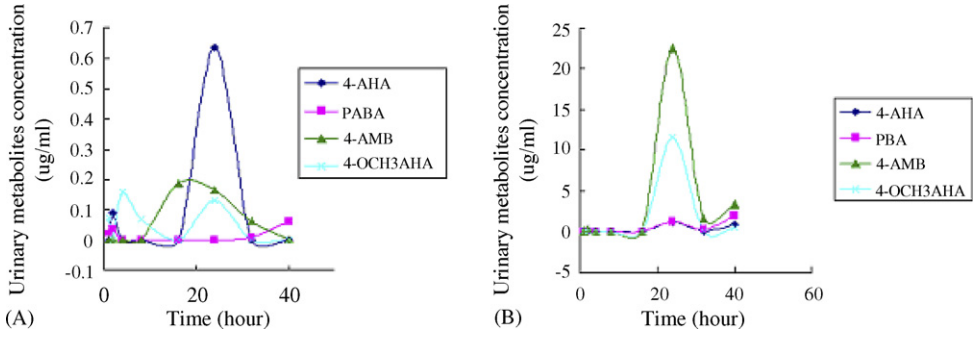


Fig. 6. Urinary metabolites of PABA levels found in a human (A) female and (B) male during percutaneous absorption.

Table 2
Urine metabolites of sample taken from human skin before and after treated with 5% PABA sunscreen cream

Mean concentration (ng/ml)[a]												
	Before			After 24 h								
	PABA			4-AHA			4-AMB			4-OCH3-AHA		
	LC-ECD		LC-UV	LC-ECD		LC-UV	LC-ECD		LC-UV	LC-ECD		
	Mean ± S.D.	Intra-day, inter-day		Mean ± S.D.	Intra-day, inter-day		Mean ± S.D.	Intra-day, inter-day		Mean ± S.D.	Intra-day, inter-day	
F1	310 ± 4.3[b]	1.4, 13	291 ± 2.9	1360 ± 41	3.0, 12	1000 ± 60	31200 ± 2152	6.9, 11	25600 ± 56	ND	ND	ND
F2	357 ± 14	3.9, 13	260 ± 5.2	690 ± 14	2.0, 3.9	680 ± 27	160 ± 1.6	0.1, 7.7	.112 ± 3.4	128 ± 16	1.3, 10	ND
F3	230 ± 6.9	3.0, 9.1	200 ± 6.0	ND	ND	ND	1930 ± 59.8	3.0, 1.3	1027 ± .51	ND	ND	ND
M1	1530 ± 37	2.4, 8.7	ND	1200 ± 26	2.2, 9.8	860 ± 34	23800 ± 238	1.0, 3.4	24400 ± 49	503 ± 15	0.3, 1.8	ND
M2	ND	ND	ND	ND	ND	ND	.560 ± 14.6	2.6, 6.8	448 ± 45	ND	ND	ND
M3	ND	ND	ND	ND	ND	ND	357 ± 1.8	0.5, 3.4	223 ± 6.7	ND	ND	ND

ND, not been detected.
[a] Number of determinations ($n = 3$).
[b] S.D., standard deviation.

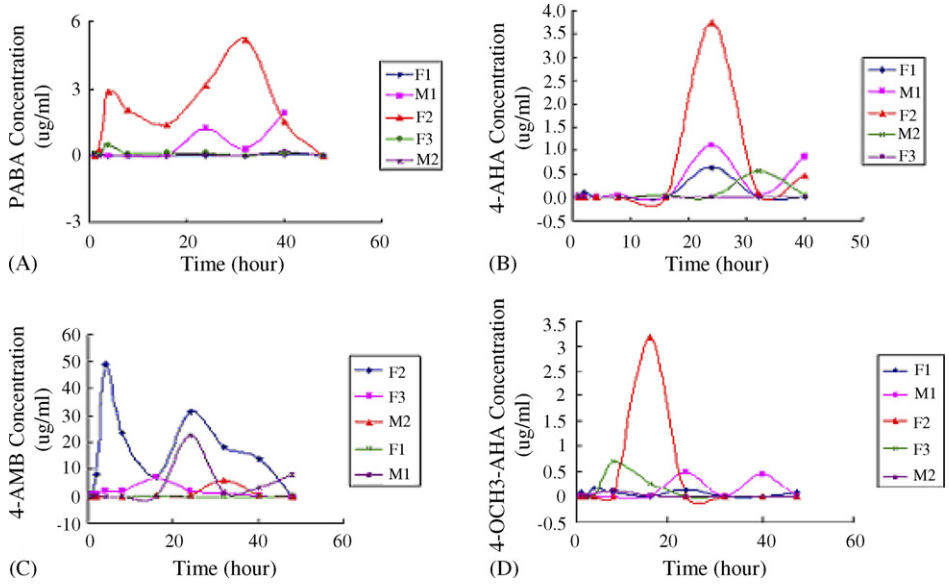


Fig. 7. Urinary excretion curves of (A) PABA and its metabolites, (B) 4-AHA, (C) 4-AMB, and (D) 4-OCH_3-AHA after application of a sunscreen cream of 5% PABA to human skin.

demonstrate that the coulometric electrode array system has become a routine technique because it enables researchers to simultaneously analyze many different compounds. Its drawbacks are that the equipment is expensive and complex to operate. A disposable carbon paste electrode in a Teflon tube was prepared for the present study. The fabrication process was inexpensive and is easily reproducible. This electrode was used in flow-through electrochemical cells to replace conventional non-disposable graphite electrodes for coulometric detection of compounds separated by HPLC.

4. Conclusions

The coulometric detector fabricated for this study was extremely simple and inexpensive. A procedure was presented here for a rapid and routine analysis of PABA and three of its metabolites in urine, based on liquid chromatography separation and subsequent coulometric detection. The detection limit was reduced to 2 ng/ml (S/N = 3) at an operational potential of +1.4 V with attenuation set at 20 nA. A major advantage of LC-EC over LC-UV detection is that LC-EC requires smaller urine samples than LC-UV does.

Acknowledgment

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