Journal of Chromatography, 143 (1977) 183—194
Biomedical Applications

CHROMBIO. 021

RAPID TRACE ANALYSIS OF BARBITURATES IN BLOOD AND SALIVA BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

U.R. TJADEN**, J.C. KRAAK and J.F.K. HUBER***

Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, Amsterdam (The Netherlands)

(Received July 14th, 1976)

SUMMARY

The performances of a number of liquid—solid systems, consisting of mixtures of water and methanol as liquid phase and methyl silica as solid phase, were investigated with respect to their use in the separation of barbiturates by high-pressure liquid chromatography (HPLC). Phase system selectivities and column efficiencies were determined.

The results were applied to the development of a rapid method for the determination of trace amounts of barbiturates in blood. The first step in the analysis, the extraction of barbiturates from blood, was also investigated and good recoveries were achieved. The extracts were analyzed by HPLC using ultraviolet detection at 220 nm. A low detection limit and high precision were obtained. An amount of 5 ng hexobarbital, for example, can be determined with a precision of ±15% and 5 μg with a precision of ± 0.3%. The time course of the concentration of hexobarbital in the serum and saliva of man after an oral administration of 400 mg is demonstrated.

INTRODUCTION

Insomnia, which is mostly a symptom of a physical or emotional disorder of man, has been successfully treated for many years with hypnotic drugs such as barbiturates. In order to obtain a greater insight into the pharmacokinetics [1] and the determination of the minimal effective and toxicological concentrations, the analysis of the drug itself and its metabolites is of great importance.

Previously, a number of analytical methods such as UV spectrophotometry and gas chromatography with flame-ionization detection [2—4] have been ap-

*The paper was presented at the conference "Biochemische Analytik", Munich, April 8—13, 1976.

**Present address: Department of Analytical Chemistry and Pharmaceutical Analysis, Gorlaeus Laboratories, State University, P.O.Box 75, Leiden, The Netherlands.
***Institute of Analytical Chemistry, University of Vienna, Währinger Strasse 38, Vienna, Austria.

plied. The former method is non-specific while the latter is mostly restricted to the determination of the drug itself at overdose levels. Some papers have described the thermionic detection of barbiturates, by means of which analyses at therapeutic levels became possible [5,6]. In order to determine the more hydrophilic metabolites, derivatization steps [7] are necessary. Such steps, however, seriously invalidate the quantitative analyses.

The low working temperatures and the many possibilities of adjusting the phase system selectivity combined with high efficiencies make high-pressure liquid chromatography (HPLC) very suitable for the analysis of thermally labile, hydrophilic and hydrophobic compounds. Because the metabolites of a drug are more hydrophilic than the drug itself, it is appropriate to choose a phase system with a hydrophobic stationary phase, in which the more hydrophilic compounds are eluted first, which permits easier detection and determination of low concentrations of metabolites.

The separation and quantitation of barbiturates in pharmaceuticals by HPLC on a strong ion exchanger has been reported previously [8]. This paper describes the separation and quantitation of barbiturates at therapeutic levels in serum and saliva by high-pressure liquid—solid chromatography with UV detection using a highly selective hydrophobic adsorbent. An improved extraction procedure for barbiturates, which is a modification of a procedure described elsewhere [6], is also presented.

EXPERIMENTAL

Apparatus

A high-pressure liquid chromatograph (Siemens SP 100) equipped with a UV detector (DuPont, Model 837), a high-pressure sampling valve (Valco CV-6-UHPa), a linear potentiometric recorder (Goerz, Servogor RE 542) and an electronic integrator (Spectra-Physics, Autolab System I) was used. Stainless-steel 316 tubing with an I.D. of 2.8 mm, an O.D. of 6.35 mm and a length of 10 cm were used for the construction of the column. In order to prevent contamination of the separation column, a pre-column (500 × 9 mm) was installed.

Chemicals and materials

In all experiments, double-distilled water and organic solvents of analytical grade (Merck, Darmstadt, G.F.R.) were used. The components for the preparation of the extraction solvent were freshly distilled.

The methyl silica was prepared from narrow-sized silica (LiChrosorb SI 60, Merck) treated with dimethyldichlorosilane, as described previously [9]. The pre-column was filled with 63—200 μm silanized silica (SI 60, Merck).

Procedures

Chromatography. In order to pack the column, 0.8 g of methyl silica was added to 5 ml of a mixture of tetrabromoethane and chloroform (sp. gr. 1.82) and dispersed ultrasonically in the liquid. The slurry was then placed in a wide-bore tube to which the column, closed at the bottom by a frit, was attached. 2,2,4-Trimethylpentane was then pumped into the tube with a high flow-rate at 1000 bar, displacing the slurry into the column. After filling, the column

was successively eluted with 100 ml of acetone and 100 ml of ethanol in order to remove the dispersion liquid and finally with 100 ml of eluent. The precolumn was filled by using a dry packing technique.

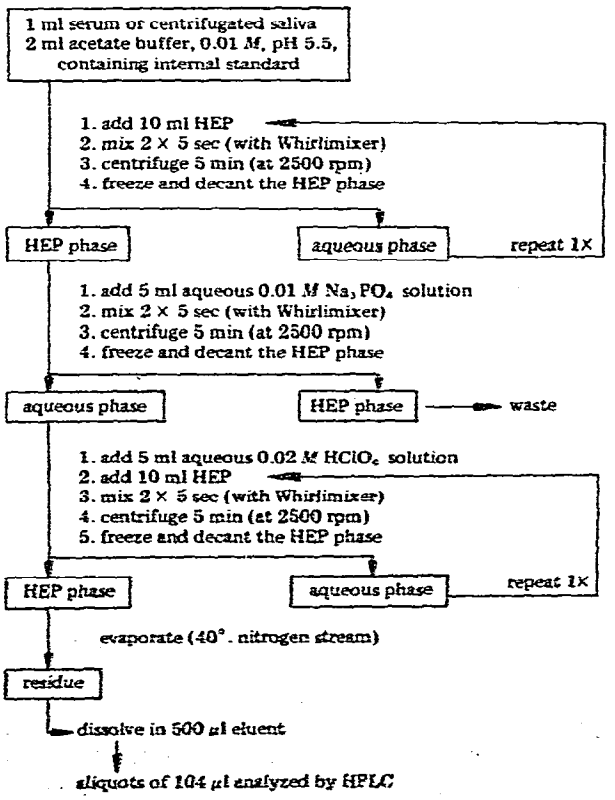
The capacity ratios were calculated from the retention times of the barbiturates and of an unretarded compound, for which potassium chromate was used. The selectivity coefficients of pairs of compounds were calculated as the ratio of their capacity ratios.

The theoretical plate height for a compound was calculated from its retention time and half the peak width at 60% of the peak height.

The samples were dissolved in the eluent and injected by means of a high-pressure sampling valve with a sample loop of 13.6 μl for the theoretical plate height measurements and of 104 μl for the quantitative determinations in serum and saliva.

Extraction. The full scheme for the extraction of barbiturates from serum and saliva with *n*-hexane—diethyl ether—*n*-propanol (49 : 49 : 2) (HEP) is outlined in Scheme 1.

In order to obtain a rapid and efficient separation of the organic and aqueous phases after mixing, the extraction mixture is centrifuged and then placed



Scheme 1. The extraction of barbiturates from serum and saliva.

in liquid nitrogen. The aqueous phase freezes within 60 sec and the organic phase can be decanted easily. The total extraction procedure takes less than 1 h.

RESULTS AND DISCUSSION

The analysis of closely related compounds such as a drug and its metabolites, present in very low concentrations in a small amount of sample, by HPLC places great demands on the magnitude of the selectivity coefficient, the capacity ratio, the theoretical plate height and the extraction procedure [10]. High selectivity coefficients, relatively small capacity ratios and small theoretical plate heights, combined with an extraction procedure with high selectivity and high recoveries for the removal of interfering compounds, are the requirements for achieving low detection limits with adequate resolution. In practice, a compromise between detection limit and resolution has to be found.

In earlier work [9], the remarkable selectivity of methyl silica towards compounds with closely related structures such as psychopharmaceuticals and sulpha drugs was reported. In order to test this type of adsorbent for the separation and determination of barbiturates, a systematic investigation with respect to column efficiency and selectivity was made. The precision of the quantitative determination of barbiturates by HPLC and the influence of the extraction procedure on the determination of barbiturates in serum were also investigated.

Phase system selectivity

The capacity ratios and selectivity coefficients of successively eluted barbiturates were measured as a function of the percentage of methanol in the mixture with water used as the mobile phase. The retention behaviour of barbiturates is dependent on pH and therefore the use of a buffer should be favourable. It was found, however, that this technique leads to non-reproducible capacity ratios and to low selectivity coefficients. Therefore, no buffer but distilled water alone was used.

The results are shown in Table I and Fig. 1. The effect of the methanol content on the capacity ratio and selectivity coefficient of barbiturates is two-fold: (i) owing to the greater lipophility of methanol compared with water, the capacity ratio decreases with increasing methanol content, and (ii) the selectivity coefficients change irregularly with the methanol content. In general, the selectivity coefficient decreases with increasing methanol content. For some pairs of compounds, however, the selectivity coefficients has a pronounced maximum or minimum at a given methanol content.

No correlation between the pK_a values of the barbiturates and their retention behaviour could be found. The systematic effect of structural increments on the log κ_i values cannot be observed clearly, mainly because of the lack of the number of systematic changes in one of the substituents. Some effects are clearly systematic: the methylene increment, according to $\log \kappa_i = a + bn$, where a and b are constants and n is the number of carbon atoms in the alkyl chain, as discussed in a previous paper [9], and the effect of a double bond in the alkyl chain, not attached directly to the ring.

The optimal composition of the eluent required to fulfil the demands of

TABLE I

CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF BARBITURATES MEASURED WITH DIFFERENT METHANOL–WATER MIXTURES AS ELUENT AND METHYL SILICA AS STATIONARY PHASE

Barbiturate	Eluent							
	25 : 75		30 : 70		40 : 50		50 : 50	
	κ_i	r_{ji}	κ_i	r_{ji}	κ_i	r_{ji}	κ_i	r_{ji}
Barbital	3.58	—	2.49	—	1.57	—	1.05	—
Heptobarbital	5.79	1.62	3.82	1.53	2.02	1.29	1.21	1.15
Phenobarbital	6.06	1.05	3.85	1.01	2.05	1.01	1.27	1.05
Allobarbital	7.35	1.21	5.25	1.36	2.72	1.33	1.61	1.27
Barotal	9.17	1.25	7.16	1.36	3.57	1.31	1.69	1.05
Brallobarbital	11.1	1.21	7.29	1.02	3.65	1.02	1.77	1.05
Butobarbital	14.8	1.34	10.3	1.41	4.71	1.29	2.25	1.27
Cyclobarbital	15.5	1.04	10.4	1.01	4.78	1.01	2.30	1.02
Hexobarbital	20.4	1.32	14.4	1.38	6.36	1.35	2.85	1.24
Mephobarbital	26.6	1.30	17.6	1.22	7.06	1.11	2.98	1.05
Heptabarbital	29.1	1.09	19.8	1.13	7.81	1.11	3.29	1.10
Vinylbital	32.3	1.11	22.3	1.13	9.03	1.16	3.61	1.10
Pentobarbital	33.6	1.04	22.9	1.03	9.39	1.04	3.69	1.02
Amobarbital	35.0	1.04	24.3	1.06	9.49	1.01	3.80	1.03
Secobarbital	48.8	1.39	34.3	1.41	12.6	1.33	4.67	1.23

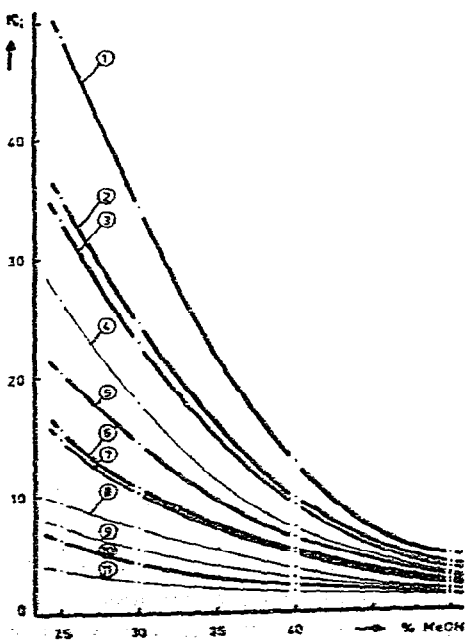


Fig. 1. Plot of the capacity ratio, κ_i, of barbiturates against the methanol content on the phase system methanol–water/methyl silica. The heavy lines represent the most common used barbiturates. 1 = Secobarbital; 2 = amobarbital; 3 = pentobarbital; 4 = methylphenobarbital; 5 = hexobarbital; 6 = cyclobarbital; 7 = butobarbital; 8 = barotal; 9 = allobarbital; 10 = phenobarbital; 11 = barbital.

small capacity ratios and high selectivity coefficients can easily be found from Table I and Fig. 1. A methanol content of about 40% seems to be a good compromise in this respect. Some pairs of barbiturates cannot be separated with the phase system investigated. However, in practice these barbiturates are seldom administred together in one dose. On the other hand, all barbiturates should be separated with sufficient resolution in order to allow accurate chromatographic identification.

Column efficiency

Some workers [11, 12] predicted an enormous improvement in column efficiency if small particles, of the order of a few micrometres, and a narrow size distribution were used. Small particles lead to large pressure drops and the lack of high-pressure pumping and injection systems delayed the development of HPLC until about 10 years ago. Since then, the improvement in column technology reported in several papers confirmed the effect of small particles on the theoretical plate height [13, 14]. In practice, good column packings can be obtained with particle sizes down to about 5 μm.

For adsorption chromatography on hydrophilic surfaces, the reported column efficiencies are generally better than those for adsorption chromatography on hydrophobic surfaces, e.g., with chemically bonded materials such as C_8 and C_{18} [15]. This effect was mainly attributed to the larger particles used on the one hand and to a slow mass transfer in the hydrophobic layer on the other. Recently, the synthesis of a short-chain modified silica, which shows excellent selectivity and efficiency, was reported [16].

In this work, a methyl silica of small particle size was used. For a number of barbiturates with capacity ratios ranging from 2 to 12, the theoretical plate height, H, was measured as a function of the mobile phase velocity, u. The results are plotted in Fig. 2.

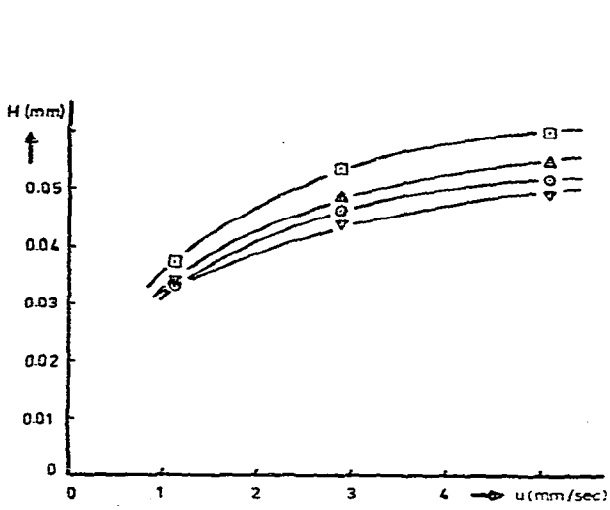
The flatness of the H versus u curves indicates a rapid mass transfer on this type of support, opposite to the effect observed on C_8 and C_{18} bonded phase materials [15]. As can be seen, the convective mixing is also small, owing to the use of small particles and an appropriate packing procedure.

This investigation demonstrates that with methyl silica, highly efficient, small-diameter columns can be prepared, which are very suitable for trace analysis. The ability of methyl silica columns to separate barbiturates is illustrated by Fig. 3, which demonstrates the separation of six barbiturates in about 2 min.

Composition of the extraction solvent

The extraction of barbiturates from serum and saliva in order to remove interfering substances and to enrich the barbiturates is an important step in this type of trace analysis.

Barbiturates are weakly acidic compounds with pK_a values ranging from 7.4 to 8.3. The distribution of acidic compounds between an organic solvent and an aqueous solution depends on the pH of the aqueous phase and on the pK_a value of the acids. The total distribution coefficient of an acid HX, defined as the ratio of the concentrations in the organic and the aqueous phases, excluding side reactions, can be expressed by



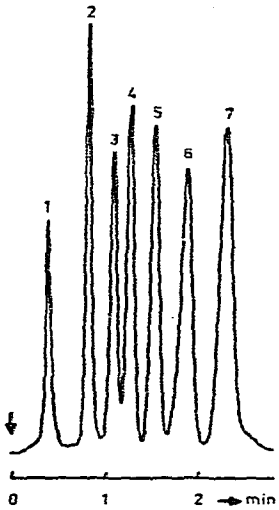


Fig. 2. H versus u curve for several barbiturates, measured on a column filled with 5-μm methyl silica as stationary phase and methanol—water (2 : 3) as mobile phase. □, Phenobarbital; △, butobarbital; ○, hexobarbital; ▽, secobarbital.

Fig. 3. HPLC separation of a test mixture of six barbiturates on methyl silica. Column, 100 × 2.8 mm; packing, methyl silica; eluent, methanol—water (1 : 1); UV detection at 205 nm. 1 = Potassium chromate; 2 = phenobarbital; 3 = barotal; 4 = butobarbital; 5 = hexobarbital; 6 = vinylbital; 7 = secobarbital.

$$K_X = \frac{[HX]_{org}}{[HX]_{aq} + [X^-]_{aq}} = \frac{1}{1 + K_a/[H^+]_{aq}} \cdot K_{HX} \qquad (1)$$

where the subscripts org, and aq refer to the organic and aqueous phases, respectively, K_{HX} is the partition coefficient to the undissociated acid HX.

The composition of the extraction solvent determines the value of K_{HX}. From eqn. 1, it can be seen that $pH \ll pK_a$ is favourable for obtaining high distribution coefficients, i.e., high recoveries. From the literature, many data [17, 18] are available on the effect of the composition of the organic phase on the distribution of barbiturates. These data show that highly lipophilic barbiturates can be extracted quantitatively from acidified solutions with non-polar solvents such as hexane and light petroleum. For less lipophilic barbiturates and the metabolites, more polar solvents such as diethyl ether and chloroform or mixtures of non-polar and polar solvents have to be used in order to obtain good recoveries.

Therefore, it was decided to use HEP as the extraction solvent in order to achieve the extraction of both the highly lipophilic and the less lipophilic barbiturates.

Blank serum extractions of untreated subjects with HEP, however, showed the presence of substances with a chromatographic retention behaviour within the group of barbiturates, as shown in Fig. 4a and 4b. In order to remove

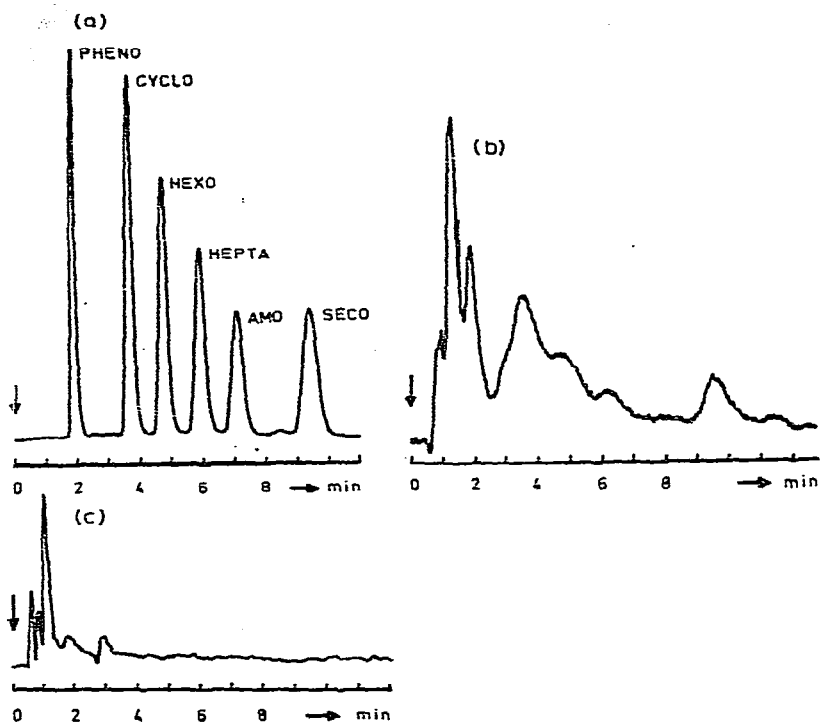


Fig. 4. Influence of the extraction procedure on the background. Column, 100 × 2.8 mm; packing, methyl silica; eluent, methanol—water (2 : 3); UV detection at 205 nm. (a) Test mixture of six barbiturates; (b) serum background before back-extraction; (c) serum background after back-extraction.

these interfering serum constituents, the extraction procedure was amended. The barbiturate-containing HEP phase was shaken with a sodium phosphate solution of pH 11.7. According to eqn. 1, a high pH value promotes the distribution of acids towards the aqueous phase. Owing to their high pK_a values, the barbiturates will be extracted completely from the organic into the aqueous phase, while a number of non-acidic compounds will remain in the organic phase and can be removed. The aqueous phase is acidified and the barbiturates are back-extracted with HEP. This extension to the extraction procedure results in a clean extract, almost without interfering substances in the elution range of the barbiturates, as shown in Fig. 4c.

Precision and linearity of the method

The precision of the quantitative determination of barbiturates by HPLC was investigated by injecting a constant volume (104 µl) of solutions of barbiturates of different concentrations (1—12 µg/ml). The UV spectra of barbiturates show a maximum at about 205 nm, but the linear dynamic range of the detector was found to be greater at 220 nm. In order to be able to cover a wide range of concentrations of barbiturates, it was decided to measure at 220 nm.

Fig. 5 shows the proportionality of peak area and injected amount of hexo-

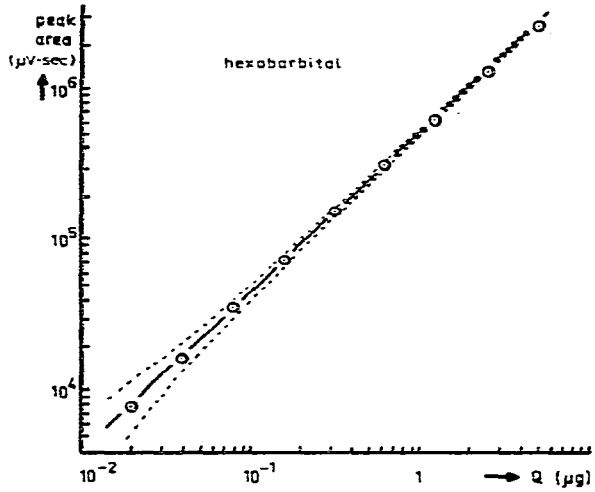


Fig. 5. Linearity and precision. The dashed lines show 3 × the standard deviation. $y = 5.18 \cdot 10^5 \cdot x - 6.20 \cdot 10^3$, where x = amount injected (μg) and y = peak area (μV·sec).

barbital measured over the range 20—5000 ng. The dashed lines show the confidence limits for ± 3 times the standard deviation (99.7% reliability). The relative standard deviation is 0.3% for 50 μg/ml, 3% for 5 μg/ml and 15% for 0.05 μg/ml. The sensitivity of the whole system, defined as the slope of the curve of the peak area versus injected amount of compound (hexobarbital), expressed in integration units (I.U.), is 5.18 μV·sec/ng, calculated by linear regression.

The linearity of the calibration graph is characterized by the correlation coefficient, which was determined to be 0.99984, indicating the very high linearity.

The standard deviation of the baseline noise, measured during the same period of time as the peak integral, was 385 μV·sec, corresponding to about 0.8 ng of hexobarbital. The detection limit of hexobarbital for a signal to noise ratio of 3 is about 2.4 ng.

The recovery and reproducibility of the extraction procedure were tested by extraction of known amounts of barbiturates of different lipophilicity added to distilled water and to blank serum. The relationship between the added and determined amounts of a number of barbiturates after extraction from distilled water and blank serum is shown in Fig. 6. The recoveries of the highly lipophilic hexobarbital and secobarbital for both water and serum ranges from 90 to 95%. For the less lipophilic phenobarbital, a smaller recovery (water 70% and serum 50%) was found.

The reproducibility in all extractions was about 3% at 10 μg/ml and 10% at 1 μg/ml, indicating that the precision of the total determination is mainly determined by the extraction. Fig. 7 shows the separation of six widely used barbiturates (300 ng of each), added to blank serum and extracted as described above.

In order to eliminate possible errors during extraction, one of these barbiturates can be chosen as an internal standard for the determination of the others.

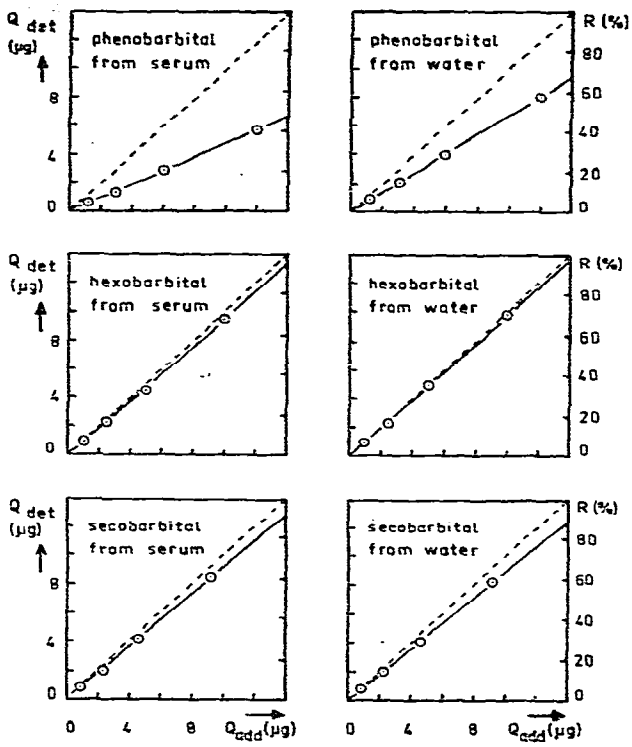


Fig. 6. Recovery of some barbiturates extracted from serum and water. The dashed lines represent 100% recovery.

Time course of concentration of hexobarbital in blood and saliva after oral administration

In order to test the method with natural samples, blood and saliva from man were examined after oral administration of 400 mg of hexobarbital in solid gelatine capsules. Blood and saliva samples were taken at increasing time intervals.

To 1 ml of serum or centrifuged saliva (5 min, 2500 rpm), 3.5 µg of amobarbital were added as internal standard and the sample was then treated as described above. The extract was analyzed by HPLC.

Figs. 8 and 9 show the chromatograms of the extracts of serum and saliva samples, respectively, from the same subject. The concentration curves obtained from another subject are shown in Fig. 10 and agree well with results obtained by other workers in similar experiments [5] using gas chromatography. From Figs. 8 and 9, it can be seen that the determination of metabolites, which are expected in the chromatogram before the barbiturate, is hindered by interfering compounds present in the extract.

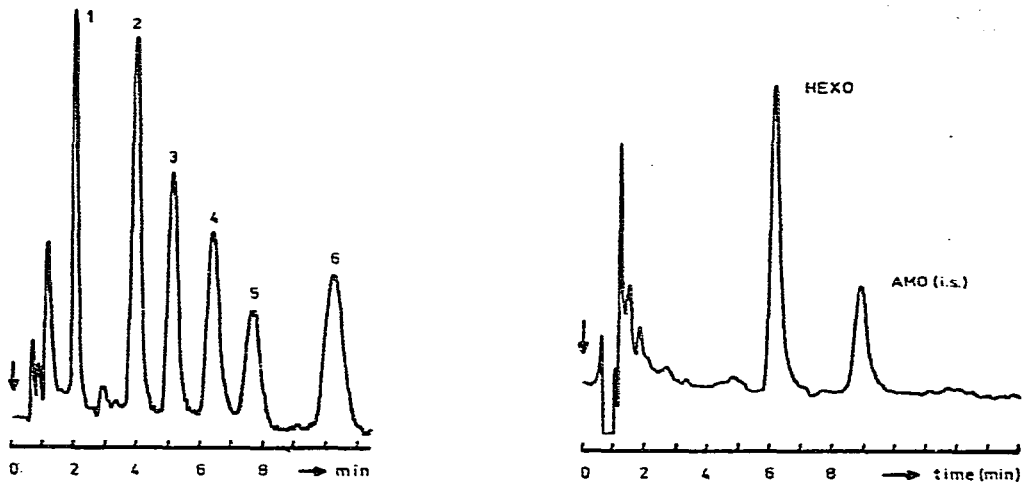


Fig. 7. Analysis of six barbiturates extracted from serum. Injection: 104 µl from 500 µl of solution obtained from 1 ml serum, to which 1.5 µg of each component was added. Conditions as in Fig. 4. 1 = Phenobarbital; 2 = cyclobarbital; 3 = hexobarbital; 4 = heptabarbital; 5 = amobarbital; 6 = secobarbital.

Fig. 8. Chromatogram of an extract from serum of man after oral administration of 400 mg of hexobarbital in solid gelatine capsules. Sample taken at 6 h after administration. Conditions as in Fig. 4. Hexobarbital peak corresponds to 160 ng; amobarbital peak corresponds to 700 ng.

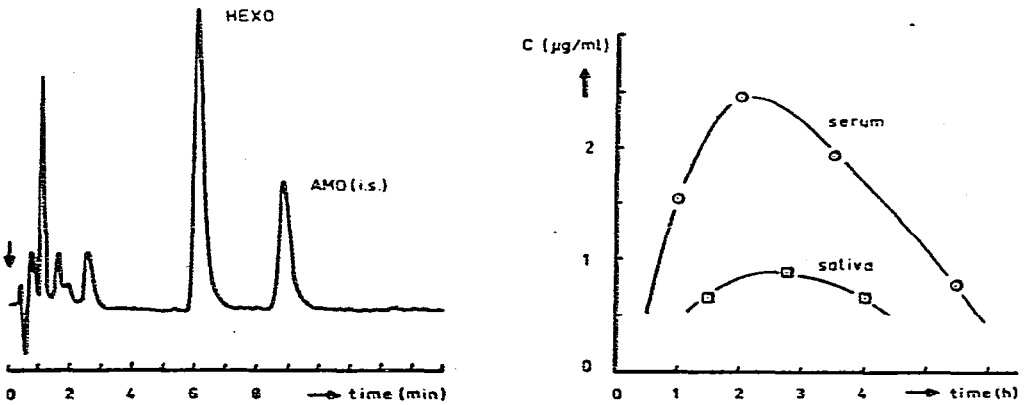


Fig. 9. Chromatogram of an extract from saliva of man after oral administration of 400 mg of hexobarbital in solid gelatine capsules. Sample taken 2.5 h after administration. Conditions as in Fig. 4. Hexobarbital peak corresponds to 140 ng; amobarbital peak corresponds to 700 ng.

Fig. 10. Hexobarbital serum and saliva concentration curves for one subject after oral administration of 400 mg of hexobarbital in solid gelatine capsules.

CONCLUSIONS

The main conclusions can be summarized as follows:

(i) Methyl silica shows excellent selectivity towards the barbiturates.
(ii) Highly efficient columns can be prepared with methyl silica as adsorbent.
(iii) Rapid trace analysis of barbiturates in body fluids at submicrogram levels is possible.
(iv) In order to determine metabolites, the extraction procedure has to be altered in order to remove interfering compounds.

ACKNOWLEDGEMENTS

The authors thank Prof. Dr. D.D. Breimer and Dr. A.G. de Boer for valuable discussions, the C.L.A.N.A.G. Institute (Zaandam, The Netherlands) for the kind gift of the pure barbiturates and Mr. F. Broekhoven for his technical assistance.

REFERENCES

1 G. Levy, in D.S. Davies and B.N.C. Prichard (Editors), Biological Effects of Drugs in Relation to their Plasma Concentrations, MacMillan, London, 1973, Ch. 6.
2 D.J. Berry, J. Chromatogr., 86 (1973) 89.
3 H.V. Street, Clin. Chim. Acta, 34 (1971) 357.
4 R.G. Cooper, M.S. Greaves and G. Owen, Clin. Chem., 18 (1972) 1343.
5 D.D. Breimer and J.M. van Rossum, J. Chromatogr., 88 (1974) 235.
6 D.D. Breimer, Thesis, University of Nijmegen, Nijmegen.
7 M. Ehrnebo, S. Agurell and L.O. Boréus, Eur. J. Clin. Pharmacol., 4 (1972) 191.
8 R.W. Roos, J. Pharm. Sci., 61 (1972) 1979.
9 J.F.K. Huber and U.R. Tjaden, in preparation; U.R. Tjaden, Thesis, University of Amsterdam, Amsterdam, 1976, Ch. 1.
10 C.A.M. Meijers, J.A.R.J.Hulsman and J.F.K. Huber, Z. Anal. Chem., 261 (1972) 347.
11 J.F.K. Huber and J.A.R.J. Hulsman, Anal. Chim. Acta, 38 (1967) 305.
12 J.H. Knox and M. Saleem, J. Chromatogr. Sci., 7 (1969) 614.
13 J.F.K. Huber, Ber. Bunsenges. Physik. Chem., 77 (1973) 159.
14 R.E. Majors, J. Chromatogr. Sci., 11 (1973) 88.
15 J.N. Done and J.H. Knox, J. Chromatogr. Sci., 10 (1972) 606.
16 J.H. Knox and A. Pryde, J. Chromatogr., 112 (1975) 171.
17 M.T. Bush, G. Berry and A. Hume, Clin. Pharmacol. Ther., 7 (1966) 373.
18 J.R. Cooper and B.B. Brodie, J. Pharmacol. Exp. Ther., 114 (1955) 109.