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TECHNICAL NOTE

TOXICOLOGY

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Sensitive Determination of Barbiturates in Biological Matrix by Capillary Electrophoresis Using Online Large-Volume Sample Stacking*

ABSTRACT: In China, some forensic cases are caused by barbiturates. Thus, the determination of trace level barbiturates in body fluid is important for the poisoning investigation. In this study, an online large-volume sample stacking (LVSS) with polarity switching in capillary electrophoresis (CE) was applied for the sensitive determination of barbiturates. This technique involves injecting a large volume of sample into a capillary and removing the sample matrix plug out of the capillary by reversing the polarity. Quantitation limit obtained was 0.048, 0.057, 0.039, and 0.015 μg/mL for secobarbital, amobarbital, barbital, and phenobarbital (signal-to-noise ratio = 9). By using LVSS, the stacking was simply achieved at 171.7-, 169.7-, 202.7-, and 169.1-fold for the above four barbiturates. The relative standard deviation values of intraday and interday were <2.11% and 4.69%, respectively. Recoveries were ranged from 83.7 to 105.2%. Finally, the trace analysis method was applied to the analysis of real forensic specimens and has achieved satisfactory results.

KEYWORDS: forensic science, secobarbital, amobarbital, barbital, phenobarbital, capillary zone electrophoresis, sample stacking, large volume

Barbiturates are substituted pyrimidine derivatives. The structures of the barbiturates are given in Fig. 1. Barbiturates are one of the most popular sedative hypnotic drugs used in clinic. They have been extensively used in the past to appease anxiety, reduce blood pressure, decelerate heart rate, reduce rapid eye movement sleep, and so on. Nowadays, although their medical application has declined, the abuse of barbiturates is widespread, and they are implicated in murder, suicide, and accidental cases (1–3). For example, in China, some forensic cases are caused by barbiturates. It was calculated that the forensic cases caused by sedative hypnotics (including barbiturates) were ranked fourth (G.-Z. Huang unpublished data) in China. Thus, the determination of trace level barbiturates in body fluid is important for the poisoning investigation, but it is challenge because of their low lethal blood levels, it is 60 μg/mL for barbital and 10 μg/mL for amobarbital and secobarbital, and could be lower in combination with alcohol and/or amphetamines (4).

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Different techniques have been developed for the determination of barbiturates. The traditional methods include gas chromatography (GC) (5), gas chromatography–mass spectrometry (GC/MS) (6,7), high-performance liquid chromatography (HPLC) with UV detection (8,9), HPLC/MS (10), micellar liquid chromatography with UV detection (4,11,12), and immunoassay (13). However, most of the methods are time consuming. They require laborious sample pretreatments and derivatization and need specialized column or equipment to improve the sensitivity. So, simple and accurate methods should be applied for the analysis of barbiturates.

Capillary electrophoresis (CE) has become a powerful separation technique because of its low solvent consumption, high resolving power, short analysis time, and simple sample pretreatment (14). It has been widely used to analyze chemicals, pharmaceuticals, and metabolites (15–17) and has gradually developed into a very active research area for the analysis of biological samples (18–21). Several CE methods have been used for the determination of barbiturates, such as capillary electrochromatography (22,23), micellar electrokinetic capillary chromatography (24–26), and capillary zone electrophoresis (CZE) (27–29). However, the sensitivity was poor and cannot be used for the analysis of trace level barbiturates in physiological samples.

Large-volume sample stacking (LVSS) is an online sample stacking method introduced by Chien and Burgi (30). This technique involves injecting a large volume of sample into a capillary. LVSS reversed electroosmotic flow (EOF) to push the matrix out of the capillary. LVSS has been used for trace level analysis of environmental and pharmaceutical samples (31–39), but so far, it has not been applied in the detection and stacking of barbiturates.

In this study, the LVSS in CZE was applied to online stacking the barbiturates in plasma. Four kinds of barbiturates (secobarbital,

amobarbital, barbital, and phenobarbital) were selected as the target drugs. To achieve lower detection limits and better stacking results, the effects of buffer conditions, applied voltage, and injection conditions were investigated.

Experimental Section

Apparatus

The experiments were performed using a P/ACE MDQ system (Beckman Coulter, Fullerton, CA) fitted with a UV detector. 32 Karat software (Beckman Coulter) on an HP personal computer (Hewlett-Packard, Palo Alto, CA) was used to gather and analyze data. Detection wavelength was set at 214 nm, and temperature of system was 25°C. Electrophoresis was performed in an untreated fused-silica capillary of 75 μm I.D. (375 μm O.D.) × 60.5 cm (50 cm effective length) (Yongnian Optical Fiber Factory, Heibei, China). An Ultrapure Water System (SG Ultra Clear system, Wasseraufbereitung und Regenerierstation Gmbh, Germany) was used to produce ultrapure water. A 320 pH meter (Mettler-Toledo Instrument Ltd., Shanghai, China) was used to measure the pH of the background electrolyte (BGE).

Chemicals

Secobarbital, amobarbital, barbital, and phenobarbital (purity >99.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sodium tetraborate ($Na_2B_4O_7$, guarantee reagent [GR]), boric acid (H_3BO_3, analytical reagent [AR]), dichloromethane (CH_2Cl_2, GR), sodium hydroxide (NaOH, AR), and hydrochloric acid (HCl, AR) were purchased from the Shanghai Chemistry Reagent Company (Shanghai, China). Methanol (CH_3OH, AR) was obtained from the First Chemical Factory of Zhenxing (Shanghai, China). Plasma samples were collected from four healthy Sprague-Dawley rats and kept frozen at −20°C until analysis.

Preparation of Buffers and Standard Solution

In this study, $Na_2B_4O_7$-H_3BO_3-CH_3OH solution was chosen as the BGE. The buffers were prepared as the following: 10–60 mM $Na_2B_4O_7$, containing 0–30% CH_3OH (v/v), was first prepared, and then, the pH was adjusted to 7.5–9.5 with 0.5 M H_3BO_3.

The standard stock solution of barbiturates (500 μg/mL) was prepared by dissolving the standards in methanol (100 μL), then diluted with ultrapure water to 10 mL. The stock solution was stored at 4°C. A series of working standard solutions were further made by diluting the stock solutions with ultrapure water.

Preparation of Standard Plasma Sample

Eighty microliters of rat plasma was spiked into Eppendorf tube, and 10 μL secobarbital, amobarbital, barbital, and phenobarbital at different concentrations (400, 300, 200, 100, 20, 10, 5, 2.5, 1, and 0.4 μg/mL) were, respectively, added into the tube to obtain a series of standard plasma samples. Then, the plasma samples were vortexed for 15 sec. After that 1 mL CH_2Cl_2 was added into plasma samples for the extraction of barbiturates. Later, the mixture in the tube was strongly shook with a vortex for 2 min and then centrifuged at $3262 \times g$ for 10 min. Barbiturates were extracted from the plasma to the organic phase. The organic layer was transferred to another Eppendorf tube, and the extraction was repeated once by adding 0.5mL CH_2Cl_2 to the residue. All of organic phases (two times) were combined together and dried using a vacuum oven at 45°C. The residue was dissolved in 100 μL ultrapure water. The final concentrations of barbiturates in the standard plasma samples were 40, 30, 20, 10, 2, 1, 0.5, 0.25, 0.1, and 0.04 μg/mL for secobarbital, amobarbital, barbital, and phenobarbital, respectively. All solutions were stored at 4°C before experiment.

For the analysis of real forensic specimens, the samples were prepared as mentioned earlier.

Procedures of LVSS

The new capillary was rinsed by 1 M NaOH, ultrapure water, 1 M HCl, ultrapure water for 20 min, respectively, and equilibrated for 30 min with the BGE. Between injections, the capillary was rinsed with the buffer for 3 min.

The stacking was achieved by LVSS method. First, a long plug of sample solution was introduced into the capillary by pressure (3.0 psi for 80 sec). Then, the sample vial was replaced with the BGE vial, and a high voltage with reversed polarity (−20 kV) was applied to push the water out of capillary by the strong cathodic EOF. Then, the zone of stacked analytes approaches the capillary inlet, the negative voltage was stopped, and the polarity was switched back to the normal configuration (20 kV) and separation was preceded.

Results and Discussions

Effect of Buffer pH

The pH of a buffer plays a very important role in the separation of ionizable analyte because it determines the extent of ionization of each individual analyte. Therefore, manipulation of buffer pH usually becomes a key strategy in optimizing a separation. The pKa values of secobarbital, amobarbital, barbital, and phenobarbital are 7.9, 7.8, 8.0, and 7.4, respectively.

In this experiment, a series of 20 mM $Na_2B_4O_7$-H_3BO_3 buffers with pH values ranging from 7.5 to 9.5 were investigated with 20 kV applied voltage and a 0.2 psi 5 sec sample injection. As shown in Fig. 2, pH has an obvious influence on the migration time and resolution of aim drugs. The resolution between secobarbital and amobarbital first increased when pH increased from 7.5 to 8.0, then decreased when pH changed from 8.0 to 9.5. The resolution of amobarbital and barbital increased gradually when the pH

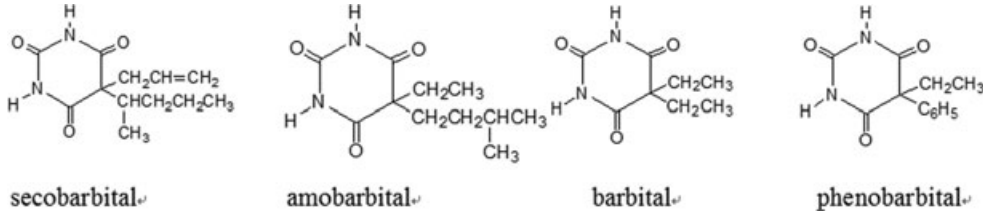

FIG. 1—*The structures of the barbiturates.*

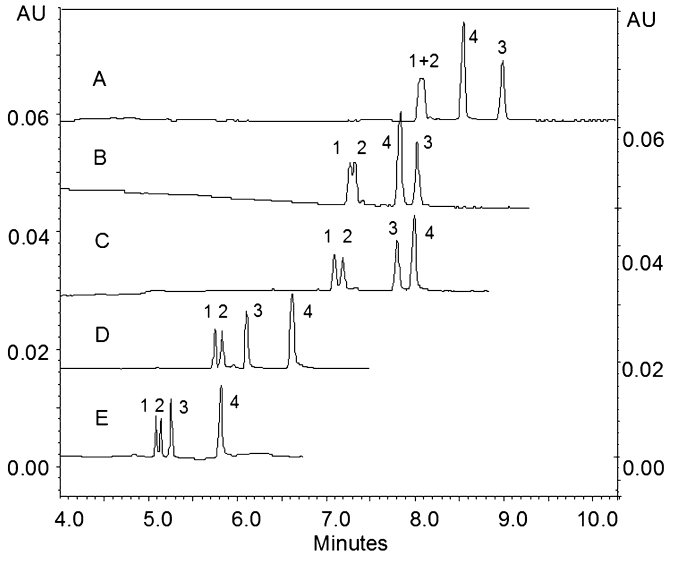

FIG. 2—*Electropherograms of barbiturates (50 μg/mL) at different pH values. (A) pH 9.5; (B) pH 9.0; (C) pH 8.5; (D) pH 8.0; (E) pH 7.5. Conditions: pH 7.5–9.5 $Na_2B_4O_7$ (20 mM)-H_3BO_3 (0.5 M), 20 kV voltage, 60.5 cm capillary (50 cm effective length), 214 nm wavelength, 25°C, 0.2 psi 5 sec sample injection. 1: secobarbital; 2: amobarbital; 3: barbital; 4: phenobarbital.*

value increased from 7.5 to 9.5. From pH 7.5 to 9.0, the resolution of barbital and phenobarbital decreased and then enhanced at higher pH. It can be seen from Fig. 2, there was no baseline separation between secobarbital and amobarbital except at pH 8.0, so pH 8.0 was chosen as the optimized pH value.

Effect of Organic Modifiers

In CZE, the addition of an organic modifier to a buffer altered the selectivity and resolution. To investigate the impact of different modifiers on separation, the most common organic solvents in CE, methanol and acetonitrile, were studied. Under the same conditions, equivalent methanol and acetonitrile were added to the BGE, respectively, the results showed that acetonitrile had little impact on resolution, but methanol increased the resolution significantly, so methanol was selected as the organic modifier solvent.

To study the influence of methanol concentration on the resolution, pH 8.0 BGE with different concentrations of methanol (0–30%, v/v) was prepared at first. The data unveiled that along with the methanol concentration increase, the resolution is also increasing. At the same time, the migration time is increasing too. To obtain better resolution and faster detection of barbiturates, 20% (v/v) methanol was chosen to provide faster separation and better resolution.

Effect of Buffer Concentration

Buffer concentration had obvious influence on the separation because it could influence the EOF and the viscosity of the electrolyte. To achieve the best resolution of the test mixtures, the effect of the concentration of buffer at pH 8.0 on separation was studied in the range of 10–60 mM (in six steps), with 20% (v/v) methanol. The variety of buffer concentration did not give any changes in the migration orders of the analytes; it had, however, significant effect on the resolutions and migration times. As expected, the migration times increased almost linearity with the increase of buffer concentration. The resolution of analytes was increased with the increasing of buffer concentration, but for barbital and phenobarbital, the resolution achieved maximum value at 40 mM and then decreased at 50 mM and elevated slightly at 60 mM. With 40 mM buffer, the resolution was the best, and the baseline separation of barbiturates was achieved. Therefore, a buffer concentration of 40 mM was chosen for the further experiments.

Effect of Applied Voltage

The applied voltage had a notable influence on separation time and resolution. Here, a buffer of 20% (v/v) methanol in 40 mM sodium tetraborate buffer (pH 8.0) was used to study the effect of voltage (12.5–25 kV). Figure 3 revealed that the applied voltage had little influence on the resolution between the analytes, but when the applied voltage increased, the migration time was reduced. It can be seen from the experimental data that the migration times of secobarbital, amobarbital, barbital, and phenobarbital decreased from 16.13, 16.55, 17.44, and 19.98 to 8.25, 8.48, 9.00, and 10.30 min, respectively, when the voltage increased from 12.5 to 25 kV. With the increasing of applied voltage, the EOF increased, thus the migration time decreased. For the sake of shorter migration time and better resolution, 20 kV was chosen for the working voltage.

Optimization of Electrode Reversing Time

The electrode reversing time is an extremely important factor that influences the effect of stacking. The reverse voltage applied in this experiment was −20 kV. By reversing the electrode for an appropriate time to pump out the sample matrix from the capillary before electrophoresis, the effect of stacking and separation was significantly improved (Fig. 4*C*). Electrode reversing time should be strictly controlled. If the reversing time of electrode is too long, most of the analytes will be discharged out of the capillary and cannot be stacked (Fig. 4*D*). If the electrode reversing time is too short, part or most of the sample matrix cannot not be pump out of

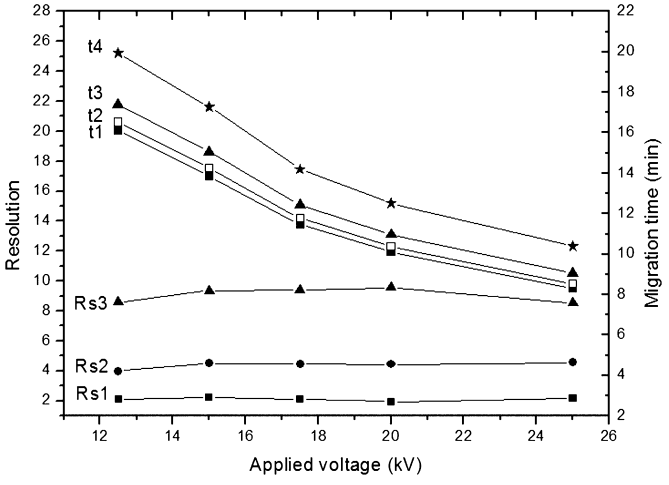

FIG. 3—*Effect of applied voltage on migration time and resolution of barbiturates. Conditions: pH 8.0 $Na_2B_4O_7$ (40 mM) (containing 20% CH_3OH, v/v)-H_3BO_3 (0.5 M). Rs1: the resolution between secobarbital and amobarbital; Rs2: the resolution between amobarbital and barbital; Rs3: the resolution between barbital and phenobarbital; t1: the migration time of secobarbital (min); t2: the migration time of amobarbital (min); t3: the migration time of barbital (min); t4: the migration time of phenobarbital (min). The other conditions are the same as those in Fig. 2.*

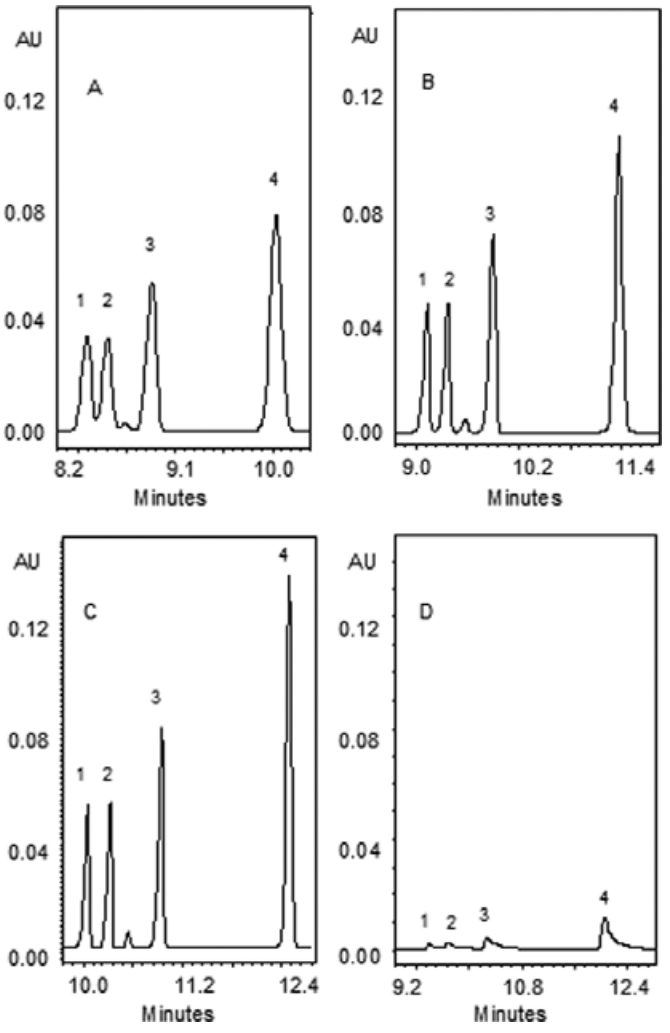

FIG. 4—*Effect of electrode reversing time on separation and stacking before electrophoresis. (A) Normal CZE without electrode reverse; (B) electrode reverse (−20 kV) for 0.2 min; (C) electrode reverse (−20 kV) for 0.4 min; (D) electrode reverse (−20 kV) for 0.6 min. Conditions: pH 8.0 $Na_2B_4O_7$ (40 mM) (containing 20% CH_3OH, v/v)-H_3BO_3 (0.5 M), 20 kV 1.4 psi 10 sec injection. The other conditions are the same as those in Fig. 2.*

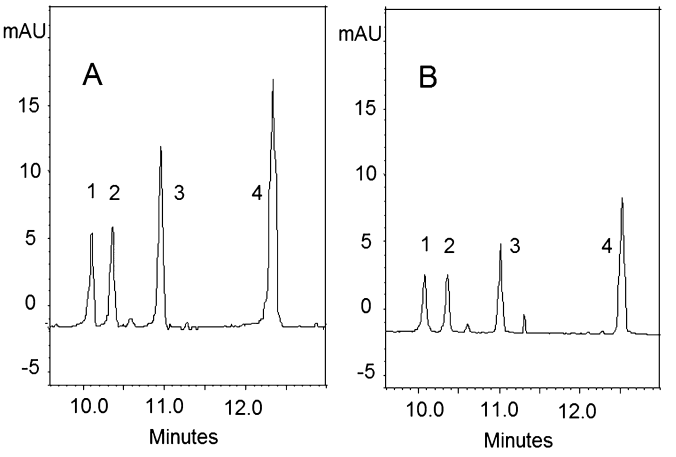

FIG. 5—*Comparisons between electropherograms of barbiturates in (A) large-volume sample stacking (0.5 μg/mL secobarbital, amobarbital, barbital, and phenobarbital, respectively) and (B) normal CZE (50 μg/mL secobarbital, amobarbital, barbital, and phenobarbital, respectively). Conditions: 3.0 psi 80 sec sample injection for (A), 0.2 psi 5 sec sample injection for (B). The other conditions are the same as those in Fig. 4.*

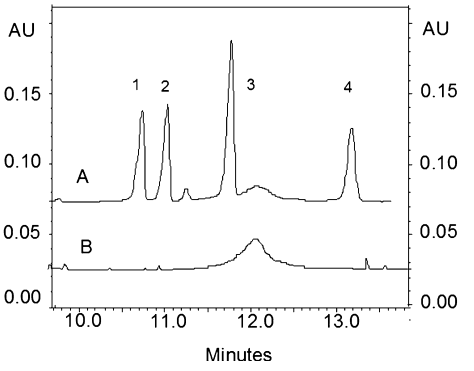

FIG. 6—*Electropherogram of spiked plasma samples. (A) 10 μg/mL secobarbital, amobarbital, barbital, and 2 μg/mL phenobarbital, respectively; (B) blank sample of plasma. Conditions: 3.0 psi 80 sec sample injection. The other conditions are the same as those in Fig. 4.*

the capillary, and the separation and stacking effect will affected (Fig. 4*B*) or even cannot reach the baseline separation (Fig. 4*A*). Many factors charged of the electrode reversing time, such as the injection time and pressure of sample, if the other conditions changed, the electrode reversing time should be changed at the same time.

Optimization of Injection Condition

Based on the examinations mentioned earlier, the buffer conditions were defined as 40 mM $Na_2B_4O_7$ (20% CH_3OH, v/v) solutions, pH 8.0. The applied voltage was set at 20 kV. To investigate the injection condition on the impact of stacking, different injection pressure (2.0–5.0 psi) based on 80 sec, with 0.5 μg/mL sample of barbital, secobarbital, amobarbital, and phenobarbital, respectively, was used to optimize the injection pressure. It was proved by experiments that the best electrode reversing time for 2.0 psi was 2.0 min, and for 2.5, 3.0, 4.0, and 5.0 psi were 2.4, 2.8, 3.4, and 3.5 min (−20 kV), respectively. The peak height of the analytes reached the highest when the injection pressure was 3.0 psi, so 3.0 psi was chosen for the further study.

Stacking Efficiency

The stacking efficiency was carefully investigated for the developed stacking method under the optimized conditions. Figure 5 compares the electropherograms obtained using LVSS methods (Fig. 5*A*) and normal CZE (Fig. 5*B*). It was clear from the electropherograms that a large increase in sensitivity was obtained using LVSS. The sensitivity in terms of peak height could be improved about 171.7-, 169.7-, 202.7-, and 169.1-fold for secobarbital, amobarbital, barbital, and phenobarbital, respectively. The sensitivity enhancement factors were calculated by simply getting the ratio of the peak heights obtained from LVSS and conventional CZE multiplying the dilution fold of the sample.

Method Validation

Specificity of Method—Under the optimized experimental conditions, Fig. 6 exhibited the peaks of barbiturates. Figure 6*A* displayed

TABLE 1—*The linearity and limits of quantitation (LOQs) of barbiturates.*

Analytes	Regression Equations	Correlation Coefficients (*r*)	LOQs(μg/mL)
Secobarbital	Y = 12289 + 47313X	0.9969	0.048
Amobarbital	Y = 9358 + 38493X	0.9999	0.057
Barbital	Y = 4361 + 63716X	0.9984	0.039
Phenobarbital	Y = 9780 + 132654X	0.9999	0.015

TABLE 2—*The intra- and interday relative standard deviation (RSD) of barbiturates at different concentrations (n = 3).*

	Spiking Level (μg/mL)		
RSD (%)	40	2	0.1
Intraday RSD (%)			
Secobarbital	1.95	0.54	0.59
Amobarbital	1.81	0.34	1.44
Barbital	1.48	0.38	0.33
Phenobarbital	2.11	0.36	0.48
Interday RSD (%)			
Secobarbital	4.26	3.81	2.75
Amobarbital	4.29	3.08	1.86
Barbital	4.31	3.39	1.19
Phenobarbital	4.69	3.18	1.68

the electropherogram of 10 μg/mL secobarbital, amobarbital, and barbital, respectively, and 2 μg/mL phenobarbital extracted from the plasma samples. Figure 6*B* was the electropherogram of a blank plasma sample, there was only one system peak, and the results indicated that no interference was identified for the targeted analytes. All of the experiments discussed earlier evidently indicated that the developed method provided good specificity for the determination of the barbiturates in plasma samples.

Linearity and Limits of Quantitation—The linearity of secobarbital, amobarbital, barbital, and phenobarbital was determined by spiking the four drugs into blank plasma. The final concentrations of secobarbital, amobarbital, barbital, and phenobarbital in the spiked plasma were 0.04, 0.1, 0.25, 0.5, 1, 2, 10, 20, 30, and 40 μg/mL, respectively. The regression equations of the curves and the correlation coefficients for barbiturates are given in Table 1, where Y is the peak area of barbiturates and X is the concentration of barbiturates. The limits of quantitation (LOQs) of secobarbital, amobarbital, barbital, and phenobarbital extracted from plasma were 0.048, 0.057, 0.039, and 0.015 μg/mL, respectively, on the basis of a signal-to-noise ratio of nine (S/N = 9). Every experiment with different concentration of barbiturate was repeated in triplicate.

Precision—To test the reproducibility of experimental results, both intraday and interday variances of peak areas of analytes were examined. The intraday values of relative standard deviation (RSD) were measured by analyzing samples with the interval of 2 h in a day for three times at three different concentrations of 0.1, 2, and 40 μg/mL, and the interday values of RSD were measured in three different days with the same three concentrations. The values of RSD of intraday for secobarbital were changed from 0.54 to 1.95%, for amobarbital were from 0.34 to 1.81%, for barbital were from 0.33 to 1.48%, and for phenobarbital were from 0.36 to 2.11%. The interday values of RSD ranged from 2.75 to 4.26% for

TABLE 3—*The recovery of barbiturates at different concentrations (n = 3).*

Spiking level (μg/mL)	Secobarbital		Amobarbital		Barbital		Phenobarbital	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
40	105.2	2.06	104.1	2.34	85.0	1.98	94.8	2.01
2	97.4	3.31	96.0	3.39	86.5	2.15	88.3	1.51
0.1	93.3	3.65	89.2	2.03	83.7	3.83	85.4	2.69

RSD, relative standard deviation.

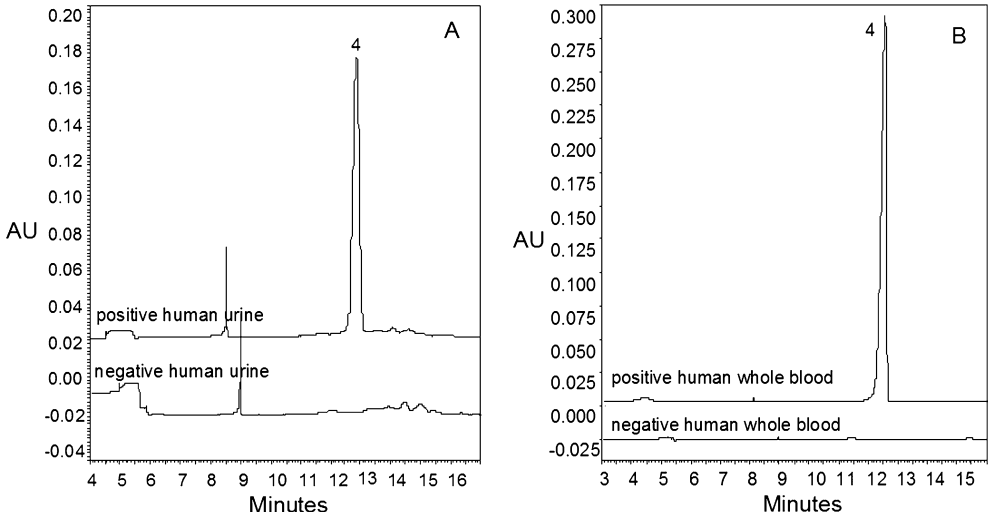

FIG. 7—*Electropherogram of (A) human urine and (B) human whole blood (please see the enlarged electropherogram in Fig. S1). Conditions: 3.0 psi 80 sec sample injection. The other conditions are the same as those in Fig. 4.*

secobarbital, 1.86 to 4.29% for amobarbital, 1.19 to 4.31% for barbital, and 1.68 to 4.69% for phenobarbital. In Table 2, all of the intraday and interday RSD values were <5%.

Recovery Assessment—The recovery assessments were performed at the same three different concentrations as intraday and interday RSD values. The recovery was calculated by the ratio value of barbiturates extracted from blank sample to the ratio of them resolved in ultrapure water directly. The recovery values of secobarbital, amobarbital, barbital, and phenobarbital extracted from plasma are shown in Table 3. It is obvious that the recoveries for secobarbital, amobarbital, barbital, and phenobarbital were higher than 80%, while the RSD values were <5%.

Application to Forensic Specimens—To evaluate the applicability of this method to forensic toxicology, two kinds of forensic specimens were investigated, including positive human urine and positive human whole blood (Fig. 7). The experimental results indicated that the developed method could be applied for the determination of barbiturates in these specimens.

Conclusion

This was the first time that the LVSS method was successfully used in the trace barbiturates in plasma and real forensic specimens. A large number of experimental results showed that this stacking method had several advantages. It provided not only wide linear range of quantitation with high precision and accuracy, but also low LOQ, good stacking effect, and simple operation. This method can be an efficient way to analyze trace level of barbiturates in plasma for forensic investigation and clinical study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

FIG. S1 The enlarged electropherogram of Fig.7B. The experimental conditions are the same as those in Fig. 7.

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