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Determination of barbiturates in urine by micellar liquid chromatography and direct injection of sample

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Abstract

A liquid chromatographic procedure for the determination of six barbiturates (barbital, diallyl barbituric acid, phenobarbital, butabarbital, amobarbital and pentobarbital) in urine samples is described. The proposed system uses a Spherisorb octadecyl-silane ODS-2 C_{18} analytical column and a guard column of similar characteristics. The UV detector was set at 240 nm. A study to select adequate composition of the micellar mobile phase for the separation of these compounds in urine samples is performed. Maximum resolution was achieved with a 0.07 M sodium dodecylsulphate-0.3% propanol at pH 7.4 eluent. Limits of detection at 240 nm were ranged between 0.13 μg ml^{-1} for diallyl barbituric acid and 2.7 μg ml^{-1} for amobarbital. The procedure allows for the determination of these compounds in 20 minutes, it does not require prior a sample preparation step and it can be very useful to the investigation of intoxication.

Keywords: Liquid chromatography; Barbital; Diallyl barbituric acid; Phenobarbital; Butabarbital; Amobarbital; Pentobarbital; Urine samples micelles; Direct injection

1. Introduction

Barbiturates are substituted pyrimidine derivatives. The basic structure common to these drugs is the barbituric acid, a substance which has no CNS activity. Replacing the two hydrogens at position 5 with alkyl, alkenyl, and/or aryl groups produces compounds with CNS activity.

Barbiturates are used principally as hypnotics in the short-term treatment of insomnia, and preoperatively to relieve anxiety and provide sedation [1]. Barbiturates have also been used for routine sedation and to relieve anxiety and provide sedation in patients with alcohol withdrawal syndrome. Barbiturates are capable of producing all levels of CNS depression, from mild sedation to hypnosis to deep coma to death. The degree of depression depends upon dosage, route of administration, and pharmacokinetics of the particular barbiturate. In addition, the patient's age, physical or emotional state, and/or the concurrent use

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of other drugs may alter the response. Barbiturates are slowly metabolised, chiefly by the microsomal enzymes in the liver. The inactive metabolites of the barbiturates are conjugated with glucuronic acid and excreted in the urine. The excreted percentage of unchanged barbiturates in urine vary between 1 and 25% of an therapeutic oral dose [1].

Barbiturates have a low therapeutic index and are therefore prone to cause toxicity. Monitoring their concentrations in body fluids is therefore essential to optimise pharmacotherapy [2]. Screening, confirmation and determination of barbiturates in body fluids are important for toxicity evaluation, for therapeutic drug monitoring and for pharmacokinetic and metabolic studies.

Typically, urine samples were screened for drugs using immunoassays [3–5]. Immunological techniques are very attractive because of their ease of performance, speed of analysis and sensitivity. However, with the exception of phenobarbital, these assays are not specific enough to monitor or identify a single compound. Due to the lack of specificity of these techniques, positive results should be confirmed in order to eliminate any false positive answer that may have resulted from the initial screening process. For that purpose many selective methods, including high-performance thin-layer chromatography (HPTLC), gas–liquid chromatography [6,7], high-performance liquid chromatography (HPLC) [8–13], gas chromatography/mass spectrometry (GC/MS) [14–19], and micellar electrokinetic capillary chromatography (MECC) [20–22] have been developed. However, most of these procedures reported require labour sample pre-treatments as protein precipitation and solvent extraction or solid phase extraction. These pre-treatments are time-consuming, increase the error sources and make the procedure more laborious.

Micellar liquid chromatography (MLC) is an alternative of reversed-phase liquid chromatography (RPLC), which employs aqueous solutions of surfactants above the micellar critical concentration as the mobile phases [23]. One of the main advantages of MLC is the possibility of determining drugs in physiological fluids without the need of a previous separation of the proteins present in the sample [24]. This paper describes a rapid RPLC procedure for determining six barbiturates (barbital, diallyl barbituric acid, phenobarbital, butabarbital, amobarbital and pentobarbital) in urine by using micellar mobile phases and direct injection of sample. This procedure allows the determination of these compounds in urine at therapeutic levels.

2. Experimental

2.1. Instrumental and measurement

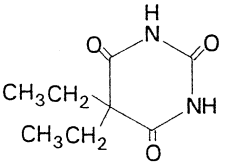
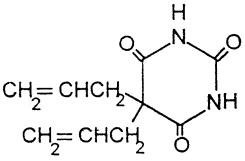
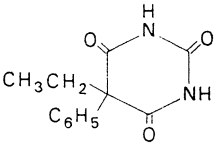
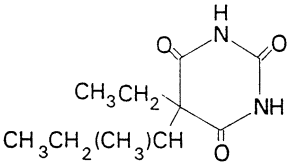
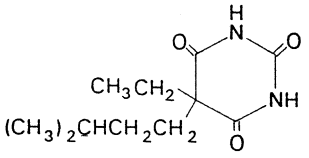
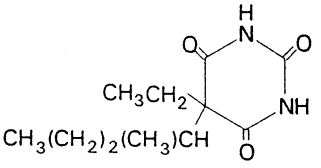
A Hewlett-Packard HP 1100 chromatograph with an isocratic pump, an UV–visible detector was used (Palo Alto, CA). Data acquisition and processing were performed on an HP Vectra XM computer (Amsterdam, The Netherlands) equipped with HP-ChemStation software from Hewlett-Packard (1996 version, Waldbronn, Germany).

The solutions were injected into the chromatograph through a Rheodyne valve (Cotati, CA) with a 20 μl loop. A Spherisorb octadecylsilane ODS-2 C_{18} column (5 μm, 120×4.0 mm i.d.) and a guard column of similar characteristics (35×4.0 mm i.d.) (Scharlau, Barcelona, Spain) were used. The mobile phase flow rate was 1 m min^{-1}. The detection was performed in UV at 240 nm. All of the assays were carried out at room temperature. The dead time value (average $t_m = 0.83$ min) was determined for each injection as the first perturbation in the chromatogram.

2.2. Reagents and standards

Mobile phases were prepared by mixing aqueous solutions of sodium dodecyl sulphate (SDS, 99%, Merck, Darmstadt) and a small amount of 1-propanol (analytical-reagent grade, Scharlau, Barcelona, Spain). The pH was adjusted to 7.4 before the addition of *n*-propanol with a 0.05 M phosphate buffer, prepared with disodium hydrogen phosphate and sodium dihydrogen phosphate (analytical reagent, Panreac, Barcelona, Spain).

Table 1
Barbiturates studies in this work

Barbiturate	Structure
Barbital	
Diallyl barbituric	
Phenobarbital	
Butabarbital	
Amobarbital	
Pentobarbital	

Stock standard solutions of barbital, diallyl barbituric acid, butabarbital, amobarbital (Sigma, St. Louis, MO), phenobarbital (kindly donated by Bayer, Barcelona) and pentobarbital (donated by B. Braun Medical, S.A.) were prepared by dissolving 10 mg of the compound in 10 ml of 0.07 M SDS solutions and they were stored in the dark at 4°C. Working solutions were prepared by dilution of the stock standard solutions in the mobile phase. Table 1 shows the structure of the barbiturates studied.

Barnstead E-pure, deionized water (Sybron, Boston, MA) was used throughout. The mobile phases and the solutions injected into the chromatograph were vacuum-filtered through 0.45 μm nylon membranes (Micron Separations, Westbore, MA).

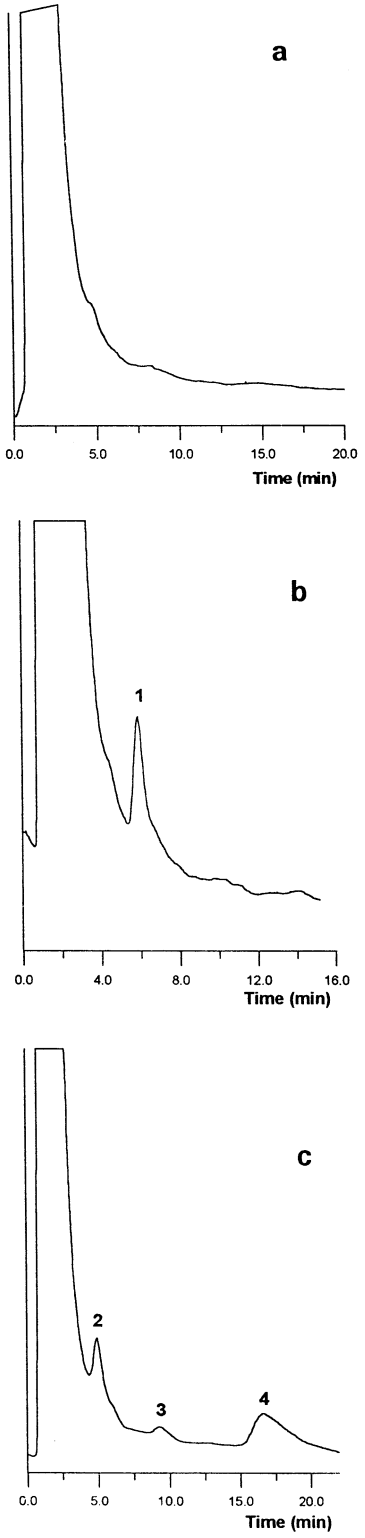
2.3. Sample preparation

Urine samples were filtered prior to use and were directly injected by triplicate onto the chromatographic system. Six standards for calibration were prepared by addition of appropriate volumes of standard solutions to 2.0 ml of urine sample (volume was completed to 4 ml with 0.07 M SDS). The concentrations of added analyte were in the range 5–40 μg ml^{-1}. Samples were prepared freshly before the injection onto the chromatographic system.

3. Results and discussion

3.1. Chromatographic studies

Barbiturates are acid compounds with different protonation constants: barbital (log $K = 7.97$), diallyl barbituric acid (log $K = 7.77$), phenobarbital (log $K = 7.4$), butabarbital (log $K = 7.9$), amobarbital (log $K = 7.8$) and Pentobarbital (log $K = 7.4$). The presence of an organized medium modifies the acid–base constants, log K, of the solubilized systems. This modification can be explained by the differences between the properties of the bulk solution and the micellar environment and by the electrostatic attractions and repulsions between the species involved and the micelle when both are charged. For anionic surfactants, there is



an increase of 0.5–3.0 in the log K value. In a previous paper, we determined the log K values potentiometrically in the presence of SDS micelles [25]. As can be expected, the log K values of barbiturates increase around 0.4 log K units in this medium.

A study to select the adequate composition of the mobile phase (pH, concentration of SDS, and modifier concentration) for the separation of barbiturates in both aqueous solutions and urine matrix was performed.

The effect of a varying mobile phase pH on the retention of the compounds was studied using a 0.15 M SDS mobile phase. The pH of the mobile phase was fixed at 3.5 and 7.4. As can be expected owing the protonation constants in aqueous solutions of the compounds, the retention factor of the compounds decreased as the mobile pH phase increased. The retention factor at pH 3.5 and 7.4 were 6.0 and 3.0 for barbital; 10.4 and 5.4 for diallyl barbituric acid; 13.7 and 6.7 for phenobarbital; 16.1 and 10.4 for butabarbital; 28.5 and 15.4 for amobarbital and 28.5 and 16.4 for pentobarbital respectively. This behaviour can be explained taking into account that at pH 3.5 the predominant form of the compound is non-ionic, while at pH 7.4 the compounds are partially ionized and, consequently, less retained due to the electrostatic repulsion between the charged compounds and the surfactant monomers absorbed into the stationary phase.

The profile of the background of the urine matrix depends on the composition of the mobile phase. The chromatogram of urine matrix shows a broad band due to the presence of the proteins at the solvent front and other smaller peaks due to the presence of endogenous compounds (Fig. 1(a)). Bonet et al. [26] indicated that in a purely micellar medium of SDS, the retention of the broad band decreases as the mobile phase pH increases and is lightly affected by the SDS concentration. In order to obtain a satisfactory chromatographic separation of the compounds in urine matrix, the pH of the mobile phase was fixed at pH 7.4 for successive experiments.

Fig. 1. Experimental chromatograms: (a) urine sample; (b) urine sample spiked with 20 μg ml^{-1} of phenobarbital (1); (c) urine sample spiked with 20 μg ml^{-1} of diallyl barbituric acid (2), butabarbital (3) and pentobarbital (4).

Table 2
Capacity factors (k') of the chromatographic peaks in mobile phases of SDS propanol at pH 7.4

SDS (M)	0.05	0.05	0.10	0.15	0.15
Propanol (%)	0.0	3.0	1.5	0.0	3.0
Barbital	6.08	2.44	2.62	3.52	1.75
Diallyl barbituric	10.71	4.39	4.28	5.42	2.73
Phenobarbital	13.90	6.12	5.41	6.35	3.47
Butabarbital	23.52	9.79	7.76	8.78	4.62
Amobarbital	35.69	14.52	10.64	13.54	6.26
Pentobarbital	55.11	15.59	11.67	21.60	6.79

Fig. 2 shows the effect of SDS concentration in the mobile phase on the retention of barbiturates. As can be observed for the highly hydrophobic compounds studied (amobarbital and pentobarbital), large changes in the retention were obtained upon increasing the surfactant concentration in the mobile phase, while for the slightly hydrophobic compounds (Barbital and Diallyl barbituric acid) the retention was scarcely modified. However, in the presence of micellar mobile phases of SDS, the efficiency values were very low and the peaks asymmetrical.

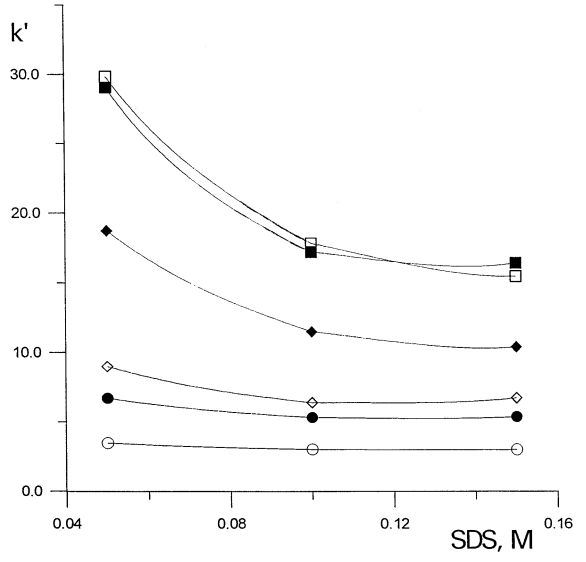


Fig. 2. Influence of SDS concentration in the mobile phase on the retention of barbiturates: ○, barbital; ●, diallyl barbituric acid; ◇, phenobarbital; ◆, butabarbital; □, amobarbital; ■, pentobarbital.

The addition of 1-propanol to the SDS mobile phase produced a reduction in the retention of barbiturates and an improvement in efficiency. Optimisation of the mobile phase composition (SDS and 1-propanol concentrations) was performed in agreement with the procedure reported by Torres Lapasió et al. [27]. In this paper, we demonstrated that the retention of a compound in a hybrid mobile phase may be described by the equation:

$$\frac{1}{k'} = A\mu + B\phi + C\mu\phi + D \quad (1)$$

where k' is the retention factor, μ is the total concentration of surfactant, ϕ is the volume fraction of alcohol and A, B, C and D are the fitting parameters. In order to obtain the fitting parameters, a factorial design (two levels with central point) was used. From the preliminary experiments, the following upper and lower limits were selected: the SDS concentration ranged from 0.05 to 0.15 M and 1-propanol concentration from 0.0 to 3.0%. The value of the pH was constant in all experiments. Table 2 shows the retention factors of the compounds obtained for the selected mobile phases. Table 3 shows the fitting parameters of Eq. (1) obtained by applying multiple linear regression. The relative difference between the calculated k values according to Eq. (1) and the experimental values expressed in percent deviation was 2.76%. Therefore, the equations can be used for further selection of optimal conditions.

Table 3
Calculated coefficients for the equations of retention (Eq. (1))

Compound	A	B	C	D
Barbital	0.5	0.7	14.5	5×10^{-3}
Diallyl barbituric	0.7	1.3	14.3	7×10^{-3}
Phenobarbital	0.9	2.4	13.1	0.03
Butabarbital	0.3	0.6	18.3	4×10^{-3}
Amobarbital	0.5	0.3	13.8	2×10^{-3}
Pentobarbital	0.8	2.3	14.8	0.02

The normalised product of the resolution was taken as the optimisation criterion [28]:

$$r = \prod_{i=1}^{n-1} \frac{S_{i,i+1}}{\left(\sum \frac{S_{i,i+1}}{n-1}\right)^{n-1}} \quad (2)$$

being $S_{i,i+1}$ the separation factor of two solutes:

$$S_{i,i+1} = \frac{t_{i+1} - t_i}{t_{i+1} + t_i} \quad (3)$$

Fig. 3 shows the resolution surface for barbiturates studied as a function of the concentration of SDS and 1-propanol. As can be observed, the global resolution decreases as the SDS and 1-propanol concentrations in the mobile phase increase. Maximum global resolution was achieved using a 0.05 M SDS-0% 1-propanol mobile phase. However, the use of this mobile phase provides low efficiency values and the retention of the large hydrophobic compounds was very large ($k = 35.7$ and 55.1 for amobarbital and pentobarbital, respectively). Using a 0.07 M SDS-0.4% 1-propanol (pH 7.4) mobile phase, the retention factors of barbiturates were adequate ($k = 2.9$, 4.8, 6.4, 10, 14.3 and 18.5 for barbital, diallyl barbituric acid, phenobarbital, butabarbital, amobarbital and pentobarbital, respectively). Using this mobile phase adequate separation between the peaks of barbiturates was achieved, but the peaks corresponding to barbital and diallyl overlapped with the end of the broad band of proteins, although they can be quantified satisfactorily. Fig. 1 shows

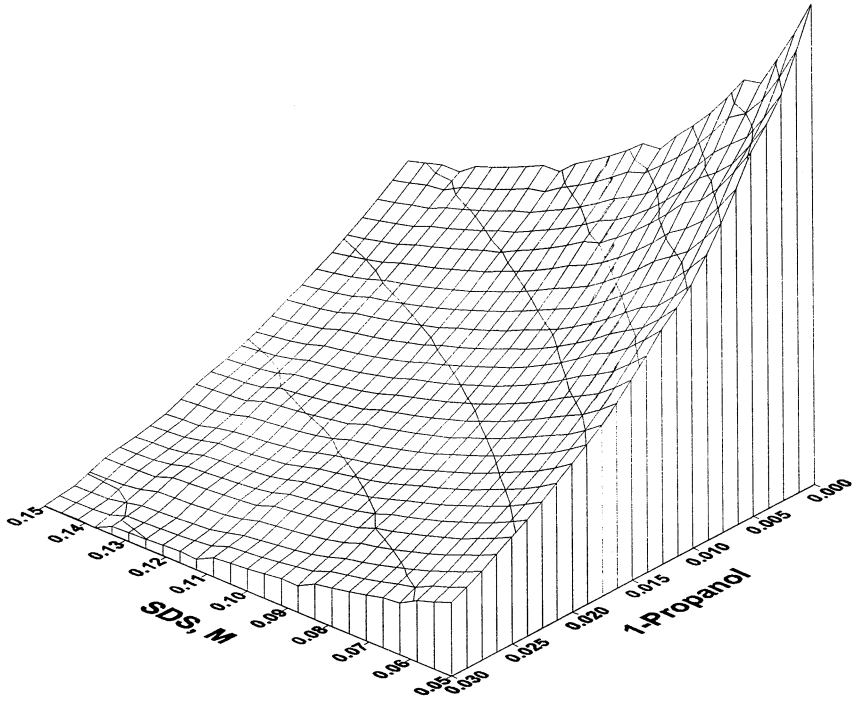


Fig. 3. Resolution surface for barbital, diallyl barbituric acid, phenobarbital, butabarbital, amobarbital and pentobarbital, as a function of the concentration of surfactant, μ, and modifier, ϕ, in the mobile phase (pH 7.4).

Table 4
Regression statistics for the calibration graphs of barbiturates in urine matrix

Compound	Peak area				Peak height			
	Slope $\pm$ ts$_s$	Intercept $\pm$ ts$_i$	SE	r	Slope $\pm$ ts$_s$	Intercept $\pm$ ts$_i$	SE	r
Barbital	0.052 ± 0.004	-0.19 ± 0.09	0.059	0.997	0.164 ± 0.017	-0.3 ± 0.6	0.152	0.998
Diallyl barbituric acid	0.059 ± 0.002	0.01 ± 0.02	0.018	0.998	0.089 ± 0.004	0.10 ± 0.05	0.043	0.996
Phenobarbital	0.107 ± 0.006	0.09 ± 0.07	0.078	0.995	0.173 ± 0.011	0.28 ± 0.14	0.144	0.993
Butabarbital	0.022 ± 0.002	-0.06 ± 0.02	0.015	0.996	0.024 ± 0.001	-0.049 ± 0.009	0.006	0.995
Amobarbital	0.009 ± 0.003	-0.01 ± 0.06	0.011	0.995	0.010 ± 0.001	-0.02 ± 0.02	0.004	0.9995
Pentobarbital	0.150 ± 0.008	-0.20 ± 0.09	0.081	0.997	0.057 ± 0.002	-0.05 ± 0.02	0.019	0.999

the experimental chromatogram of urine samples spiked with 20 μg ml^{-1} of phenobarbital (Fig. 1(b)) and diallyl barbituric acid, butabarbital and pentobarbital (Fig. 1(c)).

3.2. Analytical data

The calibration graphs of each compound in urine were obtained by injection of samples containing a varying concentration of the analytes. Peak heights and peaks areas were used as dependent variables. The concentration range studied was 5–40 μg ml^{-1}. Table 4 shows regression statistics for the calibration curves of each compound. In all cases, the calibration curves showed adequate and similar regression coefficients with peak areas and heights over the working interval, except butabarbital, amobarbital and pentobarbital that shown better regression coefficients with peak heights.

The limits of detection (LODs) in urine were calculated from the standard deviation of five-fold injections of a urine sample spiked with 2 μg ml^{-1} solution of the barbiturates barbital, diallyl barbituric acid, phenobarbital, butabarbital and pentobarbital (3σ criterion). For estimating the detection limit of amobarbital, a urine sample spiked with 8 μg ml^{-1} solution was used. The LODs obtained for the six barbiturates were: 0.63, 0.13, 0.66, 0.75, 2.7 and 0.86 μg ml^{-1} for barbital, diallyl barbituric acid, phenobarbital, butabarbital, amobarbital and pentobarbital, respectively. These values were lower than the corresponding to unchanged drug concentration excreted in the urine when therapeutic doses are used [1].

The repeatability of the method was evaluated at two concentration levels, 2 and 15 μg ml^{-1}, by preparing, for each concentration, five spiked samples of urine containing of barbital, diallyl barbituric acid, phenobarbital, butabarbital, amobarbital and pentobarbital. The precision of the method proposed, expressed as the relative standard deviation (%) was 4.5, 6.7, 6.1, 6.9, 10.1 and 12.8% (2 μg ml^{-1}) and 2.9, 3.5, 4.7, 5.6, 5.3 and 4.4% (15 μg ml^{-1}) for barbital, diallyl barbituric acid, phenobarbital, butabarbital, amobarbital and pentobarbital, respectively. These values are lower than the acceptance criteria for precision for biological samples (16% at the concentration limits and 10% at other concentration levels) [29].

3.3. Analysis of urine samples

The proposed method was applied satisfactorily to the determination of barbital, diallyl barbituric acid, phenobarbital, butabarbital, amobarbital and pentobarbital in urine samples. The results obtained for the analysis of four urine samples of healthy volunteers spiked with different amounts of barbiturates are summarised in Table 5. The recovery values were in general ranged between 90 and 108%.

The proposed method overcomes the inconvenient of the dilemma between HPLC separation and UV detection of barbiturates. The ionised forms of barbiturates are good UV chromophores, but they are poorly separated in reversed phase. The procedure reported is rapid, simple, it does not require sample pre-treatment and it can be very useful for monitoring barbitu-

Table 5
Analysis of urine samples[a]

Compound	Added (μg ml^{-1})	Found (μg ml^{-1})	Recovery (μg ml^{-1})
Barbital	45.8	43 ± 4	95 ± 9
	9.9	10.2 ± 0.2	103 ± 2
Diallyl barbituric acid	9.4	7.0 ± 0.3	75 ± 3
	2.1	2.0 ± 0.1	98 ± 5
Phenobarbital	9.4	8.5 ± 1.5	90 ± 16
	3.4	3.7 ± 0.3	108 ± 9
Butabarbital	28.1	284	100 ± 14
	5.3	5.6 ± 0.3	105 ± 6
Amobarbital	23.5	25 ± 5	104 ± 18
	8.4	8.0 ± 1.0	99 ± 12
Pentobarbital	5.7	5.25 ± 0.12	92 ± 2
	2.5	2.60 ± 0.10	104 ± 4

[a] Recovery of barbiturates added to urine samples ($n = 4$).

rates in urine at therapeutic levels and for toxicity evaluation. The procedure could be adapted for determining barbiturates in the presence of others drugs used with likely co-medication by including in the resolution optimisation procedure, the retention data of these drugs.

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References

[1] AHFS Drug Information 1997. American Society of Health-System Pharmacists, Bethesda, USA.
[2] R.F. Shaw, Barbiturates (Abused Drugs Monograph Series), Abbott Laboratories, Diagnostics Division, Irving, TX, 1988.
[3] B.E. Pape, P.L. Cary, L.C. Clay, W. Godolphin, Ther. Drug Monit. 5 (1983) 467.
[4] A.T. Watson, J.E. Manno, B.R. Manno, J. Anal. Toxicol. 7 (1983) 257.
[5] D.L. Colbert, D.S. Smith, J. Landon, A.M. Sidki, Clin. Chem. 30 (1984) 1765.
[6] J.E. Wallace, L.R. Hall, S.C. Harris, J. Anal. Toxicol. 7 (1983) 178.
[7] A. Turcant, A. Premel-Cabic, A. Cailleux, P. Allain, J. Chrom. Biomed. Appl. 18 (1982) 222.
[8] D. Berry, J. Chromatogr. 86 (1973) 89.
[9] K. Sprague, A. Poklis, J. Can. Soc. Forensic Sci. 13 (1980) 31.
[10] P. Maugin, A.A. Lugnier, A.J. Chaumont, J. Anal. Toxicol. 11 (1987) 27.
[11] M. López Rivadulla, P. Fernández, P. Rodríguez, A.M. Bermejo, L. Concheiro, Anal. Lett. 21 (1988) 2253.
[12] P.P. Rop, J. Spinazzola, A. Zahra, M. Bresson, J. Quicke, A. Viola, J. Chrom. Biomed. Appl. 71 (1988) 172.
[13] M.D. Rukhadze, G.S. Bezarashvili, M.V. Sebiskveradze, V.R. Meyer, Anal. Chim. Acta 356 (1997) 19.
[14] J. Stephenson, Lab-2000 6 (1992) 76.
[15] A.D. Barbour, J. Anal. Toxicol. 15 (1991) 214.
[16] R.H. Liu, A.M. McKeehan, B.A. Cinnamon, G. Foster, W.D. Bensley, J.G. Laugner, et al., J. Forensic Sci. 39 (1994) 1504.
[17] J. Stephenson, U. Tillmanns, GIT Fachz. Lab. 36 (1992) 130.
[18] S.J. Mule, G.A. Casella, J. Anal. Toxicol. 13 (1989) 13.
[19] R. Pocci, V. Dixit, V.M. Dixit, J. Anal. Toxicol. 16 (1992) 45.
[20] W. Thormann, P. Meier, C. Marcolli, F. Binder, J. Chromatogr. 545 (1991) 445.
[21] P. Wernly, W. Thormann, Anal. Chem. 64 (1992) 2155.
[22] K.E. Ferslew, A.N. Hagardorn, W.F. McCormick, J. Forensic Sci. 40 (1995) 245.
[23] M.J. Medina-Hernández, M.C. García Alvarez-Coque, Analyst 17 (1992) 831.
[24] M.J. Koenigbauer, J. Chromatogr. 531 (1990) 79.
[25] M. Cuenca-Benito, S. Sagrado, R.M. Villanueva-Camañas, M.J. Medina-Hernández, J. Chromatog. A. (1998) (in press).
[26] E. Bonet Domingo, M.J. Medina-Hernández, M.C. García-Alvarez-Coque, Quim. Anal. 4 (1993) 167.
[27] J.R. Torres-Lapasió, R.M. Villanueva-Camañas, J.M. Sanchis-Mallols, M.J. Medina-Hernández, M.C. García Alvarez-Coque, J. Chromatogr. 639 (1993) 87.
[28] A.C.J.H. Drouen, H.A.H. Billiet, P.J. Schoenmakers, L. De Galan, Chromatographia 16 (1982) 48.
[29] L. Huber, LC-GC Intl. 11 (2) (1998) 96.