

## Validation of an analytical methodology by gas chromatography for the assay of barbiturates in blood samples

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### SUMMARY

An analytical methodology was developed for the quantitative assay of phenobarbital, pentobarbital and butobarbital in blood samples, using aprobarbital as an internal standard. After sample cleaning by liquid-liquid extraction, the separation was achieved by liquid gas chromatography using a capillary column in pulsed splitless mode, temperature ramp, and helium as carrier gas and flame ionization detector. The methodology was selective, linear, precise and accurate. The linearity was adequate, between 20 and 100 mcg/mL, for all of three barbiturates studied. The developed methodology was applied in two forensic cases presented in the Toxicology lab of the National Institute of Legal Medicine and Forensic Sciences in Bogotá.

**Key words:** Barbiturates, gas chromatography, forensic samples, validation, flame ionization detector.

### RESUMEN

## Validación de una metodología analítica para la determinación de barbitúricos en muestras de sangre por cromatografía gaseosa con detector de ionización de llama

Una metodología analítica fue desarrollada y validada para la determinación de butobarbital, pentobarbital y fenobarbital en muestras de sangre, utilizando aprobarbital como estándar interno. Para la identificación de los diferentes fármacos se llevó a cabo una extracción líquido-líquido con posterior separación cromatográfica empleando una columna capilar en modo de inyección pulsado sin división con rampa de temperatura, helio como gas de arrastre y detector de ionización de llama. La metodología validada fue selectiva, lineal, precisa y exacta. La linealidad fue evaluada

para concentraciones entre 20 y 100 mcg/mL para cada uno de los tres barbitúricos. La metodología desarrollada se aplicó en dos casos forenses presentados en el Laboratorio de Toxicología del Instituto Nacional de Medicina Legal y Ciencias Forenses en Bogotá.

**Palabras clave:** Barbitúricos, cromatografía gaseosa, muestras forenses, detector de ionización de llama, validación.

## INTRODUCTION

Numerous cases associated with intoxications from a great variety of substances arrive at the National Institute of Legal Medicine and Forensic Sciences (NILMFC). Some of those substances are benzodiazepines, illegal recreational drugs and barbiturates. Such intoxications are related to criminal acts such as theft and sexual crimes. This is the reason why the identification and quantification of these substances is important.

In the field of forensics, several preliminary or screening tests are used for identification of these substances. The most commonly used are the immunoenzymatic methods, which in spite of their high sensitivity can show a lack of selectivity and can therefore produce false positives. For this reason, and to confirm any preliminary positive result, chromatographic techniques are used.

In the determination of barbiturates, liquid chromatography (1-4) and gas chromatography (5-9) techniques are used. Wand *et al.* reported the use of micellar capillary electrophoresis for the analysis of two barbiturates, barbital and phenobarbital (10). In this research, the results of development and validation of a gas chromatographic method for the determination of butabarbital, pentobarbital and phenobarbital in blood samples are presented. Aprobarbital was used as internal standard. For the validation of methodology we used FDA's parameters and guidelines (11) as well as some recommendations made by other authors (12, 13).

## EXPERIMENTAL PHASE OF DEVELOPMENT

### Instrumentation and materials

Analysis was performed using Hewlett Packard gas chromatography (Mod. 6890, USA) equipped with a flame ionization and nitrogen/phosphorus detector, a split/splitless injector, a series automatic and GC/HP Chemstation Data System (Mod. 7683, USA). The GC column was a HP-1 capillary column, 30 m large, 0.32 mm i.d and 0.25  $\mu\text{m}$  film thickness. Also a vortex mixer, an evaporation system Rapid-Vap<sup>®</sup> (Labconco, USA) roto-mix tubes, a Depot centrifuge, a Galaxy analytical balance (Ohaus, Germany) and glass silanized were used.

## Reagents and standards

Methanol, n-hexane, dichloromethane, n-butyl chloride, chloroform, monosodium phosphate, disodium phosphate, acetic acid, sodium hydroxide (all analytical grade). Deionized water was used throughout all experiments. The standards butabarbital, phenobarbital, pentobarbital, aprobarbital, paracetamol, nicotinamide, caffeine, ibuprofen, and aspirin were purchased from Sigma Aldrich (USA).

## Specimens

Blank preserved blood fluid, which was used for the extraction procedures, development of the methodology and validation stage, was obtained from Cruz Roja Colombiana.

## Optimization of chromatographic parameters

For this development stage, two analytical procedures reported by Anderson y Fuller (6) and Ojanpera (7) for the identification and determination of acid and neutral drugs were revised and evaluated. The chromatographic conditions and parameters used in this work are shown in Table 1.

**Table 1.** Chromatographic parameters of the analytical procedures.

Parameter	Method-1	Method-2
Column	100 % polydimehtyl siloxane 30 m, d.i. 0.32 mm, f.t. 0.25µm	5 % diphenyl 95 % methyl siloxane 30 m, d.i. 0.25 mm, f.t. 0.25µm
Mode	pulsed split-less	split-less
Gas: helium	1 mL/min	0.7 mL/min
Makeup: nitrogen	25 mL/min	293 mL/min
Hydrogen	40 mL/min	30 mL/min
Air	450 mL/min	400 mL/min
Injector temperature	250 °C	270 °C
Detector temperature	300 °C	290 °C
Oven temperature	Initial T : 80°C x 1 min	Initial T- : 70°C x 0.7 min
Oven Ramp	10°C/min to 150°C 5°C/min to 280°C (final)	20°C/min to 130°C 10°C/min to 280°C (final)

After the corresponding trials, Method 1 was selected for the subsequent validation stage considering resolution of the different substances peaks signs and the internal standard, as well as the reproducibility of selection criteria. The internal standard, aprobarbital, was used in concentration of 60 µg/mL. Table 2 shows the results for the GC system suitability parameters related to the first method.

**Table 2.** Chromatographic parameters for system suitability test.

Drug	$t_r$ (min)	Capacity factor	Number Plate N	Resolution R	Tailing factor
Aprobarbital	9,54	4,10	308252	15,44	2,32
Phenobarbital	12,89	5,89	1406672	5,73	2,41
Pentobarbital	10,79	4,77	290574	10,30	1,37
Butabarbital	9,96	4,33	252408	5,77	1,32

The obtained values for capacity factor, number plates, resolution and tailing factor for all drugs were according to pharmacopoeia requirements (14).

### SAMPLE EXTRACTION AND PURIFICATION STANDARDIZATION

In accordance with the reviewed literature, there are two keys for the best adequacy and extraction of barbiturates in biological fluids, namely, pH of the buffer and organic solvent used (5-9). For this purpose, a 2<sup>2</sup> factorial design was used to determine the best combination of these variables. The independent variable was the amount of drug extracted and the working pH values were 4.5 and 5.6. For the solvent categorical levels, butyl chloride and heptane -dichloromethane mixture (50 -50) were used. The factorial designs are shown in Tables 3 and 4.

**Table 3.** Treatments table for factorial design.

Variations	Treatment
pH 5,6 Heptane – dichloromethane	1
pH 4,5 Heptane – dichloromethane	2
pH 5,6 Butyl chloride	3
pH 4,5 Butyl chloride	4

The computed recovery differences between treatments were statistically significant (Scheffe's test,  $p = 0,05$ ). As a result of these statistical analyses, the combination of a pH 4.5 buffer and the 50/50 heptane-dichloromethane mixture was selected as the best treatment.

With this treatment, a chromatogram with fewer interfering signals was found, improving the selectivity of the methodology.

In summary, the extraction procedure established was: to 2 mL of the blood sample 0,1 mL of the internal standard solution, 3 mL of the pH 4,5 buffer and 10 mL of

extraction solvent were added. The mix was placed in the rotomixer for 15 minutes, centrifuged at 2000 rpm for 20 minutes and the organic phase removed afterwards by evaporation at 50 °C under nitrogen atmosphere. The residue was dissolved in 100 µL of methanol and 2 µL injected into GC/FID system.

**Table 4.** Percentage of recovery of barbiturates for treatments.

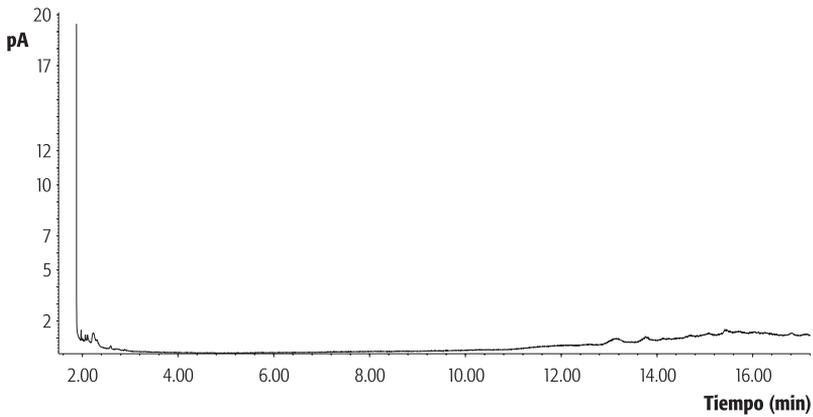
pH 5,6 Heptane - dichloromethane (50:50)			
Aprobarbital	Phenobarbital	Pentobarbital	Butabarbital
47,4 (0,57)*	65,0 (3,21)*	60,9 (2,38)*	59,7 (1,27)*
pH 4,5 Heptane - dichloromethane (50:50)			
Aprobarbital	Phenobarbital	Pentobarbital	Butabarbital
81,7 (4,62)*	87,1 (2,39)*	85,9 (3,84)*	83,9 (3,84)*
pH 5,6 Butyl chloride			
Aprobarbital	Phenobarbital	Pentobarbital	Butabarbital
38,3(3,05)*	56,8 (1,50)*	40,3 (3,14)*	48,3 (0,33)*
pH 4,5 Butyl chloride			
Aprobarbital	Phenobarbital	Pentobarbital	Butabarbital
38,4 (1,86)*	58,9 (2,34)*	41,1 (0,98)*	53,7 (3,01)*

\*Standard deviation of four determinations for each treatment.

## SAMPLE STABILITY

In accordance with the FDA guidelines (11) it is necessary to know the stability of the substances under study in the different biological matrixes selected and under different conditions. This stability depends on the nature of the substance, the type of matrix, the storage conditions, and also on the container of the matrix. The stability of substances in the selected matrix was experimentally evaluated under the freezing cycles (at -20°C) between 12 and 24 hours and thawing it at room temperature, which was repeated three times. The short-term stability was analyzed, using a 4-hour period at room temperature; the storage temperature of -20°C. The long term stability for freezing was carried out at -20°C during the period between the preparation of the first sample and the date of the last analysis, as well as in the auto-sampler tray.

The chromatogram of the blank blood sample obtained after storage at -20°C during 2 months, showed change when compared to the chromatogram obtained at the beginning of the study (Figure 1).



**Figure 1.** GC chromatogram of the blank blood sample.

Table 5 shows the results of the study expressed as a percentage of the initial concentration.

**Table 5.** Stability of substances under the experimental storage conditions adopted in this work.

Assay		Butabarbital %	Pentobarbital %	Phenobarbital %
Stock solution		98,3	99,4	94,2
Freeze and thaw stability	C <sub>1</sub>	93,4	81,1	66,3
	C <sub>2</sub>	94,2	84,2	73,8
	C <sub>3</sub>	94,3	85,7	79,4
Short-Term temperature stability	C <sub>1</sub>	89,9	86,2	80,2
	C <sub>2</sub>	93,5	89,4	80,9
	C <sub>3</sub>	96,3	91,5	86,2
Long term stability	C <sub>1</sub>	94,0	92,3	89,9
	C <sub>2</sub>	92,4	93,8	92,4
	C <sub>3</sub>	95,7	91,7	93,8
Post preparative stability (Auto sampler)	t <sub>1</sub>	96,3	97,9	92,4
	t <sub>2</sub>	97,4	92,4	89,9
	t <sub>3</sub>	99,2	95,3	81,2

C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>: 20, 60 y 100 mcg/mL. t<sub>1</sub>, t<sub>2</sub>, t<sub>3</sub>: 6, 12 y 18 hours.

We can observe a slight decrease in the concentration of the standard solution of barbiturates except in the Phenobarbital, the concentration of which is reduced by 5,8%. The internal standard, aprobarbital, showed a 0,4% decrease. We can also observe a reduction in the concentration of barbiturates facing the freezing and thawing cycles,

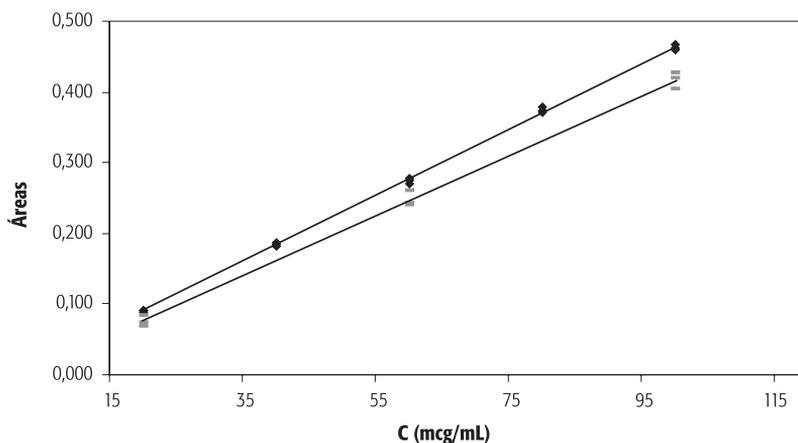
phenobarbital again being the most affected. This last evidence, but in different proportions, is repeated for the study at room temperature prior to the  $-20^{\circ}\text{C}$  storage. In the long-term study and for the three concentration levels, the instability is less marked than in the previous cases. As for the stability of the drugs in the auto-sampler of the descend equipment, the decrease of concentration became significant after 18 hours, mainly in the case of phenobarbital, an important fact which should be taken into account during the analysis of serial samples that remain on the auto-sampler tray for some time. Generally speaking, all of the stability studies carried out show that some necessary precautions must be taken for the conservation of samples in order to establish the best appropriate times for the analysis.

## VALIDATION STAGE

**Selectivity.** Figure 1 shows no signals of endogen interferences that may come from blank blood samples.

Likewise, and according to the FDA's guidelines (11), effectiveness of the chromatographic system was verified against the use of some commonly used drugs, such as ibuprofen, nicotine, caffeine and aspirin, without finding any interferences or overlapping with the chromatographic signs produced by the barbiturates under study. Retention times for such substances were 5,1, 8,3, 11,5 and 14,6 minutes respectively.

**Calibration standard curve.** In order to determine the proportionality of the answers as a function of the concentration for each one of the drugs, standard solutions for the system linearity and spiked blank blood samples for the method linearity were studied. Five and three concentration levels for the system and method were used respectively. Figure 2 shows the regression curve for phenobarbital, system and method.



**Figure 2.** Curve obtained for the linearity test of phenobarbital: 1. System (standards) 2. Method (spiked samples).

The intercept and slope as well as the experimental t values for student test, system and method, are shown in Table 6.

**Table 6.** t Values for intercept and slope, absolutes areas.

Drug	Parameter	Parameter Value		t exp	
		System <sup>1</sup>	Blood <sup>2</sup>	System <sup>1</sup>	Blood <sup>2</sup>
Butabarbital	Intercept	0,003	0,013	1,093	1,039
	Slope	0,016	0,014	416,69	76,97
Pentobarbital	Intercept	-0,020	-0,019	-1,336	-1,064
	Slope	0,019	0,018	84,73	67,41
Phenobarbital	Intercept	-0,003	-0,010	-1,393	-1,496
	Slope	0,005	0,004	135,05	42,59

<sup>1</sup> n = 15, t tab (13, 0.05): 2.16, <sup>2</sup> n = 9, t tab (7, 0.05): 2.37.

Because in all cases the experimental t-values for the intercepts were lower than the value of t in t-Student the intercept values were not significantly different from zero. Related to the slopes, they are significantly different from zero (t experimental value is higher than the critical value). Table 7 shows the results of ANOVA test for the linear regression.

**Table 7.** ANOVA of linear regression and lack-of-fit test.

Drug	F <sub>exp</sub> regression		F <sub>exp</sub> lack of fit	
	System	Blood	System	Blood
Butabarbital	173629,85	5923,57	0,658	0,363
Pentobarbital	7179,50	4543,39	1,854	1,412
Phenobarbital	18239,00	1813,66	3,073	0,040

<sup>1</sup> F<sub>tab</sub> regression: system F<sub>(1/10, 0.05)</sub>: 4.96, method (1/6, 0.05): 3.71.

<sup>2</sup> F<sub>tab</sub> lack of fit: system F<sub>(1/10, 0.05)</sub>: 4.96, method (1/6, 0.05): 3.71.

For the system, F's experimental values show significant regressions for the three drugs and non-significant linearity deviations. Similar results were found for the method.

**Minimum detectable and quantifiable concentration.** For estimate the detection limit (LOD) and the quantification limit (LOQ) the intercept and the slope of the calibration curve were used (11). The detection and quantification limits found are shown in Table 8.

**Table 8.** LOD and LOQ values for barbiturates<sup>1</sup>.

Drug	LOD (mcg/mL)	LOQ (mcg/mL)
Butabarbital	2,77	9,22
Pentobarbital	3,24	10,79
Phenobarbital	6,97	23,26

<sup>1</sup> LOD: 3a/b, LOQ: 10a/b.

**Precision.** To evaluate the repeatability and intermediate precision of the method, three concentration levels of each drug were used: 20, 60 y 100 mcg/mL. Intermediate precision was evaluated between days and between analysts. Six and three determinations were carried out for repeatability and intermediate precision, respectively. Table 9 reports the coefficients of variation (RSD) for repeatability, as well as the coefficients for each one of two analysts during the three days of assay. The global values found in all the cases were lower than 15%, values established as the maximum permissible value for bio-analytical methodologies (11).

**Table 9.** Repeatability and Intermediate precision of methodology (RSD %).

Drug	Concentration (ug/ml)	Repeatability RSD%	Analyst 1 RSD%	Analyst 2 RSD%
Butabarbital	20	10,11	8,97	10,10
	60	4,51	5,62	5,12
	100	4,45	4,13	4,49
Pentobarbital	20	7,38	7,98	8,85
	60	5,64	5,21	5,25
	100	4,51	3,75	2,77
Phenobarbital	20	12,56	11,39	11,47
	60	8,99	11,37	12,13
	100	3,88	4,36	4,82

Likewise, Table 10 shows the results for the ANOVA test for intermediate precision, using the 20 mcg/mL concentration data.

**Table 10.** Intermediate precision and analysis of variance.

Source	df	F exp			Ftab <sup>1</sup>
		Butabarbital	Pentobarbital	Phenobarbital	
Analyst	1	0,141	0,141	0,181	4,75
Day	2	0,908	0,908	0,594	3,89
Assays	2	0,052	0,052	0,480	3,89

<sup>1</sup> Analysts  $F_{(1/12, 0,05)}$ : 4,75, days / assays (2/12, 0,05): 3,89.

In accordance with the results for each one of the three barbiturates, experimental F-values are lower than tabulated F-values, showing that there are not significant differences between analysts, days and determinations.

**Accuracy.** This parameter was evaluated through the recovery percentage, replacing the Y data or the average answer values in the system regression equation obtained for each drug. The value of the experimental concentration was compared to the real concentration added to the matrix, obtaining the percentage of recovery for each drug and for each concentration level (Table 11).

**Table 11.** Accuracy of methodology through the percentage of recovery.

Drugs	C real	Y exp blood	C exp	% Recovery
Butabarbital	20	0,285	18,23	91,17
	60	0,844	54,26	90,43
	100	1,386	89,27	89,27
Pentobarbital	20	0,333	18,47	92,36
	60	1,073	57,24	95,40
	100	1,771	93,76	93,76
Phenobarbital	20	0,075	16,57	82,84
	60	0,247	53,23	88,72
	100	0,416	89,26	89,26

For each one of the barbiturates the recovery percentage is around 80%, which is a value higher than the minimum recommended by the la FDA (11) for bio-analytical methodologies (60%).

**Robustness.** The variables selected for this study were modified in order to observe the influence of these changes in the answer. In Table 12, the robustness parameters or variables studied are reported.

**Table 12.** Robustness parameters.

Parameters	Standardized Factor	Alternative Factor
Equipment	Chromatograph HP 6890	Chromatograph HP 5890 Series II
Column	HP-1 100% polydimehtyl siloxane	HP-5, 5% phenyl -95% polydimehtyl siloxane
Flow	1 mL/min	1,4 mL/min
Liner	Activate	Deactivate
Silanized material	Yes	No
Reconstitution time	Immediate	12 hours after
Injection time	Immediate	4 hours after

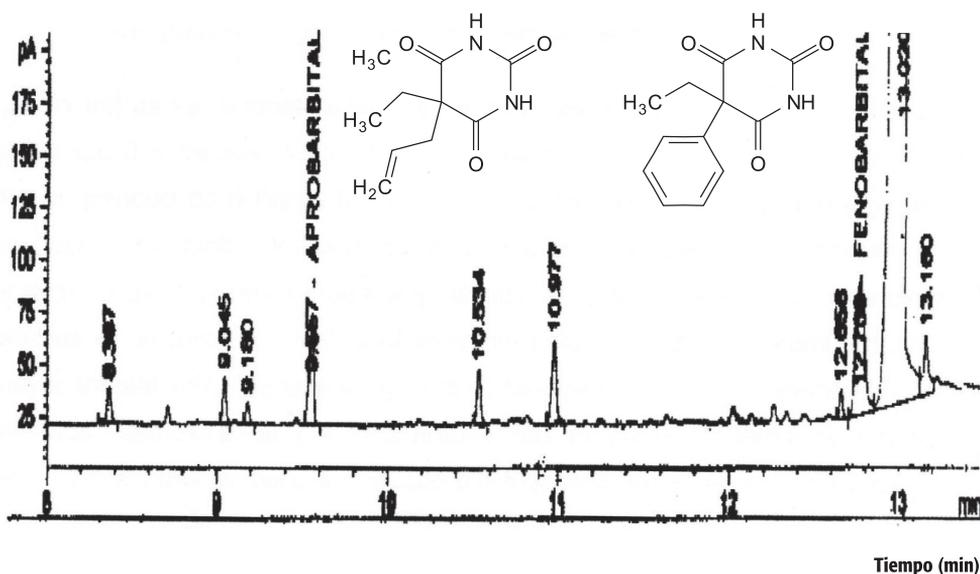
For the three barbiturates, according to Youden's method reported by Quattrochi and Laba (15) the critical factors that have to be carefully controlled during the analysis are: equipment, column, liner, material and redissolution time of the dry extract.

**APPLICATION.** To determine the usefulness of the validated methodology, we carried out a preliminary analysis of ten forensic samples that could contain some of the barbiturates under study. Of them, two showed positive results for phenobarbital with concentrations of 21 and 15,7 mcg/mL respectively. One of them showed toxic concentration (see Table 13). Phenobarbital identity was also confirmed with a second validated HPLC method. Figure 3 shows the chromatogram obtained with one of the two forensic samples.

**Table 13.** Therapeutic and toxic barbiturates concentration.

Drugs	Therapeutic concentration (mg/L)	Toxic concentration (mg/L)
Butobarbital	1 - 10	10 - 25
Pentobarbital	1 - 10	8 - 12
Phenobarbital	1 - 10	17 - 90

Plasma concentrations (16).



**Figure 3.** Blood forensic sample chromatogram.

## CONCLUSIONS

An analytical method was developed and validated for the identification and quantification of butobarbital, pentobarbital and phenobarbital in blood samples by gas chromatography with flame ionization detector and pulsed splitless. The validated bioanalytical methodology is specific, linear, precise and accurate between 20 and 100 mcg/mL for phenobarbital, pentobarbital and butobarbital. The stability of the drugs used was rigorously evaluated, mainly for samples stored at room temperature. The validated methodology, as it was demonstrated, proved useful in establishing the presence of studied barbiturates and for their quantitative determination in blood forensic samples.

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