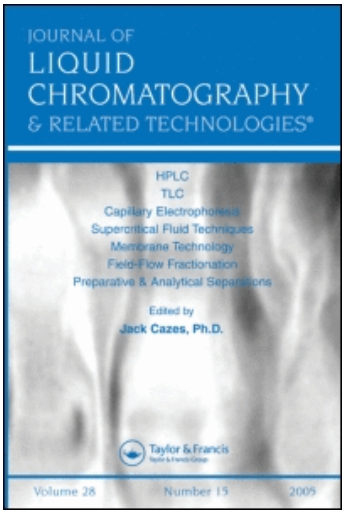

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A NEW VALIDATED METHOD FOR THE SIMULTANEOUS DETERMINATION OF A SERIES OF EIGHT BARBITURATES BY RP-HPLC

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□ *A new reversed-phase high performance liquid chromatographic (RP-HPLC) method is developed and validated for the simultaneous determination of barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone compounds in a single analytical run. The method uses a Phenosphere C_{18} (150 mm × 4.6 mm; 5 μm) column and isocratic elution. The mobile phase consisted of a mixture of methanol-water (50:50, v/v), pumped at a flow rate of 1.0 mL/min. The UV detection is set at 254 nm. The method is validated with respect to accuracy, precision (repeatability and intermediate precision), specificity, linearity, range robustness and stability of analytical solutions. All the parameters examined met the current recommendations for bioanalytical method validation. The method is specific, simple, selective and reliable for routine use in quality control analysis of barbiturates raw materials for final product release.*

Keywords allobarbitone, barbitone, cyclobarbitone, hexobarbitone, method development, method validation, methohexitone, pentobarbitone, phenobarbitone, reversed-phase liquid chromatography, secobarbitone

INTRODUCTION

Barbiturates are widely in use since the beginning of the century (barbital, 1903) especially as sedative hypnotics. With the advent of anxiolytic agents the popularity of barbiturates has suffered although they are still less costly. However, those with specialized properties such as the anticonvulsant phenobarbital continue to be commonly used.[1] In addition, abuse of barbiturates is now widespread. Due to the international nature of the illegal drug market forensic laboratories encounter

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a vast range of such compounds. Complications arise from the fact that abused barbiturates often occur as complex mixtures and other drugs and/or excipients are also present.[2] This necessitates the continued development of methods for their efficient separation and precise identification. In this work a group of eight barbiturates was selected as model mixtures (Figure 1). Barbitone (BR) is also known as 5,5-diethylbarbituric acid, colours crystals or white crystalline powder with melting point 188–192°C. It is seldom used in modern therapeutics. Allobabitone (AB) known as 5,5-diallybarbituric acid, a white crystalline powder, melting point about 173°C, dissociation constant at pka 7.8 at 25°C. Phenobarbitone (PhB) is also known as 5-ethyl-5-phenylbarbituric acid, white crystalline powder, and melting point 174–178°C, pka 7.4 at 25°C. Cyclobarbitone (CB) is known as 5-(cyclohex-1-enyl)-5-ethylbarbituric acid, melting point

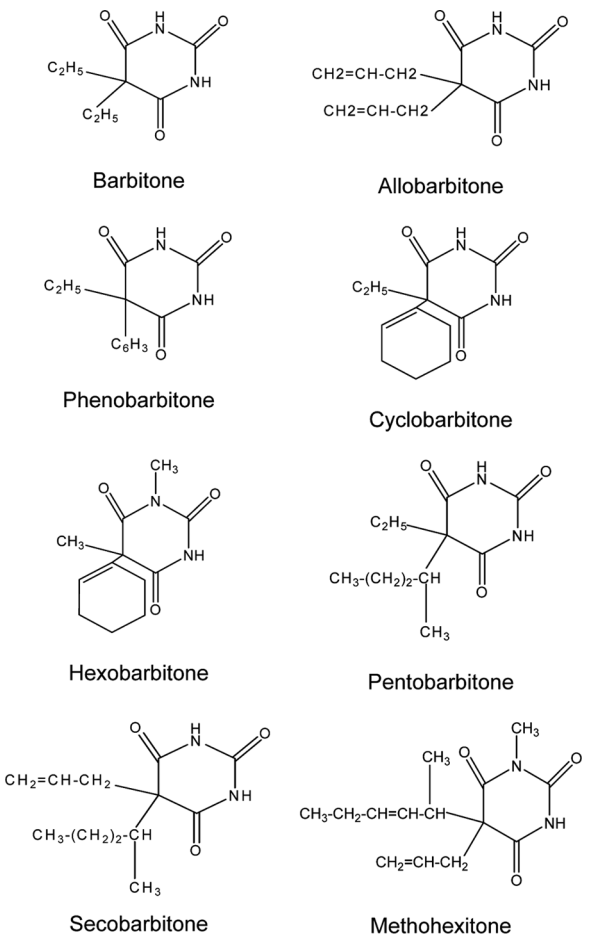

FIGURE 1 Chemical structures of the barbiturates used in this study.

171–175°C, pka 7.6 at 20°C. Hexobarbitone (HB) is known as 5-(cyclohex-1-enyl)-1,5-dimethyl barbituric acid, melting point 144–148°C, pka 8.2 at 20°C. Pentobarbitone (PB) is known as 5-(ethyl-5(1-methylbutyl) barbituric acid, melting point from 127–133°C. Secobarbitone (SB) is known as 5-allyl-5-(1-methylbutyl) barbituric acid, melting point about 100°C, very slightly soluble in water, freely soluble in ethanol and ether, also soluble in chloroform, pka 7.9 at 20°C. Methohexitone (MH) is known as ∝-(±)-5-allyl-1-methyl-5-(methypent-2-ynyl) barbituric acid. A white to faintly yellowish-white crystalline powder with melting point 92–96°C.

Some methods for the determination of some barbiturates have been reported, such as micellar liquid chromatography,[3,4] supercritical fluid chromatography,[5] gas chromatography-mass spectrometry (GC/MS),[6] thin-layer chromatography (TLC),[7,8] high-performance thin-layer chromatography (HPTLC),[9] Capillary Electrophoresis (CE),[10] high-performance liquid chromatography (HPLC),[11–14] and gas chromatography (GC),[15,16] but simultaneous determination of a series of eight barbiturate by reversed-phase HPLC and method validation has not been reported.

Furthermore, most of these procedures reported require labour sample pre-treatment and solvent extraction or solid phase extraction. These pre-treatment steps are time-consuming, increase the error sources and make the procedure more laborious.

Analytical methods validation is an important regulatory requirement in pharmaceutical analysis. In recent years, the International Conferences on Harmonization (ICH) has introduced guidelines for analytical methods validation[17] in Japan Europe and United States. The most widely applied analytical performance characteristics are accuracy, specificity, linearity, range, precision (repeatability and intermediate precision), stability of analytical solutions and robustness.

The purpose of this study was to develop and validate a rapid, accurate, simple and robust reversed-phase HPLC method for the simultaneous determination of a series of eight barbiturates (barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone) in a single chromatographic run which can be reliably used in routine quality control analysis for final drug substances release for medicinal formulations.

EXPERIMENTAL

Materials

All chemicals and reagents were of the highest purity. Methanol (HPLC-grade) and barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone were

purchased from Sigma (Gillingham, UK). Distilled water was de-ionised by using a Milli-Q system (Millipore, Bedford, MA).

HPLC Instrumentation and Conditions

A Knauer (Berlin, Germany) HPLC system equipped with a module 1000 LC pump, LC 3950 autosampler, LC 2600 photodiode-array (PDA) detector and a vacuum degasser. The data were acquired via Knauer ClarityChrom workstation data acquisition software. All chromatographic experiments were performed in the isocratic mode. The mobile phase consisted of a mixture of methanol-water (50:50, v/v). The flow rate was set to 1.0 mL/min. The injection volume was 10 µL and the detection wavelength was set at 254 nm. The chromatographic separation was carried out on a 150 mm × 4.6 mm, 5 µm C_{18} Phenosphere column obtained from Phenomenex (Macclesfield, UK).

Sample Preparation

An accurately weighted amount (0.08 g) of barbitone, (0.039 g) allobarbitone, (0.037 g) phenobarbitone, (0.07 g) cyclobarbitone, (0.048 g) hexobarbitone, (0.070 g) pentobarbitone, (0.076 g) secobarbitone and (0.07 g) methohexitone were placed in a 100 mL volumetric flask and dissolved in mobile phase (stock). A 5 mL aliquot of stock solution was diluted to 100 mL in a volumetric flask in mobile phase, yielding a final concentration of 400, 195, 185, 35, 24, 35, 38, and 35 µg/mL, respectively.

RESULTS AND DISCUSSION

Method Development

The chromatographic separation of barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone was carried out in the isocratic mode using a mixture of methanol-water (50:50, v/v) as mobile phase. The column was equilibrated with the mobile phase flowing at 1.0 mL/min for about 30 min prior to injection. The column temperature was ambient. 10 mL of standard solutions was injected automatically into the column. Subsequently, the liquid chromatographic behaviours of barbiturates were monitored with a PDA UV detector at 254 nm. Additionally, preliminary system suitability, precision, linearity, robustness and stability of solutions studies performed during the development of the method showed that the 10 µL injection volume was reproducible and the peak response was significant at the

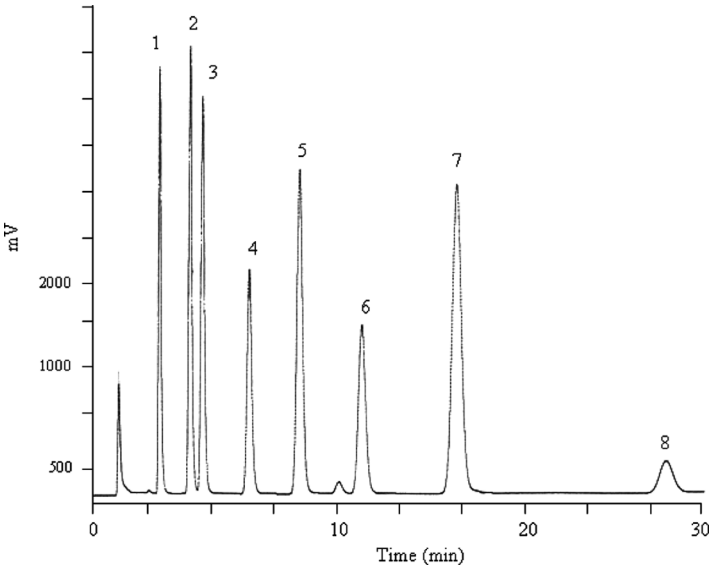

FIGURE 2 HPLC chromatogram of eight barbiturates: (1) barbitone, RT = 2.82, (2) allobarbitone, RT = 4.11, (3) phenobarbitone, RT = 4.65, (4) cyclobarbitone, RT = 6.68, (5) hexobarbitone, RT = 8.83, (6) pentobarbitone, RT = 11.52, (7) secobarbitone, RT = 15.58 and (8) methohexitone, RT = 24.63.

analytical concentration chosen. Chromatograms of the resulting solutions gave good separation and resolution (Figure 2). The analysis time for standards for all compounds was ca. 30 min.

System Suitability Test

System suitability test was developed for the routine application of the assay method. Prior to each analysis, the chromatographic system must satisfy suitability test requirements (resolution and repeatability). Peak-to-peak resolution, between each peak measured on a reference solution must be above 2. System suitability test was performed to determine the accuracy and precision of the system from six replicate injections of a solution containing 30 μg barbiturates/mL. All peaks were well resolved and the precision of injections for all preservative peaks were acceptable. The percent relative standard deviation (R.S.D.) of the peaks area responses were measured, giving an average between 0.9% and 0.36% ($n = 6$). The tailing factor (T), capacity factor (K′), theoretical plate number (N) and height equivalent to a theoretical plate (HETP) were also calculated. The results of system suitability in comparison with the required limits are shown in Table 1. The proposed method met these requirements within the accepted limits.[18,19]

TABLE 1 System Suitability Test Recommended Limits and Results of Eight Barbiturates

Parameters	Recommended Limits	Results of Barbiturates							
		1	2	3	4	5	6	7	8
Retention time (min)	–	2.82	4.11	4.65	6.68	8.83	11.52	15.58	24.63
Injection repeatability ($n=6$)	R.S.D. ≤ 1 (%, $n \geq 5$)	0.09	0.14	0.11	0.25	0.16	0.19	0.16	0.36
Resolution (Rs)	$R_s > 1.5$	–	2.56	2.06	4.06	4.30	5.38	8.12	18.10
Capacity factor (K′)	>2	2.65	2.87	3.37	5.27	7.30	9.83	13.64	22.14
Tailing factor (T)	≤2	0.625	0.750	0.750	1.000	0.887	0.875	0.875	1.000
HETP*	–	0.015	0.009	0.009	0.005	0.004	0.003	0.003	0.003
Theoretical plate (N)	>2000	2235	2491	2705	2852	3467	5023	5639	6737

*Height equivalent to a theoretical plate.

Robustness

For the determination of method robustness within a laboratory during method development a number of chromatographic parameters were evaluated, such as flow rate, column temperature, mobile phase composition, columns from different batches, and the quantitative influence of the variables were determined. For each parameter studied two injections of standard solutions were chromatographed. In all cases the influence of the parameters were found within a previously specified tolerance range. This shows that the method for determination of barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone was reproducible and robust.

METHOD VALIDATION

Linearity and Range

The linearity test was performed using five different amounts of barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, in the range 360–440, 155–230, 155–220, 5–65, 10–40 μg/mL, respectively and for pentobarbitone, secobarbitone and methohexitone 5–65 μg/mL. Solutions corresponding to each concentration level were injected in duplicate and linear regression analysis of the barbiturates peak area (y) versus barbiturates concentration (x) was calculated. The correlation coefficients ($r^2 = \geq 0.9995$) obtained for each barbiturate for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of barbiturates (Table 2).

TABLE 2 Linearity Assessment of the HPLC Method for the Assay of Barbiturates

Components	Concentration (μg/mL)[a]	Range (μg/mL)	Equation for regression line	r^2
Barbitone	400	360–440	$y = 31.08x - 10591$	0.9999
Allobarbitone	195	155–230	$y = 33.118x - 4630.3$	0.9996
Phenobarbitone	185	155–220	$y = 44.533x - 6488.2$	0.9998
Cyclobarbitone	35	5–65	$y = 44.18x + 63.3$	0.9995
Hexobarbitone	24	10–40	$y = 80.876x - 370.58$	0.9995
Pentobarbitone	35	5–65	$y = 44.76x + 76.8$	0.9997
Secobarbitone	38	5–65	$y = 44.087x + 73.167$	0.9998
Methohexitone	35	5–65	$y = 44.687x + 57.967$	0.9997

[a]Target concentration corresponding to 100%.

Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day variation). Repeatability was examined by analysing six determinations of the same batch of each component at 100% of the test concentration. The relative standard deviation (R.S.D.) of the areas of barbiturates peak were found to be less than 0.36% (Table 3), which confirms that the method is sufficiently precise.

Intermediate precision (inter-day variation) was studied by assaying five samples containing the nominal amount of barbiturates on different days. Solutions corresponding to each concentration level were injected in duplicate. The R.S.D. values across the system were calculated and found to be less than 0.45% (Table 3) for each of the multiple sample preparation, which demonstrates excellent precision for the method.

TABLE 3 Method Validation Results for Barbiturates

Validation Steps	Parameters	Results							
		1	2	3	4	5	6	7	8
Repeatability	R.S.D. (%, $n = 6$)	0.22	0.13	0.19	0.35	0.20	0.11	0.19	0.26
Int. precision									
Day 1	R.S.D. (%)	0.22	0.18	0.24	0.17	0.09	0.11	0.22	0.19
Day 2	R.S.D. (%)	0.27	0.22	0.18	0.13	0.14	0.39	0.27	0.45
Standard stability (24 h data)	Change in response factor (%)	0.10	0.09	0.13	0.15	0.11	0.14	0.12	0.13
System suitability	R.S.D. (%, $n = 6$)	0.18	0.18	0.23	0.16	0.18	0.32	0.22	0.29

TABLE 4 Recovery Studies of the HPLC Method for the Assay of Barbiturates

	Applied Concentration (% of Target) ($n=3$)		
Components	50 (%)	100 (%)	150 (%)
Barbitone	99.98 (±0.14)[a]	99.92 (±0.17)	99.88 (±0.27)
Allobarbitone	99.97 (±0.21)	100.00 (±0.32)	99.84 (±0.35)
Phenobarbitone	99.93 (±0.16)	99.92 (±0.27)	99.80 (±0.17)
Cyclobarbitone	99.92 (±0.17)	100.00 (±0.32)	100.00 (±0.15)
Hexobarbitone	99.82 (±0.37)	99.92 (±0.44)	99.85 (±0.17)
Pentobarbitone	99.98 (±0.23)	98.94 (±0.30)	99.72 (±0.25)
Secobarbitone	99.84 (±0.09)	99.97 (±0.27)	99.79 (±0.48)
Methohexitone	99.95 (±0.39)	100.00 (±0.21)	99.84 (±0.25)

[a]The coefficient of variation.

Accuracy/Recovery Study

Recovery studies may be performed in a variety of ways depending on the composition and properties of the sample matrix. In the present study, three different solutions were prepared with a known added amount of pure barbiturate compounds to give a concentration range of 50–150% of that in a test preparation. These solutions were injected in triplicate and percent recoveries of response factor (area/concentration) were calculated (Table 4).

Specificity and Selectivity

Injections of the blank were performed to demonstrate the absence of interference with the elution of the barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone barbiturates. These results demonstrate (Figure 3) that there was no interference from the other compounds and, therefore, confirm the specificity of the method.

Stability of Analytical Solutions

Sample solutions were chromatographed immediately after preparation and then re-assayed after storage at room temperature for 24 h. The results given in Table 3 showed there was no significant change (<0.16% response factor) in barbiturate concentrations over this period.

Measurement of Robustness

Analytical methods developed for use in quality control laboratories ideally are robust. Retention time for the analytes of interest will not

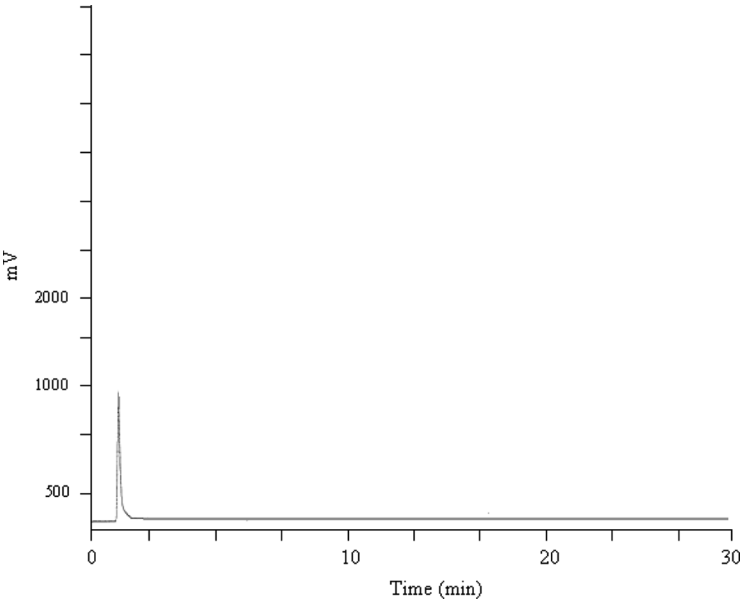

FIGURE 3 HPLC chromatogram of the blank run.

change significantly from day-to-day or from laboratory-to-laboratory if the method is considered robust. To determine the robustness of the chromatographic methodology developed for barbiturates, experimental conditions were purposely altered and chromatographic characteristics were evaluated. The effected temperature was also studied. Standard solutions were prepared and injected at early 20°C and again at 27°C. In all cases studied, the retention times of these compounds (barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone) were remains same 2.83, 4.12, 4.66, 6.68, 8.83, 11.51, 15.57 and 24.64 min, respectively (Figure 4). The coefficient of variation for retention time was lass then 1%. Good separation was always achieved, indicating that the analytical method remained selective for all components under the measured conditions.

System Suitability

A system suitability test was performed to determine the accuracy and precision of the system by injecting six replicate injections of barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone standard solutions. The R.S.D. of the peak areas responses was measured. The R.S.D. for barbiturates was less then (0.33%) as can be seen in Table 3.

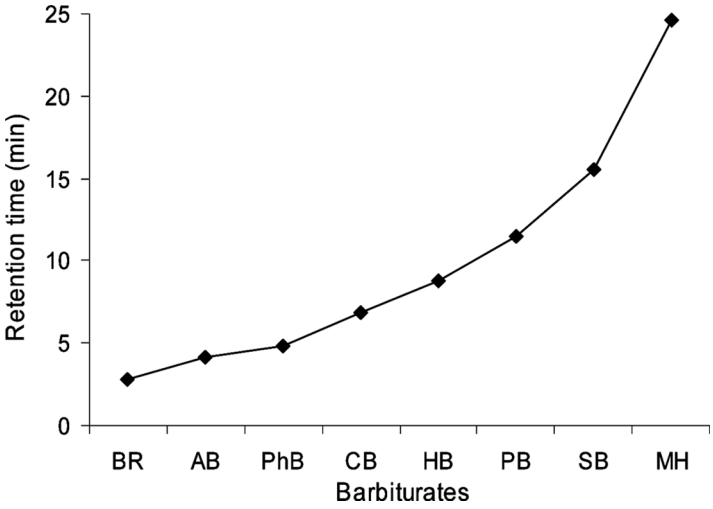

FIGURE 4 Retention times (min) of barbiturates: barbitone (BR), allobarbitone (AB), phenobarbitone (PhB), cyclobarbitone (CB), hexobarbitone (HB), pentobarbitone (PB), secobarbitone (SB), and methohexitone (MH).

CONCLUSION

A new RP-HPLC method with UV spectrophotometric detection was developed successfully for the simultaneous determination of a series of eight barbitone, allobarbitone, phenobarbitone, cyclobarbitone, hexobarbitone, pentobarbitone, secobarbitone and methohexitone compounds. The method was validated and the results obtained were accurate and precise with R.S.D. $< 1\%$ in all cases and no significant interfering peaks were detected. The method is specific, simple, selective and reliable for routine use in quality control analysis of barbiturates raw materials for final product release.

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