Journal of Chromatography, 427 (1988) 172–180
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4124

Note

Column liquid chromatographic analysis of barbiturates in biological fluids

POK PHAK ROP, J. SPINAZZOLA, A. ZAHRA, M. BRESSON, J. QUICKE and A. VIALA*

Laboratoire Interrégional de Police Scientifique de Marseille, 2 Rue A. Becker, 13224 Marseille Cedex 2 (France)

(First received October 15th, 1987; revised manuscript received January 7th, 1988)

The barbiturates in addition to their therapeutic application as hypnotics, anticonvulsants and anaesthetics, are often used as drugs of addiction, sometimes alone or associated with other drugs or alcohol. Therefore they are involved in many accidental and suicidal poisonings, especially in France [1]. During the last ten years 20% of the necropsic samples analysed in our laboratory contained barbiturates.

The determination of barbiturates in biological fluids, particularly in blood, is of great interest in forensic toxicology and clinical pharmacology and numerous procedures have been reported. Thin-layer chromatography [2,3], UV spectrophotometry [2–4], gas chromatography [5–13], gas chromatography coupled with mass spectrometry [12,13], high-performance liquid chromatography (HPLC) [14–22] and immunoassays [23–28] have been applied. Prior isolation from biological samples was usually necessary, using organic solvent extraction, or non-ionic resin absorption [29–31], or simple deproteinization for phenobarbital [18]. The methods are often time-consuming and sophisticated.

This study was undertaken in order to develop a rapid, simple and sensitive procedure for the identification and quantification of barbiturates in blood and urine using HPLC after deproteinization or extraction. The analysis was carried out on the most frequently encountered barbiturates in France.

EXPERIMENTAL

Reagents and glassware

All reagents were of analytical-reagent grade. Acetonitrile (RS per HPLC), methanol (RS per HPLC) and diethyl ether (RPE) were obtained from Carlo

0378-4347/88/$03.50

Erba (Milan, Italy), perchloric acid (Normapur), trisodium phosphate (Normapur) and methylene chloride (Normapur) from Prolabo (Paris, France) and distilled water ("for injection") from Laboratoires Aguettant (Lyon, France). All glassware was washed with 3% RBS 25 biodegradable solution obtained from Biolyon (Dardilly, France), then rinsed with distilled water and dried before use. All glass centrifuge tubes were rinsed first with acetone, then with the extraction solvent.

Standards

Barbiturates currently used as drugs in France examined were amobarbital, barbital, butalbital, butobarbital, cyclobarbital, heptabarbital, hexobarbital, methohexital sodium, phenobarbital, proxibarbital, secobarbital and vinylbital. Barbiturates not currently used as drugs in France examined were aprobarbital (possible internal standard), butabarbital, mephebarbital, pentobarbital, thialbarbital and thiopental. The internal standard was methaqualone. All the barbiturates were kindly supplied by the respective manufactures and methaqualone by Houdé-ISH Laboratoires (Paris, France).

Stock solutions of each drug were prepared in methanol at a concentration of 1 mg ml^{-1} and stored at 4 °C. They were diluted to 0.1 mg ml^{-1} for preparing the calibration standards. Methaqualone (or aprobarbital) was used as an internal standard.

Apparatus and chromatographic parameters

The chromatographic analysis was performed on a component system consisting of a Waters Model 510 pump, a Waters Model WISP 712 automatic sample injection module and a Waters Model 490 programmable multi-wawelength detector, set at 0.1–0.01 a.u.f.s. and at 230 nm for thiopental and thialbarbital analysis and 198 nm for the other barbiturates. The detector was monitored with a Waters 840 data and chromatography control station. A μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm, ambient temperature) was connected to the detector. The mobile phase was acetonitrile–distilled water (35:65, v/v) at a flow-rate of 1 ml min^{-1}.

Procedure

Whole blood, serum, plasma. Into a 5-ml haemolysis tube were pipetted 50 μl of the internal standard (methaqualone or possibly aprobarbital) methanolic solution (0.1 mg ml^{-1}). Following evaporation to dryness, 0.5 ml of the sample to be analysed and 100 μl of 40% perchloric acid were added. After vortexing for 1 min, the mixture was centrifuged for 10 min at 2800 *g*, then 20 μl of the supernatant were injected into the chromatograph for analysis. A calibration graph was simultaneously run under same conditions. The ratio between the peak area of the sample drug and that of the internal standard was calculated and plotted against the concentration of the drug after analysis of blank samples spiked with increasing concentrations of each drug (2, 4, 10 and 20 μg ml^{-1}) and a constant amount of the internal standard (methaqualone or aprobarbital, 5 μg).

Urine. A 0.5-ml volume of urine was pipetted into a 25-ml centrifuge tube,

followed by 2 ml of 0.02 *M* perchloric acid. The unconjugated fractions of barbiturates were extracted with 6 ml of diethyl ether. After shaking for 10 min, the liquid was centrifuged for 10 min at 2800 *g*. Exactly 5 ml of the solvent phase were transferred into a centrifuge tube containing 50 μl of the internal standard solution (0.1 mg ml^{-1}) and evaporated to dryness at 45°C*. The residue was dissolved in 100 μl of mobile phase, vortexed for 30 s and centrifuged or filtered. A 20-μl volume of the clear liquid was injected into the chromatograph. A calibration graph was run under the same conditions as for the blood fluids from blank urine spiked with drugs.

The above urine extraction procedure can be applied to 0.5-g samples of gastric content homogenates or to blood for determining the barbiturates whose retention times are short (proxibarbital, barbital). A subsequent purification may be necessary (see the procedure indicated under *Selectivity*).

RESULTS AND DISCUSSION

Table I and Fig. 1. show the retention times and a chromatogram of several barbiturates and methaqualone after evaporation of the methanolic standard solutions and injection of the residues dissolved in the mobile phase.

Fig. 2 shows chromatograms for a blank of whole blood: (A) after deproteinization, (B) after spiking with phenobarbital, amobarbital, secobarbital and methaqualone and deproteinization, (C) after diethyl ether extraction followed by purification, (D) after spiking with barbital, phenobarbital, butalbital, amobarbital and secobarbital, before diethyl ether extraction and purification, methaqualone being added in the last ether phase, and (E) after spiking with proxibarbital, barbital, phenobarbital, amobarbital and aprobarbital, before diethyl ether extraction and purification. Fig. 3 shows a chromatogram from a necropsic blood sample from an abuser who had taken barbiturates and alcohol shortly before his death (murder by fire arm).

After choosing the determination wavelengths and the internal standards, the validity of the method was exclusively tested on whole blood and especially with phenobarbital, amobarbital and secobarbital, because these barbiturates are the most commonly encountered in cases of poisoning in France and in therapeutic uses. Further, the analysis of plasma, serum and urine is easier.

Choice of detection wavelength

As pointed out by Kraak and Crombeen [32] and Stout and De Vane [33], interferences may be observed at low wavelengths. We tested five wavelengths (198, 220, 230, 240 and 254 nm) to choice that which gave the best response, because the volume injected (20 μl) is low, considering the volume of the deproteinized liquid (600 μl). The results obtained are given in Table II. For most barbiturates 198 nm was selected; for thialbarbital and thiopental, 230 or 240 nm

*When aprobarbital was used as internal standard, 10 μl of the standard solution (0.1 mg ml^{-1}) had to be added directly to the sample because its extraction parameters are similar to those of the other barbiturates.

TABLE I

RETENTION TIME OF DRUGS

Flow-rate: 1 ml min^{-1}.

Drug	Retention time (mean of ten injections) (min)	Retention time relative to methaqualone (internal standard)	Retention time relative to aprobarbital (internal standard)
Proxibarbital	3.80	0.22	0.28
Barbital	4.50	0.26	0.33
Mephebarbital	6.30	0.37	0.46
Phenobarbital	7.10	0.41	0.52
Cyclobarbital	7.30	0.43	0.54
Butobarbital	7.48	0.44	0.55
Butalbital	8.00	0.47	0.59
Butabarbital	8.00	0.47	0.59
Heptabarbital	9.70	0.57	0.71
Hexobarbital	10.14	0.60	0.74
Amobarbital	11.20	0.66	0.82
Pentobarbital	11.50	0.68	0.84
Vinylbital	12.35	0.73	0.90
Secobarbital	13.50	0.79	0.99
Aprobarbital	13.60	0.80	1.00
Methaqualone	17.00	1.00	1.25
Thialbarbital	20.50	1.20	1.50
Thiopental	21.00	1.23	1.54
Methohexital sodium	27.00	1.59	1.98

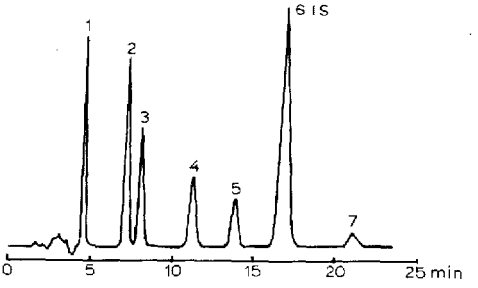

Fig. 1. Chromatogram of barbiturates and methaqualone after direct injection of a solution into the mobile phase with detection at 198 nm. Peaks: 1 = barbital; 2 = phenobarbital; 3 = butalbital; 4 = amobarbital; 5 = secobarbital; 6 = methaqualone; 7 = thiopental (200 ng of each compound).

gave better results. We ascertained that the interferences at low wavelengths depend on the composition of the mobile phase. No inferference occurred using the described procedure for the barbiturates and the other drugs tested. The baseline

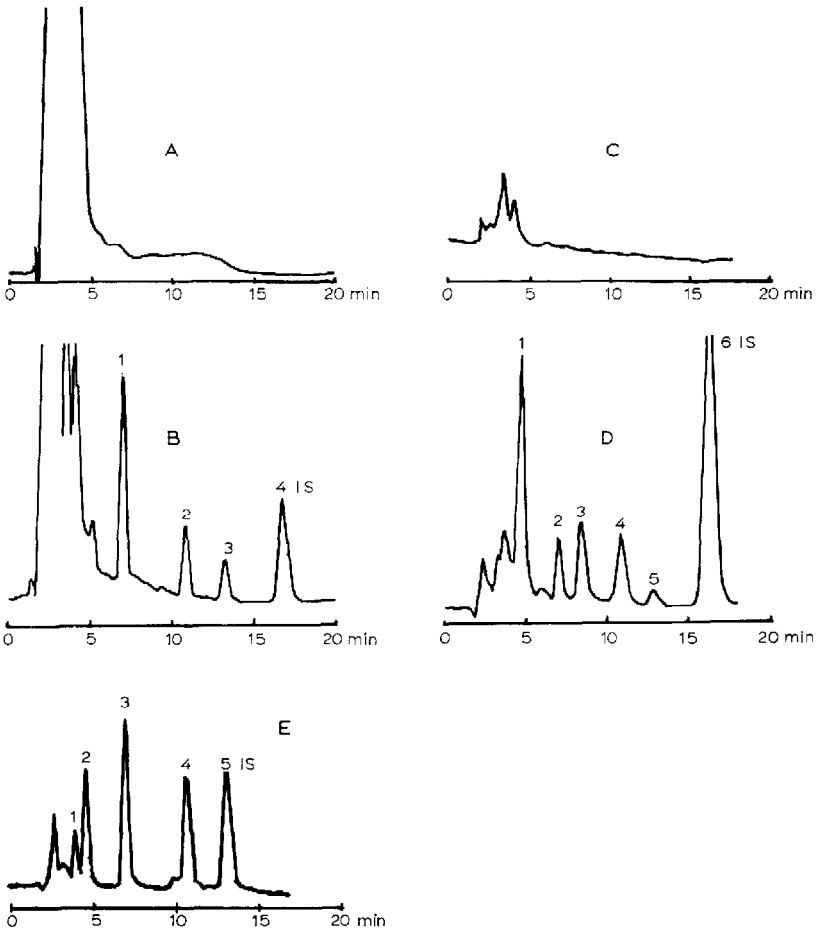

Fig. 2. Chromatograms of (A) blank of whole blood after deproteinization; (B) blank of whole blood (0.5 ml) spiked before deproteinization with 5 μg of (1) phenobarbital, (2) amobarbital, (3) secobarbital and (4) 5 μg of methaqualone as internal standard; (C) blank of whole blood after diethyl ether extraction and purification; (D) blank of whole blood (0.5 ml) spiked with 0.5 μg of (1) barbital, (2) phenobarbital, (3) butalbital, (4) amobarbital and (5) secobarbital before diethyl ether extraction and purification and (6) 5 μg of methaqualone added to the last ether phase; (E) blank of whole blood (0.5 ml) spiked with 0.5 μg of (1) proxibarbital, (2) barbital, (3) 1 μg of phenobarbital, (4) amobarbital and (5) aprobarbital before diethyl ether extraction and purification.

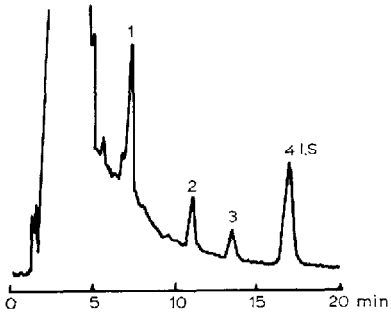

Fig. 3. Chromatogram of post-mortem blood; concentrations found were (1) 4 μg ml^{-1} for phenobarbital, (2) 7.20 μg ml^{-1} for amobarbital and (3) 9.20 μg ml^{-1} for secobarbital with (4) methaqualone as internal standard.

TABLE II

DETECTION RESPONSE AT 0.1 a.u.f.s. OF THE TESTED DRUGS AT DIFFERENT WAVELENGTHS

+ + + = Very good response; + + = good response; + = poor response; ± = bad response.

Drug	198 nm	220 nm	230 nm	240 nm	254 nm
Barbiturates currently used as drugs in France:					
Amobarbital	+ + +	+ +	+ +	+ +	+
Barbital	+ + +	+ +	+ +	+ +	+
Butalbital	+ + +	+ +	+ +	+ +	+
Butobarbital	+ + +	+ +	+ +	+ +	+
Cyclobarbital	+ + +	+ +	+ +	+ +	+
Heptabarbital	+ + +	+ +	+ +	+ +	+
Hexobarbital	+ + +	+ +	+ +	+ +	+
Methohexital sodium	+ + +	+ +	+ +	+	±
Phenobarbital	+ + +	+ +	+ +	+ +	+
Proxibarbital	+ + +	+ +	+ +	+ +	+
Secobarbital	+ +	+ +	+ +	+ +	+
Vinylbital	+ + +	+ +	+ +	+ +	+
Barbiturates not currently used as drugs in France:					
Aprobarbital	+ + +	+ +	+ +	+	+
Butabarbital	+ + +	+ +	+	±	±
Mephebarbital	+ + +	+ + +	+ +	+	±
Pentobarbital	+ + +	+ +	+	±	±
Thialbarbital	+ +	+ +	+ + +	+ + +	+ +
Thiopental	+ +	+ +	+ + +	+ + +	+ +
Internal standard:					
Methaqualone	+ + +	+ + +	+ + +	+ + +	+ + +

instability which may be observed at 198 nm depends on the sensitivity adopted; at 0.01 a.u.f.s. the analysis did not exhibit any difficulties.

Choice of internal standard

As possible internal standards we tested some barbiturates (aprobarbital, butabarbital mephebarbital and pentobarbital), not currently used in France, and methaqualone, classed as "Narcotic–Table B" in France and therefore not used as a sedative or hypnotic drug. Considering the respective retention times (Table I), we chose methaqualone because it is well separated from the sample barbiturates. However, the use of aprobarbital, which also gives a good response, is possible, except for the determination of secobarbital (similar retention times).

Recovery

The percentage extraction of phenobarbital, amobarbital and secobarbital (2 and 4 $\mu g\ ml^{-1}$) was measured using the deproteinization conditions described and methaqualone as the internal standard. For the assay, the tested drugs were

TABLE III

WITHIN-DAY AND BETWEEN-DAY REPRODUCIBILITY

Drug	Concentration ($\mu g\ ml^{-1}$)	Coefficient of variation (%)	
		Within-day	Between-day
Phenobarbital	2	13.30	6.22
	4	7.10	9.80
	10	8.00	13.60
	20	4.60	6.80
Mean		8.25	9.10
Amobarbital	2	16.00	12.40
	4	10.30	9.70
	10	9.20	12.00
	20	6.30	10.80
Mean		10.45	11.22
Secobarbital	2	18.00	5.70
	4	3.40	12.60
	10	4.90	8.00
	20	8.50	5.60
Mean		8.70	7.80

added before the deproteinization procedure and the internal standard was added to the supernatant after deproteinization. For the blank, drugs and internal standard were added together to the supernatant, after deproteinization. Peak-area ratios of the assay chromatograms were compared with those obtained from the blanks. The mean recoveries of the three tested compounds were greater than 86%.

Calibration

The linear regression parameters for the calibration graphs were determined; the relationships were linear between 2 and 20 $\mu g\ ml^{-1}$. The correlation coefficients were 0.994 for phenobarbital, 0.998 for amobarbital and 0.999 for secobarbital.

Reproducibility

The reprocucibility of the analysis (six determinations) was tested for phenobarbital, amobarbital and secobarbital with methaqualone as internal standard. The results are given in Table III. The within-day coefficients of variation were between 3.4 and 18% with respective mean values of 8.25, 10.45 and 8.70%. The between-day coefficients of variation over a period of two weeks were between 5.6 and 13.60% with respective mean values of 9.10, 11.22 and 7.80%.

Detection limits

The detection limits for quantitative determination were 0.2–0.4 $\mu g\ ml^{-1}$, depending on the barbiturate.

Selectivity

Chromatograms of blank whole blood obtained with only the deproteinization procedure showed no background interference from endogenous constituents (Fig. 2A, except for proxibarbital (retention time, $t_R = 3.80$ min) and barbital ($t_R = 4.50$ min). In such an eventuality, the following procedure has to be adopted: extraction with an organic solvent (diethyl ether or methylene chloride) from acidic medium, back-extraction into dilute alkaline phase (0.01 *M* Na_3PO_4), as described by Mangin et al. [22], and re-extraction into an organic solvent after acidification of the alkaline layer; the last solvent phase is removed into a centrifuge tube containing 50 µl of internal standard solution (0.1 mg ml^{-1}) and evaporated to dryness*; the residue is dissolved in 100 µl of mobile phase and 20 µl are injected into the chromatograph. Under these conditions, no interference from endogenous compounds was observed (Fig. 2C).

Several drugs were also tested for possible interferences. No interference was noted with morphine, codeine, ethylmorphine, papaverine, noscapine, cocaine, lidocaine, dextromoramide, pentazocine, clomipramine, desmethylclomipramine, imipramine, desipramine, trimipramine, desmethyltrimipramine, amitriptyline, nortriptyline, levomepromazine, chlorpromazine, diazepam, nordiazepam, clotiazepam, aspartam, salicylic acid, acetylsalicylic acid, benzoic acid or saccharin. Proxibarbital and barbital cannot be determined in samples that also contain acetaminophen ($t_R = 4$ min) or caffeine ($t_R = 4.2$ min), which are extractable under the same conditions.

Remarks

For the determination of thialbarbital and thiopental, whose retention times are long, the flow-rate of the mobile phase can be increased to 2 ml min^{-1}, with UV detection at 230 nm. This method is also useful for the determination of methaqualone, taking a barbiturate with a shorter retention time than the internal standard.

CONCLUSION

The proposed procedure provides sufficient sensitivity, reproducibility, selectivity and rapidity for the determination of barbiturates. It is suitable for both clinical pharmacology and forensic toxicology.

ACKNOWLEDGEMENTS

The authors express their gratitude to the manufacturers of the tested barbiturates and to Houdé-ISH Laboratoires for providing the standard compounds.

*If aprobarbital is used as the internal standard, 10 µl of the standard solution (0.1 mg ml^{-1}) may be added directly to the sample before the first extraction.

REFERENCES

1 J.P. Benais, R. Gherardi and M. Rudler, J. Med. Légale Droit Med., 28 (1985) 375-379.
2 A. Heyndrickx and M. Van Der Heede, J. Eur. Toxicol., 8 (1975) 14-20.
3 D. Biou and M. Pays, Toxicol. Eur. Res. 2 (1979) 7-23.
4 C.B. Walberg, Clin. Toxicol., 18 (1981) 879-885.
5 D.J. Berry, J. Chromatogr., 86 (1973) 89-105.
6 S.Y.R. Sun and D.J. Hoffmann, J. Pharm. Sci., 68 (1979) 386-388.
7 J. Vincent, C. Feurstein, M. Gavend and J. Faure, Clin. Chim. Acta, 93 (1979) 391-399.
8 D.N. Pillai and S. Dilli, J. Chromatogr., 220 (1981) 253-274.
9 A. Turcant, A. Premel-Cabil, A. Cailleux and P. Allain, J. Chromatogr., 229 (1982) 222-226.
10 T. Villen and I. Petters, J. Chromatogr., 258 (1983) 267-270.
11 J.E. Wallace, L.R. Hall and H. Steven, J. Anal. Toxicol., 7 (1983) 178-180.
12 B.K. Tang, B. Yilmaz and W. Kalow, Biomed. Mass Spectrom., 11 (1984) 462-465.
13 F. Susanto, S. Humfeld and H. Reinauer, Fresenius Z. Anal. Chem., 323 (1986) 168-172.
14 U.R. Tjaden, J.C. Kraak and J.F.K. Huber, J. Chromatogr., 143 (1977) 183-194.
15 H.R. Schulten and D. Kummler, Anal. Chim. Acta, 113 (1980) 253-267.
16 R. Gill, A.A.T. Lopes and A.C. Moffat, J. Chromatogr., 226 (1981) 117-123.
17 H.L. Levine, M.E. Cohen, P.K. Duffner, K.A. Kustas and D.D. Shen, J. Pharm. Sci., 71 (1982) 1281-1283.
18 M. Khalil and C. Gonnet, Analusis, 11 (1983) 513-519.
19 G. Nicot, G. Lachatre, J.P. Valette, L. Merle and Y. Nouaille, Thérapie, 39 (1984) 361-367.
20 R. Gill, A.L. Moffat, R.M. Smith and T.G. Huzdely, J. Chromatogr. Sci., 24 (1986) 153-159.
21 J. de Jong, M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 381 (1986) 431-437.
22 P. Mangin, A.A. Lugnier and A.J. Chaumont, J. Anal. Toxicol., 11 (1987) 27-30.
23 R. Cleeland, R. Davis, J. Heveran and E. Grunberg, J. Forensic Sci., 20 (1975) 45-57.
45 T.S. Sulkowski, G.D. Lathrop, J.H. Meritt, J.H. Landez and E.R. Noe, J. Forensic Sci., 20 (1975) 524-536.
25 E.L. Slightom, J. Forensic Sci., 23 (1978) 292-303.
26 A.T. Waston, J.E. Manno and B.B. Manno, J. Anal. Toxicol., 7 (1983) 257-261.
27 D.L. Golbert, D.S. Smith, J. London and A.M. Sidki, Clin. Chem., 30 (1984) 1765-1769.
28 J. Ojanperä, E. Vuori, R. Nieminen and A. Pentillä, J. Forensic Sci., 31 (1986) 707-709.
29 R. Truhaut, E. Bonnet-Kannapell, J.P. Dupeyron and P. Fabiani, Ann. Falsif. Expert. Chim., 70 (1977) 661-673.
30 M. Stajic, Y.H. Caplan and R.C. Backer, J. Forensic Sci., 24 (1979) 722-731 and 732-744.
31 Y.H. Caplan, R.C. Backer, M. Stajic and B.C. Thomson, J. Forensic Sci., 24 (1979) 745-751.
32 J.C. Kraak and J.P. Crombeen, J. Liq. Chromatogr., 5 (Suppl. 2) (1982) 273-304.
33 S.A. Stout and C.L. De Vane, J. Chromatogr., 285 (1984) 500-508.