Use of a Statistically Designed Experimental Approach to Optimize the Propylketal Derivatization of Barbiturates

Mark M. Kushnir*,[1] and **Francis M. Urry**[2]

[1]ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT 84108 and [2]Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84112

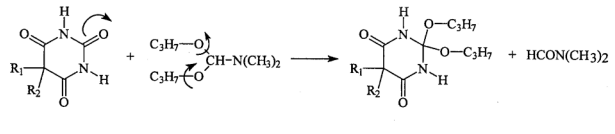
Abstract

The derivatization of barbiturates with dimethylformamide dipropylacetal and dimethylformamide diisopropylacetal is studied with respect to the optimization of reaction recovery and reliability. A second-order orthogonal experimental design is utilized in order to obtain regression equations for the reaction recovery dependence on the derivatization solution composition, incubation temperature, and time for amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital. Regression equations for the effect of incubation temperature and time on the derivative recovery and the optimum conditions for derivatization recoveries are obtained. Differences in the phenomena of the derivative formation are evaluated between the two derivatizing reagents and the barbiturates. Based on the analysis of the obtained equations, it is concluded that the dipropylketal derivative of barbiturates is superior in comparison with diisopropylketal when considering the milder conditions of the reaction, absence of sudden changes in the recovery with a variation in the derivatization parameters, and reliability for the simultaneous testing of the barbiturates. A method for the routine testing of the barbiturates by gas chromatography–mass spectrometry in urine specimens is included.

Introduction

A common problem for high-volume laboratories is the compatibility of different assays that are combined on the same gas chromatograph (GC). The assays for the underivatized barbiturate drug class are not compatible with other assays on nonpolar capillary GC columns. Barbiturate derivatization is an effective way to overcome the problem. Published methods for barbiturate analysis typically involve alkylation and ketal formation. Several reviews on the fundamentals of derivatization chemistry for barbiturates in chromatographic analysis have been published (1–4). Alkylation can be accomplished with dimethyl sulfate (5), diazomethane (6), quaternary ammonium reagents (7), and methyl iodide (8). A common alkylation method involves the pyrolysis of barbiturate quaternary amine salts during injection into the instrument (9). The disadvantages of the method are a narrow linear range, a harmful action of the reagents and reaction by products on the GC column, and the presence of more than one derivatization product for each barbiturate. Derivatization by alkyl halide (8,10) requires the use of quaternary ammonium hydroxide as the catalyst. This reaction also produces by-products harmful to the GC column that eventually lead to column deterioration. Some improvement in method performance can be achieved by using cleanup extraction after alkyl iodide derivatization (10). Another promising group of reagents for barbiturate derivatization are the dimethylformamide dialkyl acetal compounds. The reagents have been commonly used for on-column derivatization (11) and it was assumed that the product of the reaction was *N*-alkyl barbiturate, but Venturella (12) showed that the derivative was the barbiturate dialkylketal. The proposed mechanism of the reaction is presented in Figure 1. The derivative formation takes place on carbon 2 because of its polarization, which is promoted by the electron-donor properties of two alkyl groups found in the para position and its location between two electronegative nitrogen atoms in the ortho positions, respectively.

Because derivatization recovery and products depend on the reaction conditions, optimization of the reaction parameters is critical for the adequate performance and reliability of the analysis. The objectives of this study were to find the optimal condi-



Barbiturate	R_1	R_2
Amobarbital	C_2H_5–	$(CH_3)_2CH(CH_2)_2$–
Butalbital	CH_2=$CHCH_2$–	$(CH_3)_2CHCH_2$–
Pentobarbital	C_2H_5–	$CH_3(CH_2)_3(CH_3)CH$–
Phenobarbital	C_2H_5–	Phenyl–
Secobarbital	CH_2=$CHCH_2$–	$CH_3(CH_2)_2(CH_3)CH$–

Figure 1. Reaction of barbiturate dipropylketal derivative formation and structures of the barbiturate derivatives.

* Author to whom correspondence should be addressed.

tions for the ketal derivative formation of butalbital, amobarbital, pentobarbital, secobarbital, and phenobarbital and to evaluate the difference between two derivatizing reagents—dimethylformamide dipropylacetal (DMFDPA) and dimethylformamide diisopropylacetal (DMFDIPA).

Experimental

Chemicals, equipment, and supplies

Hexane, ethyl acetate, isopropanol, chloroform (all OPTIMA grade), and dimethyl sulfoxide (certified ACS grade) were obtained from Fisher Scientific (Pittsburgh, PA). The derivatizing reagents DMFDPA and DMFDIPA were purchased from Aldrich (Milwaukee, WI).

All other chemicals were of analytical grade. An extraction solvent was prepared by mixing hexane with ethyl acetate to a ratio of 19:1. Disposable borosilicate glass culture tubes (16 × 100 mm) with Teflon-lined plastic closures from Fisher Scientific were used for sample extraction and derivatization. Derivatization experiments were carried out using a dry block heater (Pierce, Rockford, IL). Miscellaneous supplies included a shaker, vortex mixer, Drummond pipet set, an adjustable pipet with disposable pipet tips, and glass autosampler vials with crimp caps.

Standards

Amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital were obtained from Sigma (St. Louis, MO). Pentobarbital d_5 and phenobarbital d_5 were purchased from Radian International LLC (Austin, TX) for use as internal standards. A combined barbiturate standard containing amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital was prepared in methanol at a concentration of 12 ng/µL.

Internal standard

Analytical method

The internal standard for the method precision study contained pentobarbital d_5 and phenobarbital d_5 in methanol at a concentration of 5 ng/µL each.

Recovery experiments

The dipropylketal (DPK) derivative of pentobarbital d_5 was used as the internal standard for the derivatization experiment. The internal standard was prepared by aliquoting 1 mL of the 5-ng/µL pentobarbital d_5 methanolic solution into an empty tube. The methanol was evaporated at room temperature, 75 µL of dimethyl sulfoxide (DMSO) and 125 µL DMFDPA were added, and the tube was vortexed and then incubated in a heating block at 120°C for 30 min. The DPK pentobarbital d_5 derivative was extracted from the solution (as will be described) and reconstituted in 1 mL of ethyl acetate.

GC–MS analysis

Instrumental analysis was conducted using a Hewlett-Packard (HP) (Palo Alto, CA) 5890 GC equipped with an HP 5970 mass-selective detector when operating in the electron-ionization mode. The GC was equipped with a 7673 A autosampler and a DB-5ms capillary column (12.5-m × 0.25-mm i.d., 0.25-µm film thickness) (J&W Scientific, Folsom, CA). The injection-port temperature was 240°C. The column temperature was held at 120°C

Table I. Experimental Matrix and Derivatization Recovery for Butalbital, Amobarbital, Pentobarbital, Secobarbital, and Phenobarbital DIPK and DPK Derivatives.

				DIPK derivative (%recovery)					DPK derivative (%recovery)				
Experiment	x_1	x_2	x_3	Buta*	Amo†	Pento‡	Seco§	Pheno**	Buta	Amo	Pento	Seco	Pheno
1	–1	+1	+1	87	86	87	80	85	89	90	91	87	90
2	+1	+1	+1	81	75	81	70	71	76	71	77	70	70
3	–1	–1	+1	62	64	62	63	63	10	12	13	12	8
4	+1	–1	+1	34	37	34	38	33	3	3	4	3	2
5	–1	+1	–1	94	74	94	91	72	40	39	45	40	29
6	+1	+1	–1	62	53	62	61	35	9	10	12	11	6
7	–1	–1	–1	13	15	13	14	7	2	2	2	2	2
8	+1	–1	–1	2	2	2	2	2	1	0	1	1	0
9	0	0	0	75	59	72	77	63	14	15	12	18	10
10	0	0	0	65	65	66	92	64	10	17	20	13	21
11	0	0	0	80	68	78	81	71	24	23	24	25	16
12	1.35	0	0	66	66	63	70	61	18	16	20	19	12
13	–1.35	0	0	82	79	82	81	85	52	50	39	56	38
14	0	1.35	0	81	77	81	82	81	89	88	89	89	8
15	0	–1.35	0	4	4	4	5	2	2	2	2	2	1
16	0	0	1.35	77	63	77	74	61	60	46	64	64	40
17	0	0	–1.35	21	28	21	24	13	2	2	2	2	1

* Buta, butalbital.
† Amo, amobarbital.
‡ Pento, pentobarbital.
§ Seco, secobarbital.
** Pheno, phenobarbital.

for 1 min, ramped at 20°C/min to 200°C, held for 1 min, ramped at 40°C/min to 280°C, and then finally held for 1 min. The transfer-line temperature was held at 280°C. Helium with a linear velocity of 50 cm/s was used as the carrier gas. The injection volume was 1 µL. The ion fragments monitored for the ketal derivatives of barbiturate were *m/z* 252 and 210 for butalbital, *m/z* 240 and 198 for amobarbital, *m/z* 240 and 198 for pentobarbital, *m/z* 245 and 203 for pentobarbital d_5, *m/z* 252 and 210 for secobarbital, *m/z* 288 and 246 for phenobarbital, and *m/z* 293 and 251 for phenobarbital d_5.

Sample preparation

Analytical method

To 2 mL of urine, 100 µL of the combined internal standard, 2 mL of a 0.3M NaH_2PO_4 buffer (pH 5.0), and 5 mL chloroform were added. The tubes were shaken for 10 min and centrifuged at 2500 rpm for 5 min, and then the aqueous solution was aspirated and discarded. The organic phase was transferred to a new set of tubes and evaporated to dryness under nitrogen. The residues were reconstituted with 75 µL of DMSO and 125 µL of the derivatizing reagent, and the tubes were capped. The tubes were incubated in a heating block at 120°C for 30 min to obtain DPK derivatives and at 150°C for 25 min to obtain diisopropylketal (DIPK) derivatives. After the derivatization, 1 mL of a 0.10M phosphate buffer (pH 8.3) and 5 mL of a hexane–ethyl acetate mixture were added to each tube. The tubes were then shaken for 10 min and centrifuged for 5 min. The organic phase was transferred to new tubes, evaporated under a stream of nitrogen at room temperature, and the residues were reconstituted with 200 µL of ethyl acetate.

Recovery experiments

The experiments were performed in order to study the reaction recovery of DPK and DIPK derivatives of barbiturates using the derivatizing reagents DMFDPA and DMFDIPA. The ratio of the DMSO and derivatizing reagent (v/v), incubation temperature, and time used in the experiments are listed in an experimental matrix in a coded form (Table I). The derivative cleanup extraction was similar to that described by Barbour (13). A 200-µL volume of the combined barbiturate standard was aliquoted into each tube, and the methanol was evaporated at room temperature. DMSO and the derivatizing reagent were added to the tubes, and then the tubes were vortexed and placed into a heating block. In order to stop the reaction, the tubes were immersed into an isopropanol/dry ice bath for 30 s. After the derivatization, 50 µL of the pentobarbital d_5 DPK derivative (internal standard), 1 mL of the phosphate buffer, and 5 mL of the hexane–ethyl acetate mixture were added to each tube. The tubes were shaken for 10 min and centrifuged for 5 min. The organic phase was transferred to new tubes and evaporated at room temperature under a stream of nitrogen. The residues were reconstituted with 200 µL of ethyl acetate, transferred into autosampler vials, and injected with 1 µL aliquot into the GC–MS. The recovery of the derivative was calculated as the ratio of the amount of derivatized barbiturate versus the amount of barbiturate initially aliquoted into a tube (2400 ng each).

Table II. Variation Ranges for the Experimental Matrix

Parameter	z_1	z_2		z_3
		DMFDPA	DMFDIPA	
Zero level	1:1	140	115	15
Variation step	0.5	15	15	8
+1.35	1.68:1	160	136	26
+1	1.5:1	155	130	23
–1	1:1.5	125	100	7
–1.35	1:1.68	120	94	4

Experimental design for recovery study

The parameters most significantly affecting the recovery of the derivatization reaction included in this study and the variation ranges for each parameter (Table II) were chosen based on preliminary experiments. The evaluated parameters were the derivatization solution composition (z_1), incubation temperature (z_2), and time (z_3). A second-order orthogonal experimental design (14–16) with parameters varied on three levels was used to determine a mathematical approximation for the reaction recovery as a function of each of the three parameters. The variables (z_i) were coded to a uniform scale with the formula:

$$x_i = (z_i - z_i^0) / \varnothing z_i \qquad \text{Eq. 1}$$

where x_i is the coded value of a variable, z_i is the real value of a variable, z_i^0 is the zero level of a variable, and $\varnothing z_i$ is the range of a variation.

Seventeen experiments were carried out in duplicate, and derivatization recovery (R) was determined for each of the barbiturates (Table I). Preferable conditions were those that resulted in a maximum conversion of a barbiturate to its derivative. The recovery values that were obtained in the experiments were used to calculate a second-order polynomial for recovery as a function of the reaction parameters:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{23}x_2x_3 + b_{13}x_1x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \qquad \text{Eq. 2}$$

where x_1 is the ratio of the DMSO/derivatizing reagent, x_2 is the incubation temperature, and x_3 is the incubation time.

The polynomials facilitated the calculation of the recovery at any conditions within the experimental area. The polynomials were reduced to a canonical form (15,16) in order to obtain visual interpretation of the results and determine the nature of the obtained recovery surfaces. The canonical form of a polynomial was:

$$R - R_s = B_{11}X_1^2 + B_{22}X_2^2 \qquad \text{Eq. 3}$$

where B_{11} and B_{22} are the coefficients of the equation, R_s is the recovery in the stationary point, and X_1 and X_2 are the reaction parameters.

The transformation to the canonical form was performed in three steps: (*a*) finding the coordinate of a stationary point; (*b*) transferring an origin of the coordinate to the stationary point; and (*c*) rotating the axes in order to make them parallel with the

axes of the response surface (15). The stationary point coordinates were found by taking partial derivatives ($\delta R/\delta x_i = 0$) and solving the system of equations. Transferring the origin of the coordinate system to a stationary point and rotating the axes of the system were performed by the methods of linear algebra.

A surface type represented by an equation depends on the signs at the equation coefficients. Depending on the signs, the surface can be an elliptic paraboloid with minimum or maximum in the center, a hyperbolic paraboloid with the saddle point in the center, or a stationary ridge (15). The curvature of a surface is determined by the absolute values of the coefficients B_{11} and B_{22}. The surface is steeper in the direction of a variable with the greater value. A free term in the equation (R_s) represents the response in the surface stationary point. Process stability depends on a type of surface and its slope. At low values of the coefficients, output does not change significantly with a change in the parameter. At the greater values, even a small change in a parameter produces a significant change in the recovery.

Results and Discussion

The coefficients of the second-order polynomials (Eq. 3) for the recovery dependence on the parameters were calculated for each barbiturate. A Student t-test was used to provide the basis for a decision on the significance of the coefficients of the polynomials. The values of the Student t-test are given in Table III. All the variables with t-test values less than the reference number ($t_{0.10(2)}$ = 2.92) were eliminated from the equations. The polynomials for the recovery dependence on the experimental conditions for DPK and DIPK derivatives are presented in Table IV. The polynomials were assessed statistically with a Fisher test. The Fisher test values for all the equations (Table V) were less than the reference number ($F_{0.10(10,2)}$ = 99.4) and indicate the validity of the equations.

An analysis of the regression equations showed that the terms at the linear effects for both derivatizing reagents had similar values and signs. This represented the similarity in the parameter

Table III. Student t-test Values for the Coefficients of the Regression Equations

	DIPK derivative					DPK derivative				
Parameter	Buta*	Amo†	Pento‡	Seco§	Pheno**	Buta	Amo	Pento	Seco	Pheno
x_0	39.2	48.5	39.8	32.6	49.1	16.1	26.1	21.0	21.3	18.8
x_1	4.7	5.7	6.1	3.6	8.0	3.8	10.1	4.1	5.4	4.5
x_2	15.2	17.2	19.8	11.3	17.8	12.2	21.4	15.8	15.4	15.8
x_3	8.1	10.6	10.5	5.9	14.2	8.0	12.7	10.2	10.2	9.9
x_1x_2	0.1	0.4	0.6	0.3	0.2	0.6	0.2	0.6	0.2	0.1
x_2x_3	4.0	3.9	5.2	4.1	3.1	4.9	8.3	5.8	5.8	7.5
x_1x_3	0.5	0.3	0.7	0.3	0.2	0.6	0.2	0.6	0.2	0.1
x_1^2	3.4	3.7	3.3	3.5	4.4	0.6	0.9	0.1	0.1	0.7
x_2^2	5.7	6.8	5.7	5.5	7.6	3.0	4.1	3.3	1.8	4.4
x_3^2	5.2	6.3	5.2	5.1	7.7	0.2	1.4	0.6	1.1	0.3

* Buta, butalbital.
† Amo, amobarbital.
‡ Pento, pentobarbital.
§ Seco, secobarbital.
** Pheno, phenobarbital.

Table IV. Regression Equations and Extreme Recovery Coordinates for Barbiturate Derivatization

Barbiturate	DIPK derivative	DPK derivative
Butalbital	$Y = 87.0 - 8.50x_1 + 27.2x_2 + 14.5x_3 - 8.60x_2x_3 - 7.70x_1^2 - 11.0x_2^2 - 12.0x_3^2$; $x_1 = -0.55$, $x_2 = -1.16$, $x_3 = -0.19$	$Y = 25.0 - 8.50x_1 + 27.0x_2 + 17.8x_3 + 13.0x_2x_3 + 6.20x_2^2$; $x_1 = -1.35$
Amobarbital	$Y = 74.0 - 7.70x_1 + 23.1x_2 + 14.2x_3 - 6.30x_2x_3 - 6.50x_1^2 - 12.0x_2^2 - 11.0x_3^2$; $x_1 = -0.59$, $x_2 = -0.86$, $x_3 = -0.40$	$Y = 24.0 - 12.6x_1 + 26.6x_2 + 15.8x_3 + 12.4x_2x_3 + 7.40x_2^2$; $x_1 = -1.35$
Pentobarbital	$Y = 80.0 - 8.50x_1 + 27.2x_2 + 14.5x_3 - 8.60x_2x_3 - 7.70x_1^2 - 13.0x_2^2 - 12.0x_3^2$; $x_1 = -0.55$, $x_2 = -0.96$, $x_3 = -0.40$	$Y=31.0 - 7.10x_1 + 27.7x_2 + 17.9x_3 + 12.2x_2x_3 + 7.50x_2^2$; $x_1 = -1.35$
Secobarbital	$Y = 86.0 - 7.90x_1 + 24.8x_2 + 12.9x_3 - 10.8x_2x_3 - 10.1x_1^2 - 15.6x_2^2 - 14.7x_3^2$; $x_1 = -0.39$, $x_2 = -0.74$, $x_3 = -0.17$	$Y = 30.0 - 9.40x_1 + 26.0x_2 + 17.5x_3 + 12.0x_2x_3$; $x_1 = -1.35$
Phenobarbital	$Y = 74.0 - 10.2x_1 + 22.7x_2 + 18.1x_3 - 4.75x_2x_3 - 7.50x_1^2 - 13.0x_2^2 - 13.0x_3^2$; $x_1 = -0.68$, $x_2 = -0.78$, $x_3 = -0.55$	$Y = 25.0 - 7.30x_1 + 25.2x_2 + 16.0x_3 + 15.0x_2x_3 + 7.50x_2^2$; $x_1 = -1.35$

influence on the derivatization recovery. In addition to the effect of a single parameter, the equations for DIPK derivatives had single-parameter nonlinear terms and also terms representing the interrelationship between the parameters. The greatest absolute values among the coefficients (Table IV) had linear terms for the reaction temperature. This indicated that temperature was the most important factor affecting the recovery with both derivatizing reagents. The presence of the interrelationship and second-order terms for the derivatization temperature and time represented the mutual influence of the parameters on each other and the nonlinearity of the effects. The second-order terms of a single parameter were approximately twice as high as the interrelationship terms. The major difference between the regression equations for the DPK and DIPK derivatives was the different signs at the coefficients of the second-order parameter effects and the more significant nonlinearity of the equations for DIPK derivative recovery.

The choice of the solvent that will be used during the derivatization is very important. DMSO promotes the reaction by binding cations and leaving nucleophilic particles in a nonsolvated form (17). The regression equations showed similar effects of derivatization solution composition on the barbiturate recovery for both derivatizing reagents. As the solvent–derivatizing reagent ratio decreased, the recovery increased. Comparison of the equations for the DIPK derivatives showed no significant difference for the derivatizing solution composition influence on the reaction recovery between the barbiturates. Among the DPK derivatives, the influence of the derivatization solution composition was stronger for amobarbital than the other barbiturates. The major difference between the equations for different derivatives was the presence of the second-order terms for the derivatizing solution influence on the recovery of the DIPK derivatives. The optimum composition for the DMSO–DMFDIPA ratio was calculated by taking partial derivatives ($\delta R/\delta x_{1i} = 0$) and solving the equations for x_{1i}. For the DPK derivatization, there was a linear correspondence between the solution composition and recovery. In order to increase recovery at a given incubation temperature and time, it was necessary to decrease the DMSO–derivatizing reagent ratio. In general, the derivatization solution composition had less influence on the reaction yield compared with the incubation temperature and time. The calculated conditions for optimum recovery are presented in Table IV.

DIPK derivatives

In order to evaluate the interrelationship of the parameters, the regression equations (Table IV) were reduced to a canonical form (equation 3). Figure 2 is a plot of the dependence of the recovery surfaces for butalbital, secobarbital, and phenobarbital DIPK derivatives on incubation temperature and time. The surfaces for amobarbital and pentobarbital were similar to that of butalbital. The canonical equations analysis showed that all the surfaces for DIPK derivatization were elliptic paraboloids with a maximum in the center. In the equations for all the barbiturates (Table VI), both equation terms were negative. This represented a decrease in the derivative recovery with any change in the reaction temperature or time relative to the optimum values. The different magnitudes of the coefficients suggested that a change would affect each barbiturate recovery differently. Such phenomena can be explained as an incomplete formation of the derivatives at incubation temperatures and times below the optimum values, thus supplanting the effect to the decomposition of the deriva-

Table V. Fisher Test Values for the Agreement Between the Regression Equations and Experimental Results

Barbiturate	Derivative	
	DIPK	DPK
Butalbital	9.09	6.00
Amobarbital	16.17	10.57
Pentobarbital	9.27	5.07
Secobarbital	5.47	8.54
Phenobarbital	16.24	3.68

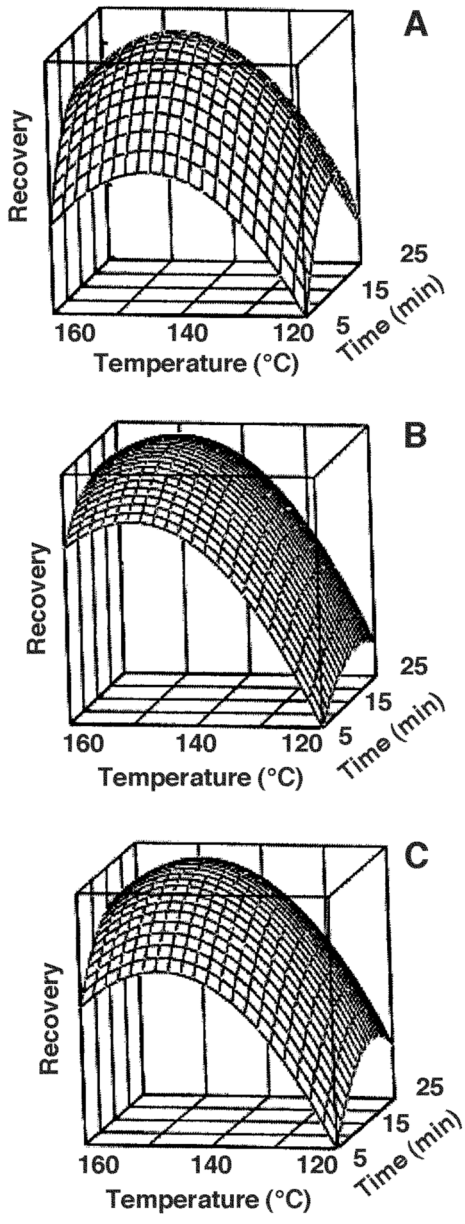


Figure 2. Dependence of the recovery of the following DIPK derivatives on incubation temperature and time: (A) butalbital, (B) secobarbital, and (C) phenobarbital.

tives at temperatures and times above optimum values. Most likely, the decomposition was a consequence of a steric hindrance between two isopropyloxy groups bound to carbon 2 (Figure 1).

The optimal values for the derivatizing solution composition, incubation temperature, and time were different among the barbiturates. Because apexes of the recovery surfaces were observed at a combination of different temperatures and times among the compounds, conditions optimal for the recovery of one barbiturate DIPK derivative would not be optimal for the other barbiturates.

DPK derivatives

Figure 3 is a plot of the dependence of the recovery surfaces for butalbital, secobarbital, and phenobarbital DPK on incubation temperature and time. Recovery surfaces for amobarbital and pentobarbital were similar to that of butalbital. All the canonical equations for DPK derivatives had one negative and one positive term. These equations corresponded to the surface of a hyperbolic paraboloid with a saddle point in the center. The surfaces corresponding to the experimental field were a part of one of the branches of the hyperbolic paraboloids. The surfaces had the same tendency for the incubation temperature influence on the recovery for all of the barbiturates. Similar values of the coefficients in the equations for butalbital, amobarbital, and pentobarbital represented an equivalent impact of the incubation temperature and time on the recovery. The major increase in the recovery was observed with an increase in the incubation temperature and (to a significantly smaller extent) an increase in the incubation time. The secobarbital and phenobarbital derivatization phenomena were different from the other barbiturates. The phenobarbital DPK recovery surface had a significantly steeper slope compared with the other barbiturates. The slope of the recovery surface for secobarbital was flatter than for other barbiturates in the direction of the incubation temperature and significantly steeper than the other barbiturates in the direction of the incubation time.

In order to obtain secobarbital DPK recovery comparable with the derivatives of the other barbiturates, it was necessary to use a combination of the maximum incubation temperature and time. The recovery surfaces for DPK derivatives do not have apexes within the experimental area; therefore, an increase in the incubation temperature and time did not produce a negative impact on the recovery as occurred for DIPK derivatives.

An analysis of the canonical equations showed that within the same derivatizing reagent, there were similar phenomena for the influence of the derivatizing solution composition, incubation temperature, and time for butalbital, amobarbital, and pentobarbital. Phenomena for phenobarbital and secobarbital derivatization by both reagents were different from the other barbiturates. The recovery surface analysis for DIPK derivatives showed that the reaction was more kinetically controlled, which is demanding for maintaining the optimal incubation conditions. The recovery of DIPK derivatives (Figure 2) would decline with any variation in temperature, time, and derivatization solution composition relative to the optimal values. The derivatization by DMFDPA was more rugged for the simultaneous quantitative analysis of barbiturates. The difference in the derivatization phenomena for butalbital, amobarbital, and pentobarbital from secobarbital and phenobarbital suggests that for reliable quanti-

Table VI. Canonic Equations for Recovery of Barbiturates DIPK and DPK Derivatives Depending on Incubation Temperature and Time

Barbiturate	DIPK derivative	DPK derivative
Butalbital	R-110 = $-8.5X_2^2 - 17X_3^2$	R-36 = $10X_2^2 - 4.2X_3^2$
Amobarbital	R-100 = $-8.3X_2^2 - 15X_3^2$	R-49 = $11X_2^2 - 3.5X_3^2$
Pentobarbital	R-110 = $-8.5X_2^2 - 17X_3^2$	R-45 = $11X_2^2 - 3.4X_3^2$
Secobarbital	R-110 = $-10X_2^2 - 21X_3^2$	R-30 = $6X_2^2 - 6X_3^2$
Phenobarbital	R-110 = $-10X_2^2 - 16X_3^2$	R-37 = $13X_2^2 - 4X_3^2$

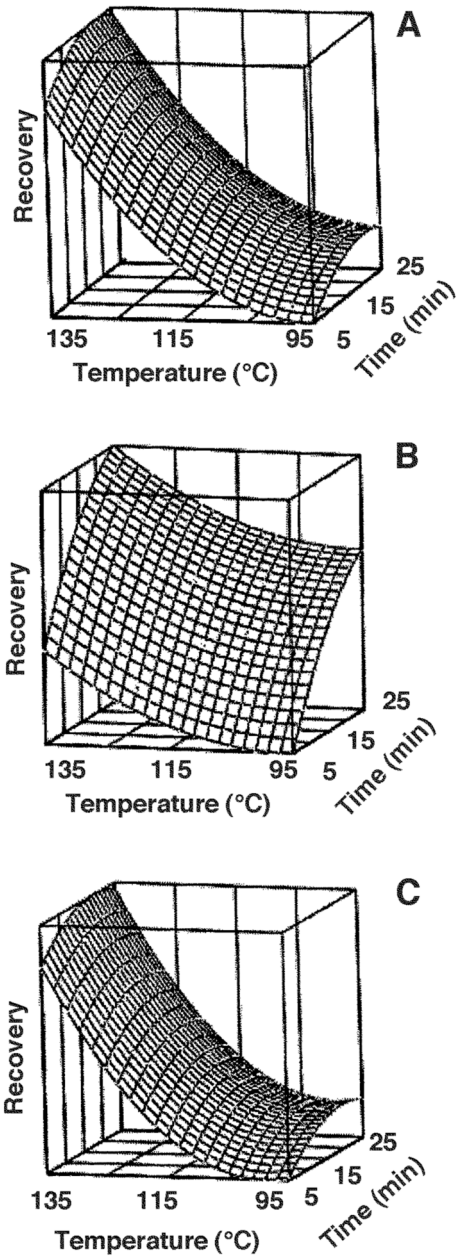


Figure 3. Dependence of the recovery of the following DPK derivatives on incubation temperature and time: (A) butalbital, (B) secobarbital, and (C) phenobarbital.

tation it is preferable to have separate internal standards for the two groups of barbiturates.

Precision study

Figure 4 shows the total ion chromatogram of a spiked urine sample extracted by the described method and derivatized to produce DPK derivatives of the barbiturates. A summary of the method precision study for both derivatizing reagents is presented in Table VII. The internal standards used for the assay were pentobarbital d_5 for butalbital, amobarbital, pentobarbital, and secobarbital and phenobarbital d_5 for phenobarbital. The method precision for phenobarbital was calculated by using both of the internal standards. Calibration was performed with every run using standards at 120, 300, and 1200 ng/mL. Limits for the ion mass ratios were established by the 300-ng/mL calibrator with the criterion of maintaining the quantitative ion mass ratio of the controls and samples within ± 20%. Consistent within-run quantitative ion mass ratios and day-to-day relative peak area ratios were observed for the calibrators and samples. All of the experiments for the precision study were performed three times and the mean of the obtained results presented. Within-run precision was determined by analyzing urine samples containing known concentrations of the barbiturates. Between-run precision was determined by analyzing the samples over three days. For butalbital, amobarbital, pentobarbital, and secobarbital, acceptable results for quantitation were observed with pentobarbital d_5 as the internal standard. Significant improvement in the precision for phenobarbital was observed by incorporating phenobarbital d_5 as the internal standard. Imprecision for secobarbital was greater than for the other barbiturates, with some improvement observed with the use of the DPK derivative. The comparison of results between the derivatizing reagents showed a more consistent performance for the method using DMFDPA derivatization. The results of the precision evaluation supported conclusions derived from the regression equations analysis. The assay using DPK derivatives has been used in a high-volume drug-testing laboratory for several years and appears to be reliable and trouble-free.

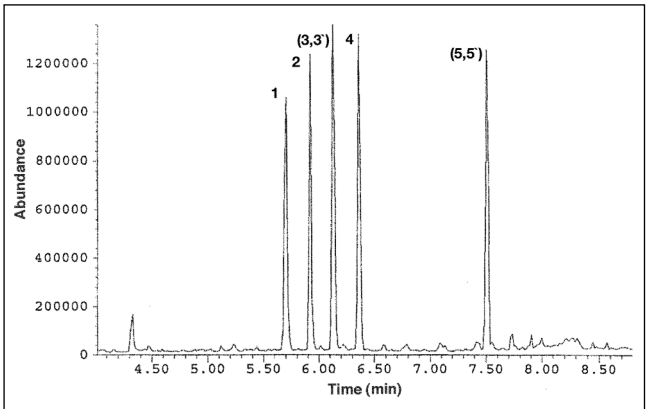


Figure 4. Total ion chromatogram of the extracted urine sample (DPK derivatives) spiked with 1000 ng/mL of (1) butalbital, (2) amobarbital, (3) pentobarbital, (4) secobarbital, and (5) phenobarbital and 250 ng/mL of the internal standards (3`) pentobarbital $d_5$ and (5`) phenobarbital d_5.

Conclusion

The obtained regression and canonical equations were used to predict the reliability of the derivatization reactions and to calculate the recovery of the derivatives at any set of the reaction conditions. An analysis of the equations showed that DPK derivatives are superior to DIPK with the consideration of milder conditions of derivatization, absence of sudden changes in recovery with variation in reaction conditions, and expected reliability for the simultaneous testing of multiple barbiturates. The equations predicted that any variations from the optimum values of the temperature and incubation time during DMFDIPA derivatization would reduce reaction recovery. The probable reason for the lower reliability of DIPK derivatization is the steric hindrance between two isopropyloxy groups bound to carbon 2 in the barbiturate molecule. The barbiturate DIPK derivative recovery was more susceptible to variations because of changes in the reaction parameters and required more precise control over the reaction temperature and time than for DPK derivatives. The difference between the derivatization phenomena for secobarbital and phenobarbital from the other barbiturates suggests the preference of having separate internal standards for reliable quantitation of the barbiturates. Results of the precision study for barbiturates analysis with both derivatizing reagents support the conclusions obtained from the theoretical analysis of the regression and canonical equations.

Table VII. Method Precision for Simultaneous Analysis of Barbiturates as DPK and DIPK Derivatives

	Butalbital*		Amobarbital*		Pentobarbital*		Secobarbital*		Phenobarbital*		Phenobarbital†	
Derivative	**DPK**	**DIPK**	**DPK**	**DIPK**	**DPK**	**DIPK**	**DPK**	**DIPK**	**DPK**	**DIPK**	**DPK**	**DIPK**
300 ng/mL												
Mean (ng/mL)	291	304	306	323	302	295	281	294	331	342	302	313
Within-run CV (%)	3.1	5.1	3.4	4.7	2.4	3.2	4.9	13.0	7.8	7.6	2.2	4.0
Between-run CV (%)	4.3	6.1	4.0	6.0	2.8	5.7	6.8	12.1	8.5	12.2	2.2	7.9
3000 ng/mL												
Mean (ng/mL)	2912	2985	2913	2966	3049	2864	2825	3219	3326	2882	2854	2934
Within-run CV (%)	1.4	2.3	2.5	5.4	2.8	4.1	4.7	11.2	7.3	10.4	1.4	6.3
Between-run CV (%)	3.0	4.8	5.2	7.8	3.8	6.0	5.0	10.0	11.1	16.8	2.6	7.8

* Pentobarbital d_5 as the internal standard.
† Phenobarbital d_5 as the internal standard.

References

1. J.D. Nicolson. Derivative formation in the quantitative gas chromatographic analysis of pharmaceuticals, part 11; a review. *Analyst* **103:** 193–222 (1978).
2. D.R. Knapp. *Handbook of Analytical Derivatization Reactions.* John Wiley & Sons, New York, NY, 1979, pp. 639–54.
3. A. Hulshoff and A.D. Forch. Alkylation with alkyl halide as derivatization method for the gas chromatographic determination of acidic pharmaceuticals. *J. Chromatogr.* **220:** 275–311 (1981).
4. H.H. Maurer. Identification and differentiation of barbiturates, other sedative-hypnotics and their metabolites in urine integrated in a general screening procedure using computerized gas chromatography–mass spectrometry. *J. Chromatogr.* **530:** 307–26 (1990).
5. H.F. Martin and J.L. Driscoll. Gas chromatographic identification and determination of barbiturates. *Anal. Chem.* **38:** 345–46 (1966).
6. M.G. Horning, K. Lertratangkoon, J.Nowlin, W.G. Stillwell, R.N. Stillwell, T.E. Zion, P. Kellaway, and R.M. Hill. Anticonvulsant drug monitoring by GC-MS-COM techniques. *J. Chromatogr. Sci.* **12:** 630–35 (1974).
7. H. Roseboom and A. Hulshoff. Rapid and simple clean-up and derivatization procedure for the gas chromatographic determination of acidic drugs in plasma. *J. Chromatogr.* **173:** 65–74 (1979).
8. R.H. Liu, A.M. McKeehan, C. Edwards, G. Foster, W.D. Bensley, J.G. Langner, and A.S. Walia. Improved gas chromatography/mass spectrometry analysis of barbiturates in urine using centrifuge-based solid-phase extraction, methylation, with d_5-pentobarbital as internal standard. *J. Forensic Sci.* **39:** 1504–14 (1994).
9. E. Brochmann-Hansen and T.O. Oke. Gas chromatography of barbiturates, phenolic alkaloids, and xanthine bases: flash-heater methylation by means of trimethylanilinium hydroxide. *J. Pharm. Sci.* **58:** 370–71 (1969).
10. R. Meatheral. GC–MS confirmation of barbiturates in blood and urine. *J. Forensic Sci.* **42:** 1160–70 (1997).
11. A.S. Christophersen and K.E. Rasmussen. Glass capillary column gas chromatography of barbiturates after flash-heater derivatization with dimethylformamide dimethylacetal. *J. Chromatogr.* **192:** 363–74 (1980).
12. V.S. Venturella, V.M. Gualario, and R.E. Lang. Dimethylformamide dimethylacetal as a derivatizing agent for GLC of barbiturates and related compounds. *J. Pharm. Sci.* **62:** 662–68 (1973).
13. A.D. Barbour. GC–MS analysis of propylated barbiturates. *J. Anal. Toxicol.* **15:** 214–15 (1991).
14. G.E.P. Box, W.G. Hunter, and J.S. Hunter. *Statistics for Experiments. An Introduction to Design Data Analysis and Model Building.* John Wiley & Sons, New York, NY, 1978.
15. D.C. Montgomery. *Design and Analysis of Experiments,* 2nd ed. John Wiley & Sons, New York, NY, 1983, pp. 189–354.
16. G. Taguchi. *System of Experimental Design,* Vol. I and 2. Kraus International, New York, NY, 1987.
17. J. Mathiew and R. Panico. *Mechanism of Organic Reactions.* Hermann, Paris, France, 1972, Chapter 11.

Manuscript accepted December 8, 2000.