CHROM. 19 243

LIQUID CHROMATOGRAPHIC DETERMINATION OF BARBITURATES USING A HOLLOW-FIBRE MEMBRANE FOR POSTCOLUMN pH MODIFICATION

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SUMMARY

A sensitive, high-performance liquid chromatographic method is described for the determination of barbiturates by postcolumn pH modification. The barbiturates (barbital, phenobarbital, hexobarbital and amobarbital) were separated on a C_{18} column using a mixture of methanol and water as an eluent. Then the pH of the eluent was raised to 10 by introducing ammonia or ammonium ion through a sulphonated hollow-fibre membrane inserted between the column and the detector. The detection was based on the primarly ionized barbiturates at 240 nm. At barbiturate concentrations of 2.0 μg/ml, the within- and between-experiment precision (relative standard deviation) was 0.65–3.28 and 0.76–1.90%, respectively. The limits of detection were about 0.5–2.5 ng at a signal-to-noise ratio of 3. The method was applied to the determination of amobarbital in saliva.

INTRODUCTION

Barbiturates are a widely abused class of drug and there is an increasing need to identify them in forensic science laboratories. Several high-performance liquid chromatographic (HPLC) methods[1–31] have been developed for the determination of these drugs in pharmaceutical preparations and in body fluids. The separation of barbiturates is performed mainly on a reversed-phase column, while three methods of detection have been employed: (a) native ultraviolet (UV) detection at 195–254 nm[2,4,6,8–10,12–30]; (b) UV detection at 240 nm due to primarily ionized species (the un-ionized barbiturates have very weak absorbances, but their ionized forms have an UV absorption maximum at around 240 nm)[3,7,11]; (c) UV and fluorometric detection after precolumn labeling with 2-naphthacyl bromide[5] and 4-bromomethyl-7-methoxycoumarine[1]. The disadvantage of method (a) is that considerable interference in the chromatograms by endogenous compounds in body fluids can occur with detection at a low wavelength, while with detection at an high wavelength a sensitive detection cannot be attained. Method (c) needs tedious derivatization procedures, and has not been applied to biological samples. Method (b) has an advantage over

(a) because of less interference by endogenous compounds, however it requires an additional pump, mixing tee and reaction coil for postcolumn reaction. In order to solve this problem, a resin-based reversed-phase column and an alkaline mobile phase were used for the determination of pentobarbital in plasma. However, barbiturates which are weakly acidic show excellent separation on a reversed-phase column using a neutral mobile phase. During the preparation of this manuscript, Jansen et al.[32] reported an HPLC method which employed only one pump for the separation and postcolumn pH modification. However, this method requires the regeneration of an ion-exchange column for postcolumn pH modification after ca. 20-h use.

Ion-exchange membranes have been used as suppressors in ion chromatography[33-35]. Recently, Davis and Peterson[36] reported the application of a hollow-fibre membrane to a postcolumn reactor for HPLC, and Hwang and Dasgupta[37] used hollow-fibre membrane reactors for the determination of aqueous peroxides by flow injection analysis. In previous papers[38,39] we reported the HPLC assays of β-lactamase inhibitors in serum and urine using a hollow-fibre postcolumn reactor.

Here we report an HPLC method for the determination of barbiturates using a sulphonated hollow-fibre membrane. The use of a C_{18} column and the hollow-fibre membrane immersed in ammonium hydroxide solution allows sensitive detection at 240 nm. The application of this method to the determination of amobarbital in saliva is also described.

EXPERIMENTAL

Reagents and materials

Barbital, calcium phenobarbital, hexobarbital and sodium amobarbital were kindly donated by Nihon Shinyaku (Kyoto, Japan), Dainippon Pharmaceutical (Osaka, Japan), Sankyo (Tokyo, Japan) and Teikoku Chemical Industry (Osaka, Japan). The structures and the names of the barbiturates used are illustrated in Fig. 1. Other chemicals of analytical reagent grade were obtained from Nakarai (Kyoto,
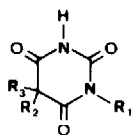

	R_1	R_2	R_3
Barbital	H	C_2H_5	C_2H_5
Phenobarbital	H	C_2H_5	⬡
Hexobarbital	CH_3	CH_3	⬡
Amobarbital	H	C_2H_5	$CH_2CH_2CH(CH_3)_2$

Fig. 1. Structures of barbiturates used.

Japan) and used without further purification. Deionized, glass-distilled water and glass-distilled methanol were used for the preparation of the HPLC eluent.

The sulphonated hollow-fibre membrane (AFS-2 fiber) was obtained from Dionex (Sunnyvale, CA, U.S.A.). A Baker-10 SPE system (J. T. Baker, Phillipsburgh, NJ, U.S.A.) with a 3-ml octadecyl disposable column was used for the pretreatment of saliva samples.

Chromatography

The experimental set-up used is shown in Fig. 2. The following HPLC system was used: a Model 655 liquid chromatograph (Hitachi, Tokyo, Japan) for delivering the eluent; a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a 100-μl loop for the loading of the samples; a Model 638-41 variable-wavelength UV monitor equipped with a 17-μl flow-through cell (Hitachi) for detection; a sulphonated hollow-fibre membrane (15 cm × 0.3 mm I.D.) immersed in ammonium hydroxide solution as a reactor. A reversed-phase C_{18} column (15 cm × 4 mm I.D.) packed with Develosil ODS-5 (5 μm) (Nomura, Seto, Aichi, Japan) was protected by a guard column (3 cm × 4 mm I.D.) packed with the same material. The eluents were as follows: A, water–methanol (1.5:1, v/v) for the separation of barbiturates; B, water–methanol (1:1, v/v) for the assay of amobarbital in saliva. The flow-rate was 0.8 ml/min. The detection was performed at 240 nm. The sulphonated hollow-fibre membrane was treated as reported previously[39] and was attached to suitable connecting fittings. It was immersed in a 50-ml beaker containing a 0.05 M ammonium hydroxide solution and inserted between the column and the detector. The pH of the eluent after the detector was 10.2. All separations and postcolumn reactions were carried out at ambient temperature.

Comparison of detection methods

The peak broadening due to the postcolumn reactor was estimated by the following methods: A, without a postcolumn reactor; B, with an open-tubular postcolumn reactor; C, with a sulphonated hollow-fibre postcolumn reactor. Detection was performed at 240 nm. For method B, the following devices were used: a double plunger pump (NP-DX-2; Nihon Seimitsu Kagaku, Tokyo, Japan) for delivering the postcolumn reagent (0.01 M ammonium hydroxide solution) at a flow-rate of 0.2 ml/min; a mixing tee and a reaction coil of 15 cm × 0.5 mm I.D. PTFE tubing as a reactor. For method C, the sulphonated hollow-fibre postcolumn reactor (15 cm × 0.3 mm I.D.) immersed in 0.05 M ammonium hydroxide solution was used. For methods B and C, the pH of the eluent after the detector was 10.2. A 10-μl portion of barbiturate solution (barbital, 5 μg/ml; phenobarbital and hexobarbital, 10 μg/ml; amobarbital, 20 μg/ml) was introduced into the chromatograph under the conditions
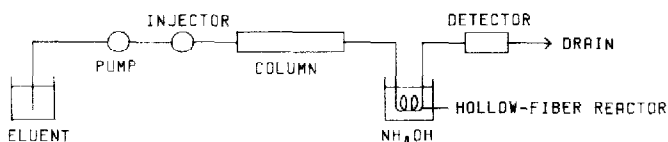

Fig. 2. The experimental set-up used.

described above. The dispersion, σ_t^2, and the number of theoretical plates, N, of each peak were calculated.

Pretreatment of saliva samples

Mixed saliva samples were obtained by chewing 100 cm² Parafilm. The samples spiked with amobarbital were centrifuged at 3000 g for 5 min. To a 1.0-ml portion of the supernatant, 0.1 ml of hexobarbital solution (5 μg/ml) was added and then the sample was applied to the extraction column (Baker-10 SPE). The column was washed three times with 3 ml water and eluted with 0.8 ml methanol. A 50-μl aliquot of the eluent was loaded onto the column.

RESULTS AND DISCUSSION

Reaction conditions

It is well known[3,40] that the monoanionic forms of barbiturates (in basic solutions, pH $\approx$ 10) have a strong absorption at around 240 nm. After separation of barbiturates using a C_{18} column, the pH of the eluent was raised to 10 using a sulphonated hollow-fibre membrane immersed in ammonium hydroxide solution (which is inserted between the column and the detector). The membrane permitted inward transport of ammonia or ammonium ion, resulting in a change in pH of the eluent to $\approx$ 10.

The reaction conditions were examined by using the flow injection technique. The length of the hollow-fibre reactor was fixed at 15 cm. Fig. 3 shows the effect of
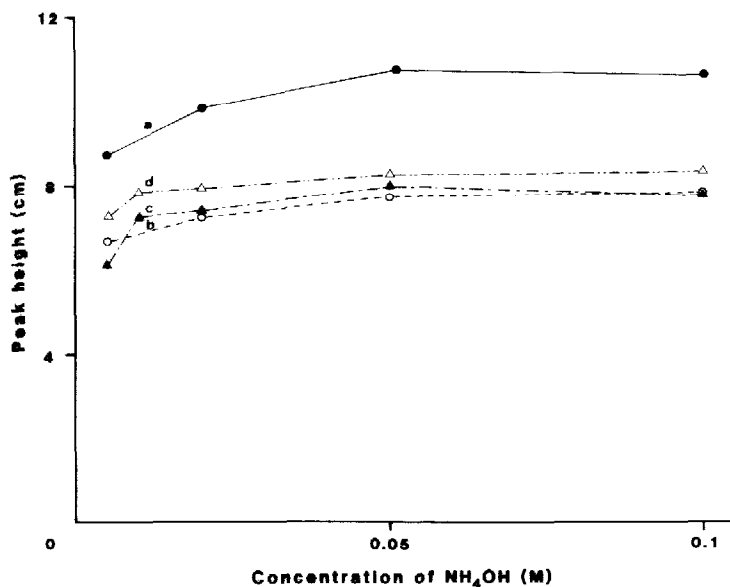

Fig. 3. Effect of the ammonium hydroxide concentration on the UV response due to the monoanionic forms of the barbiturates in flow injection analysis. A 10-μl portion of the barbiturate solution (10 μg/ml) was injected at a hollow-fibre length of 15 cm. Carrier solution: eluent A. Detection: 240 nm. Sensitivity: 0.128 a.u.f.s. (a) Barbital; (b) phenobarbital; (c) hexobarbital; (d) amobarbital.

the concentration of ammonium hydroxide solution on the UV absorbance of the ionized barbiturates. The ammonium hydroxide concentration was varied from 0.005 to 0.1 M, when the pH of the eluent after the detector changed from 9.0 to 10.5. As shown in Fig. 3, the maximum and constant peak height was obtained at $\geqslant 0.05$ M ammonium hydroxide.

Thus, the postcolumn reaction conditions selected for the assay of barbiturates were 0.05 M ammonium hydroxide and a 15-cm hollow fibre.

Comparison of detection methods

The band broadening in the three detection methods A–C was compared with respect to the dispersion, σ_t^2, and the number of theoretical plates, N; method A, without a postcolumn reactor; B, with an open-tubular postcolumn reactor; C, with a sulphonated hollow-fibre postcolumn reactor. Table I gives σ_t^2 and N for the three methods. The increases in the dispersion due to the open-tubular and hollow-fibre postcolumn reactors were 5.2–28.1 and 2.9–9.4 s^2, respectively. The calculated number of theoretical plates indicated 16–41 and 4.5–17% loss of resolution due to these reactors, respectively.

Fig. 4 shows the chromatograms of barbiturates obtained by three detection methods; parts A–C correspond to detection methods A–C. In method A, barbiturates were detected from their native UV absorption at 240 nm by raising the detector response eight-fold compared with that of methods B and C. In methods B and C, barbiturates were sensitively detected at 240 nm following postcolumn ionization; method C was 4.5- to 33-times more sensitive compared with method A. The peak heights of barbiturates in method C were 1.4- to 1.7-times higher than in method B. This is due to the fact that the band broadening in the hollow-fibre postcolumn reactor is much less than that in a conventional postcolumn reactor, as described above.

Precision and linearity

Table II shows the within- and between-experiment precision of the assays of barbiturates by the absolute amount method. The eight-point calibration graph of peak height *versus* concentration for barbiturates was linear in the concentration range 0.05 50 μg/ml and passed through the origin; correlation coefficient 0.999. The limits of detection were about 0.5–2.5 ng at a signal-to-noise ratio of 3.

TABLE I

BAND BROADENING IN THE POSTCOLUMN REACTOR

σ_t^2 and N are the total dispersion in the system and the calculated number of theoretical plates.

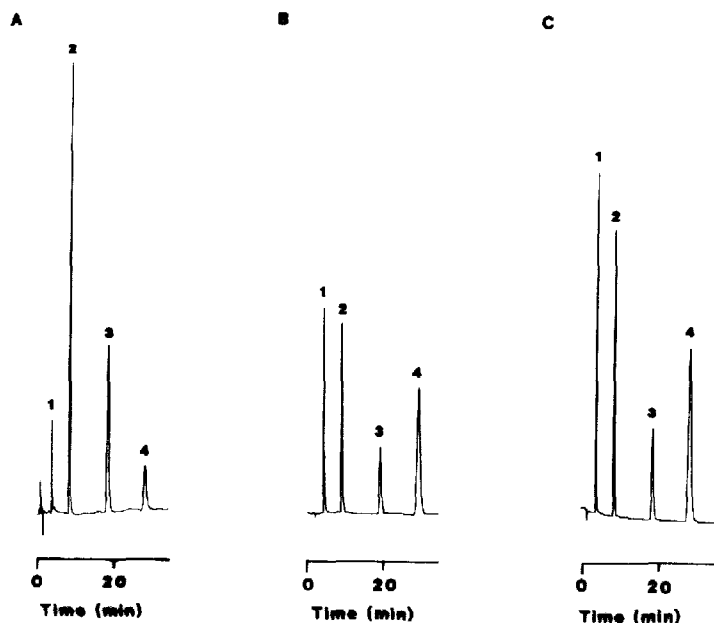| Barbiturate	Method A		Method B		Method C	
	σ_t^2 (s^2)	N	σ_t^2 (s^2)	N	σ_t^2 (s^2)	N
Barbital	9.2	4930	14.4	2920	12.1	4080
Phenobarbital	36.0	5810	49.4	4390	41.7	5380
Hexobarbital	183	6030	208	5050	192	5580
Amobarbital	437	6490	465	5450	442	6200

Fig. 4. Comparison of the three detection methods for barbiturates. (A) Without a postcolumn reactor; (B) with an open-tubular postcolumn reactor; (C) with a sulphonated hollow-fibre postcolumn reactor. Detection was performed at 240 nm. Peak assignments: 1 = barbital (5 μg/ml); 2 = phenobarbital (10 μg/ml); 3 = hexobarbital (10 μg/ml); 4 = amobarbital (20 μg/ml). Injection volume: 10 μl. Sensitivity: (A) 0.004 a.u.f.s.; (B and C), 0.032 a.u.f.s.

Application to saliva samples

On the basis of the aforementioned results we attempted to apply the method described to the determination of amobarbital in saliva. Fig. 5A shows a chromatogram obtained for a solid-phase extract of a control saliva sample; a chromatogram of a control saliva sample spiked with amobarbital and hexobarbital (internal standard), treated in the same manner as in A, is shown in Fig. 5B. Amobarbital and

TABLE II

ACCURACY AND PRECISION OF THE ASSAY OF BARBITURATES

The concentration of each compound was 2.0 μg/ml. The values given are means ± coefficient of variation (C.V.) (%).

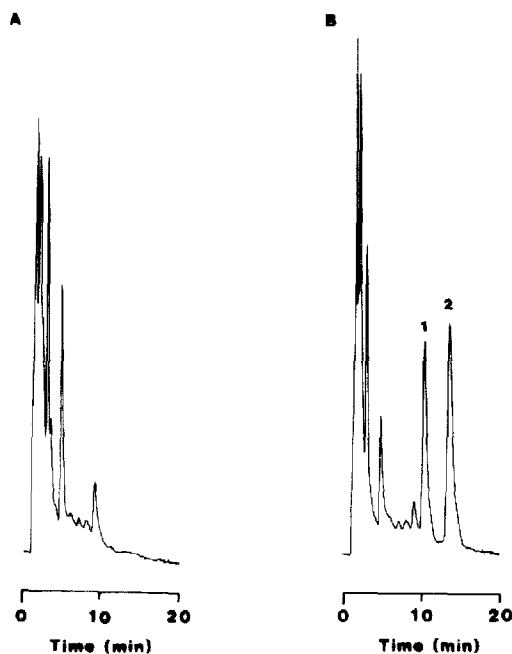| Assay No.	Concentration found [mean (μg/ml) ± C.V. (%)]			
	Barbital	Phenobarbital	Hexobarbital	Amobarbital
Within-experiment (n = 5)				
1	1.99 ± 0.65	1.96 ± 1.43	2.00 ± 1.52	1.97 ± 0.90
2	2.02 ± 0.66	2.01 ± 0.69	2.02 ± 2.07	2.04 ± 2.66
3	2.00 ± 0.68	2.00 ± 0.90	1.97 ± 2.11	1.98 ± 3.28
Between-experiment (n = 3)	2.00 ± 0.76	1.99 ± 1.33	2.00 ± 1.26	2.00 ± 1.90

Fig. 5. Chromatograms of control saliva (A) and control saliva spiked with amobarbital (B). Saliva samples spiked with amobarbital were treated according to the procedures described in Experimental. Peak assignments: 1 = hexobarbital (0.5 µg/ml, internal standard); 2 = amobarbital (0.5 µg/ml). Injection volume: 50 µl. Sensitivity: 0.008 a.u.f.s. For other conditions, see Experimental.

hexobarbital were completely separated from the background components of saliva. Table III shows the within- and between-experiment precision of the assay of amobarbital in saliva by the peak-height ratio method. The six-point calibration graph constructed by the peak-height ratio method for amobarbital was linear in the concentration range 50–2000 ng/ml. The detection limit was 20 ng/ml at a signal-to-noise ratio of 3 with a 50-µl injection.

It has been reported[41] that the concentration of amobarbital in saliva is above 200 ng/ml, 50 h after a single oral administration of an amobarbital tablet (120 mg).

TABLE III

ACCURACY AND PRECISION OF AMOBARBITAL ASSAY IN SALIVA

Saliva samples spiked with amobarbital (0.5 µg/ml) were treated according to the procedures described in Experimental. Values are the ratios of peak height of amobarbital to that of hexobarbital (0.5 µg/ml).

Assay No.	Peak height ratio
Within-experiment (n = 5)	
1	1.27 ± 1.62%
2	1.33 ± 0.63%
3	1.32 ± 1.61%
Between-experiment (n = 3)	1.31 ± 2.46%

The proposed method will be applicable to pharmacokinetic studies of barbiturates or the monitoring of barbiturate levels in cases of abuse.

ACKNOWLEDGEMENTS

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