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Note

Selective on-line sample handling for the determination of barbiturates in urine by liquid chromatography with precolumn technology

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Despite their widespread replacement with benzodiazepines in the field of hypnotic formulations, barbiturates are still being prescribed extensively. Monitoring of barbiturate concentrations in body fluids is important because of their therapeutic width, which is quite small [1]. In addition, barbiturates are frequently used for suicidal attempts, hence barbiturate screening is quite common in forensic research.

Gas chromatographic (GC) and reversed-phase column liquid chromatographic (LC) methods for the determination of barbiturates were recently reviewed by Gupta [2] and by Heusler [3]. The use of LC in the determination of barbiturates in body fluids suffers from the major drawback that non-selective low-wavelength UV detection has to be used, which hampers on-line analysis of real samples. In principle, the selectivity can be somewhat improved by determining the barbiturates in their anionic form at 254 nm [4–6]. However, we preferred to incorporate the selectivity in the sample handling step by on-line solid-phase extraction of the barbiturate anions using an anion-exchange resin. Interfering inorganic ions are eliminated in the same way as has been demonstrated recently in the trace analysis of phenol in surface water [7], viz. via a solid-phase extraction procedure involving a hydrophobic sorbent.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) LC system consisting of two Model 410 pumps (one of them equipped with a home-made membrane pulse dampener),

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an MCS 670 tracer valve-switching unit and a Model 200 programmer, was used in combination with a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 757 UV detector operated at 220 nm. Chromatograms were analogue recorded using a Kipp & Zonen (Delft, The Netherlands) BD 8 recorder and processed manually.

Stationary phases and columns

Sample clean-up was performed on home-made 2 × 4.6 mm I.D. and 5 × 3.0 mm I.D. stainless-steel precolumns [8] packed by hand, using a syringe containing a slurry of the 10-μm styrene—divinylbenzene copolymer PRP-1 (Hamilton, Reno, NV, U.S.A.) in methanol and pre-swollen 11-μm quaternary ammonium anion-exchange resin Aminex A-28 (Biorad, Richmond, CA, U.S.A.) in aqueous buffer, respectively.

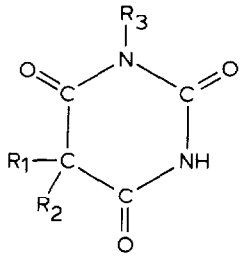
A 100 × 3.0 mm I.D. glass cartridge prepacked with 5-μm Chromspher C_{18} (Chrompack, Middelburg, The Netherlands) served as the analytical column.

Chemicals

Analytical-grade sodium acetate, acetic acid, ammonium acetate, ammonium hydroxide (25%) and HPLC-grade methanol were obtained from J.T. Baker (Deventer, The Netherlands). Pharmaceutical-grade barbiturates were kindly supplied by the Academic Hospital of the Free University; their structures and pK_a values are given in Table I. Stock solutions of the barbiturates (2 mg/ml) were prepared in methanol, stored at 4°C and diluted with an aqueous 0.05 *M* sodium acetate—acetic acid buffer (pH 5.0) prior to use.

TABLE I

STRUCTURES OF THE BARBITURATES USED AS TEST COMPOUNDS



Derivative	R_1	R_2	R_3	pK_a	Supplier
Butobarbital	C_2H_5	*sec.*-Butyl	H	8.0	Brocacef*
Hexobarbital	CH_3	1-Cyclohexenyl	CH_3	8.2	Brocacef
Amobarbital	C_2H_5	3-Methylbutyl	H	7.9	OPG**
Secobarbital	Allyl	1-Methylbutyl	H	7.9	OPG

*Maarssen, The Netherlands.
**Utrecht, The Netherlands.

LC-grade water, prepared from demineralized water using a Milli-Q (Millipore, Bedford, MA, U.S.A.) water purification system, was used for all standard solutions and eluents. The eluents were degassed under vacuum in an ultrasonic bath. The pH of the buffer solutions was adjusted using a Philips

(Eindhoven, The Netherlands) PW 9409 pH meter, either before (acetate buffer; pH 5.0) or after (ammonia—ammonium acetate buffer; pH 9.5) the addition of methanol.

Urine samples were filtered over a 1.2-μm membrane filter and injected directly into the LC system. Their pH should be in the range 4—7.

General procedure

Final experiments were performed using the set-up shown in Fig. 1. The procedure is as follows. A standard barbiturate mixture or a urine sample is transferred by pump A from the 25-μl injection loop to the PRP-1 precolumn using 1 ml of an aqueous 0.05 *M* acetate buffer. Here, sorption of the barbiturates and of various organic contaminants occurs, while inorganic constituents are flushed to waste. Since all anions can interfere with sorption of the barbiturates on the anion-exchange resin, the excess of acetate ions is flushed out using 3.5 ml of a weak ($2 \cdot 10^{-3}$ *M*) aqueous acetate buffer. Next, pump A is switched to deliver 3 ml of $4 \cdot 10^{-3}$ *M* ammonia buffer—methanol (50:50) and the barbiturates, now in their anionic form, are eluted from the PRP-1 precolumn towards the Aminex A-28 anion-exchange precolumn, on which they are trapped as a narrow zone, while neutral organic compounds are flushed to waste. Finally, the barbiturates are neutralized and desorbed by eluting the Aminex precolumn in the forward-flush mode via pump B with 400 μl of 0.1 *M* acetate buffer—methanol (50:50) and separated on the C_{18} analytical column with subsequent UV detection at 220 nm.

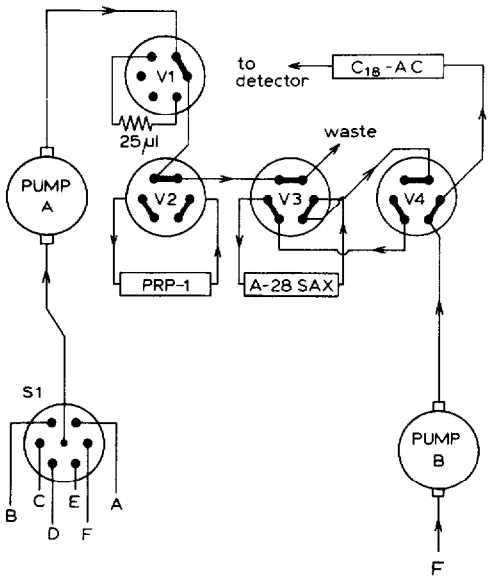


Fig. 1. Experimental set-up for the on-line sample clean-up and determination of barbiturates in urine. S: low-pressure solvent selection valve; V: high-pressure switching valve. A: 0.05 *M* sodium acetate buffer (pH 5.0); B: $2 \cdot 10^{-3}$ *M* sodium acetate buffer (pH 5.0); C: $4 \cdot 10^{-3}$ *M* ammonia buffer (pH 9.5)—methanol (50:50); D: methanol—water (80:20); E: water; F (pump B): 0.1 *M* sodium acetate (pH 5.0)—methanol (50:50). Precolumns: 2 × 4.6 mm I.D. (PRP-1) and 5 × 3.0 mm I.D. (Aminex A-28). Flow-rate of pump A, 2 ml/min (1 ml/min when the ion exchanger was switched in-line).

TABLE II

GENERAL PROCEDURE USING THE SET-UP OF FIG. 1

1. Trapping of the barbiturates on PRP-1 using a 0.05 *M* acetate buffer (pH 5.0)
2. Flushing of PRP-1 with $2 \cdot 10^{-3}$ *M* acetate (pH 5.0)
3. Transfer of barbiturates towards Aminex A-28 with $4 \cdot 10^{-3}$ *M* ammonia buffer (pH 9.5)—methanol (50:50)
4. Flushing Aminex A-28 with ammonia buffer—methanol (50:50)
5. Forward-flush desorption from Aminex A-28 towards the C_{18} analytical column using 0.1 *M* acetate buffer (pH 5.0)—methanol (50:50).
6. Regeneration of PRP-1 with methanol—water (80:20)
7. Flushing both precolumns with water

During the separation step on the C_{18} analytical column, the PRP-1 precolumn is regenerated on-line via pump A with 6 ml of methanol—water (80:20) and, finally, both precolumns are flushed with 3 ml of water.

In order to increase sample throughput, the next run is started while the actual separation is still in progress. The general procedure has been summarized in Table II; details concerning the valve-switching programme will be made available on request.

RESULTS AND DISCUSSION

Characteristics of the precolumns

Breakthrough experiments were carried out according to the procedure described in ref. 9, using a 0.2–1 μg/ml standard solution of butobarbital (pH 5.0). This is the barbiturate showing least retention on the PRP-1 precolumn. At a flow-rate of 2 ml/min the breakthrough volume on the 2 × 4.6 mm I.D. PRP-1 precolumn was found to be 22 ml.

The choice of eluent used for desorption of the barbiturates from the PRP-1 precolumn and their subsequent sorption on the Aminex anion exchanger was found to be rather critical. The use of $4 \cdot 10^{-3}$ *M* ammonia buffer—methanol (50:50) provided both a sufficiently low desorption volume (2.5 ml) for the most highly retained barbiturate, secobarbital, from the PRP-1 precolumn, and sufficient retention of the anionic barbiturates on the Aminex anion exchanger.

TABLE III

GENERAL PERFORMANCE OF THE AUTOMATED BARBITURATE ANALYSIS USING 25-μl STANDARD SOLUTIONS

Compare Table II and the set-up of Fig. 1.

Compound	Repeatability* (n = 11) (% R.S.D.)	Linearity (r)** (n = 7)	Detection limit*** (ng)	Recovery* (%)
Butobarbital	2.5	0.9999	1	95—103
Secobarbital	3.5	0.9998	2	97—105

*Barbiturate level 2 μg/ml.
**Linearity range 0.06—60 μg/ml barbiturate.
***Signal-to-noise ratio = 3:1.

In order to reduce anion competition on the Aminex as much as possible, ammonium acetate instead of ammonium chloride was used; the pH was kept at 9.5. Although at this pH 1—5% of the barbiturates are not dissociated, rapid adjustment of the dissociation equilibrium will prevent any noticeable loss.

It should be mentioned here that retention on the anion exchanger appeared to be highly dependent on flow-rate; the breakthrough volume of butobarbital, in its anionic form, at 0.5 ml/min was almost three times higher than at 1 ml/min (27 vs. 10 ml). We found a breakthrough volume of 10 ml to be amply sufficient for our purposes and used a flow-rate of 1 ml/min in all further experiments.

A 0.1 *M* acetate buffer was found to be satisfactory for the efficient and quantitative desorption of barbiturates from the anion exchanger towards the C_{18} analytical column by < 250 μl of eluent at 0.4 ml/min. In order to avoid hydrophobic interaction on the organic matrix of the anion-exchange resin, and to provide an adequate elution profile on the C_{18} analytical column, the aqueous acetate buffer was diluted with an equal volume of methanol.

Performance and application

With the experimental set-up shown in Fig. 1 and the general procedure described in Table II, analytical data were collected for butobarbital and seco-

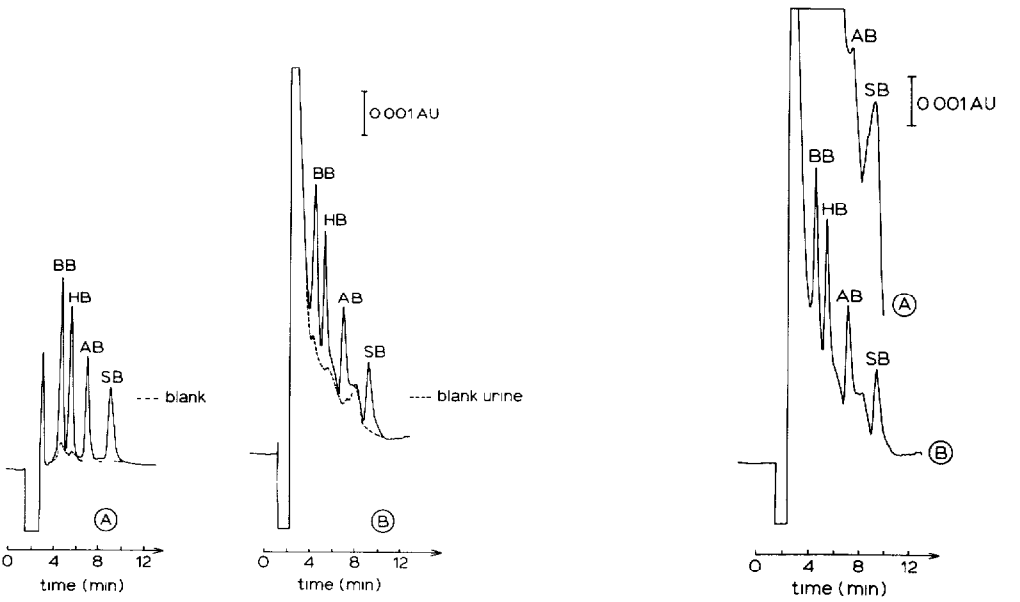


Fig. 2. Chromatograms of 25 μl of (A) a 0.05 *M* acetate buffer and (B) a urine sample, both either with (——) or without (- - - -) barbiturates (spiking level, 1 μg/ml). For procedure, see the set-up of Fig. 1 and Table II. Detection by UV at 220 nm; 0.01 a.u.f.s. Peaks: BB = butobarbital; HB = hexobarbital; AB = amobarbital; SB = secobarbital. Other conditions as in Fig. 1.

Fig. 3. (A) Chromatogram obtained after loading of a 25-μl spiked urine sample on a 2 × 4.6 mm I.D. precolumn packed with PRP-1 using a flush of 6 ml of a 0.05 *M* acetate buffer (pH 5.0) and direct elution with the eluent (F, pump B; cf. Fig. 1) to the analytical column. Other conditions as in Fig. 2. (B) Same chromatogram as in Fig. 2B. Peaks: BB = butobarbital; HB = hexobarbital; AB = amobarbital; SB = secobarbital.

barbital. The results, which are presented in Table III, clearly demonstrate the potential of the method. One should note that, in spite of the repeated sorption/desorption process, the recovery is rather close to 100%.

In Fig. 2, chromatograms for a blank, a standard solution, and a spiked and blank urine sample are shown. Repeated injection of spiked urine samples showed a good repeatability for all four test solutes, as can be seen from Table IV. The recoveries relative to those for standard samples are also satisfactory. Some minor interferences show up in Fig. 2B; however, these are not expected to influence the accuracy of the barbiturate determination, because the barbiturate concentrations will at least be ten-fold higher in real samples as compared to the spiking level in the present experiment. In addition, recoveries are close to 100%.

TABLE IV

APPLICATION OF THE AUTOMATED BARBITURATE ANALYSIS TO 25-μl SPIKED URINE SAMPLES

Compare Table II and Fig. 1; spiking level, 1 μg/ml.

Barbiturate	n	Recovery* (%)	Repeatability (% R.S.D.)
Butobarbital	5	95	4.5
Hexobarbital	4	97	1.1
Amobarbital	4	98	1.0
Secobarbital	5	91	3.2

*Relative to the recovery of standard samples as given in Table III.

The dramatic gain in selectivity obtained by the dual-precolumn approach as compared to a single-precolumn clean-up is demonstrated in Fig. 3. Chromatogram A was obtained after loading the PRP-1 precolumn with 25 μl of spiked urine, flushing with 6 ml of 0.05 *M* acetate buffer, and direct elution of the barbiturates from the precolumn with 0.1 *M* acetate buffer—methanol (50:50) to the analytical column. Chromatogram B was obtained using the dual-precolumn technique. As a consequence of the high selectivity of the latter method, the detection limits in urine are of the same order of magnitude as with standard solutions, i.e. 1—2 ng for 25-μl loop injections.

CONCLUSIONS

Using two sample clean-up precolumns in series, with cleaning action being based on the removal of, first, inorganic and, then, organic compounds, urine samples can be analysed for barbiturates with such selectivity that simple UV detection at 220 nm can be achieved. Modification of the method according to the procedure outlined in ref. 6, which allows detection at 254 instead of 220 nm, may well produce even cleaner chromatograms. The present method, which is fully automated, is linear over at least three orders of magnitude and displays good repeatability. Recoveries in urine are close to 100% at therapeutic concentrations.

In principle, apart from barbiturates, many other biologically active acidic compounds can also be determined with the present method, provided their pK_a values are in the range 1—10, simply by adjusting the pH of the buffer solution that serves as transfer eluent from the first precolumn to the second one. Because of the peak compression that occurs upon transfer from the PRP-1 to the Aminex A-28 precolumn, one is not limited to compounds that display large breakthrough volumes on the PRP-1 precolumn. If necessary, a larger PRP-1 precolumn can be chosen, as was demonstrated earlier in the determination of phenol [7].

Finally, substitution of the anion exchanger by a cation-exchange resin should allow basic compounds to be analysed in a manner similar to the one reported here. This will provide an alternative to the approach of ref. 10, where inorganic interferences were precipitated off-line and organic contaminants filtered out on-line using a PRP-1 precolumn.

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