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Improved Gas Chromatography/Mass Spectrometry Analysis of Barbiturates in Urine Using Centrifuge-Based Solid-Phase Extraction, Methylation, with d_5-Pentobarbital As Internal Standard

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ABSTRACT: Effective solid-phase extraction, derivatization, and GC/MS procedures are developed for the simultaneous determinations of butalbital, amobarbital, pentobarbital, and secobarbital, using a deuterated pentobarbital (d_5-pentobarbital) as the internal standard. Buffered (pH 7) urine samples were extracted with Bond Elute Certify II™ cartridge. Iodomethane/tetramethylammonium hydroxide in dimethylsulfoxide was used for methylation, while a HP 5970 MSD equipped with a 13 m J & W DB-5 column (5% phenyl polysiloxane phase) and the Thru-Put Target® software package were used for GC/MS analysis and data processing. This protocol was found to be superior, in both chromatographic performance characteristics and quantitation results, over a liquid-liquid extraction procedure without derivatization using hexobarbital as the internal standard. Extraction recoveries observed from control samples containing four barbiturates range from 80% to 90%. Good one-point calibration data are obtained for all four barbiturates in the 50 to 3200 ng/mL range. Interestingly, the one-point calibration data for pentobarbital are inferior to the other three barbiturates—due to interference from the internal standard (d_5-pentobarbital). The calibration data of pentobarbital are best described by a hyperbolic curve regression model. Precision data (% CV) for GC/MS analysis, over-all procedure, and day-to-day performance are approximately 2.0%, 6.0%, and 8.0%, respectively. With the use of a 2 mL sample size, the attainable detection limit is approximately 20 ng/mL.

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Quantitative analysis of underivatized barbiturates by gas chromatography (GC)-based methods often encounters difficulties caused by the adsorption of these polar compounds onto the column materials as reported in 1970 [*1,2*]. In a later study [*3*], the reported day-to-day retention index percent CV data for barbiturates were significantly larger than those for other drugs included in the same study (0.008 to 0.017 versus 0.005 to 0.006). A recent report [*4*] also showed relatively high percent CV (approximately 7 to 10%) data on precision studies. Neither studies adopted derivatization and the chromatograms included in these two articles showed substantial peak tailing. In 1987, we adopted the U.S. Navy's procedure for the simultaneous confirmatory analysis of butalbital, amobarbital, pentobarbital, and secobarbital [*5*], but often experienced difficulties. Problems encountered included (a) poor chromatographic peak shape; (b) inconsistencies in GC/MS results of replicates that were injected with different length of delay following extract reconstitution; and (c) difficulties in reproducing (within ±20%) the quantitative results of these analytes in three controls included in each analytical batch. These problems are often serious if the injector insert and the column have not been freshly maintained.

This article reports the integration and critical evaluation of the following approaches aiming for the establishment of an effective and reliable protocol for high-volume confirmatory testing of barbiturates (butalbital, amobarbital, pentobarbital, secobarbital) in urine samples: (a) selection of a deuterated analog of an analyte as the internal standard for all four analytes [*6*]; (b) a centrifuge-based solid-phase extraction approach [*7,8*]; and (c) methylation of the analytes [*1,9,10*]. In addition, we report two very important observations, that is, (a) a deuterated analog of the analyte is not necessary the best internal standard and (b) a calibration model, that fully accounts for the underlying ion fragmentation mechanism, provides improved quantitation results.

With more than five years of experience conducting GC/MS analysis of the subject barbiturates under routine and high-volume settings, we have concluded that the hereby reported protocols are superior to other procedures known to the authors. The reproducibility, linearity, and recovery data are reported to support the effectiveness of the hereby described protocol for the intended use.

Materials and Methods

Standards, Reagents, and Controls

Butalbital, amobarbital, pentobarbital, and secobarbital were purchased from Sigma (St. Louis, MO). The internal standard d_5-pentobarbital (0.1 mg/mL in methanol) was purchased from Radian (Austin, TX). The derivatization reagents and solvent—tetramethylammonium hydroxide (24% in methanol), iodomethane, and dimethylsulfoxide—were purchased from Eastman Kodak (Rochester, NY), Mallinckrodt (Paris, KY), and Aldrich (Milwaukee, WI), respectively. Analytichem Bond Elute Certify II™ columns were obtained from Varian (Harbor City, CA).

0.1 M Acetate buffer (pH 7) was prepared using 13.6 g sodium acetate in 1-L solution which was adjusted to pH 7.0 with 1 N NaOH and 1 N HCl.

A sample size of 2 mL was adopted for the protocol. Standard and control solutions were prepared in urine using 0.1 mg/mL stocks. Internal standard was incorporated in each sample (50 μL of 10 μg/mL d_5-pentobarbital stock) at a 250 ng/mL concentration level. Each operation batch also includes a 200 ng/mL solution serving as the one-point calibration standard.

Solid-Phase Extraction

The entire extraction and derivatization process is schematically shown in Fig. 1. Column conditioning, washing, and eluting steps were processed in a centrifuge to facilitate batch operation. A Polypropylene Insert for "Mini" Scintillation Vials (Wheaton, Millville, NJ) was used to connect the column and a 15 mL centrifuge tube for collection of the liquid forced through the column by centrifugal force. The derivatized product in the organic phase was decanted after freezing the lower aqueous layer in a dry ice/isopropanol bath. This practice, preferred over the Pasteur pipetting procedure, generates a more complete and uniform phase transfer. For a batch size of 24 samples, the entire extraction/derivatization procedure can be completed in a 2 to 3 h period.

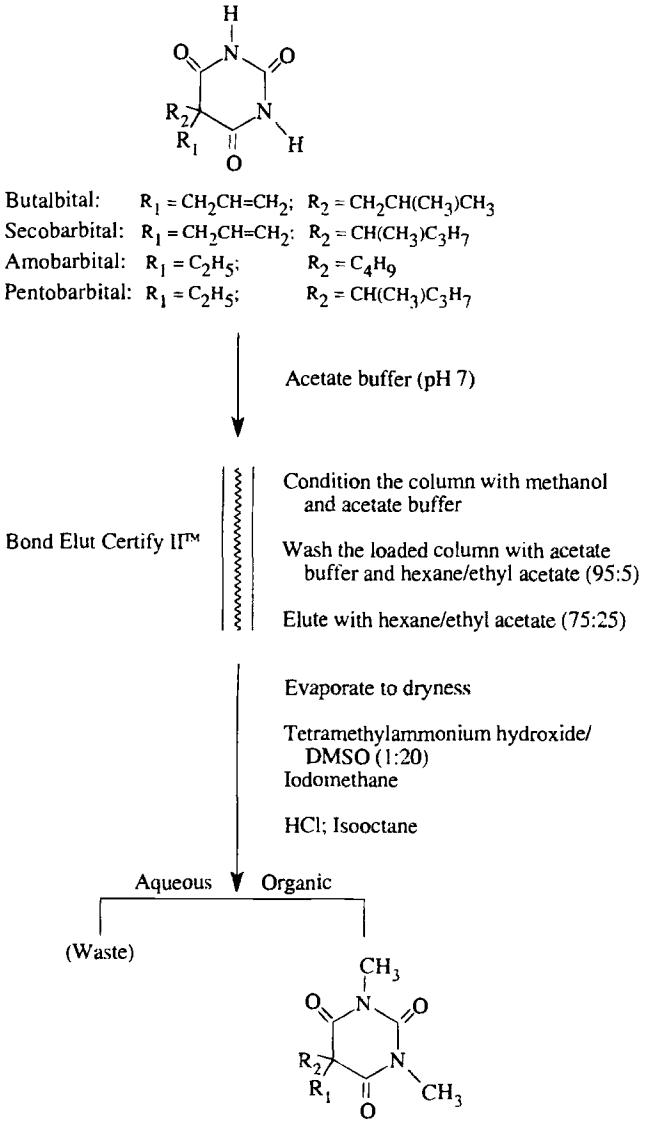

FIG. 1—*Solid-phase extraction and derivatization scheme.*

GC/MS Analysis

GC/MS Analysis was performed using a HP 5890 Gas chromatograph interfaced to a HP 5970 (Hewlett-Packard, Palo Alto, CA) mass selective detector (MSD). The gas chromatograph was equipped with a 13-m J & W (Folsom, CA) DB-5 (5% phenyl polysiloxane phase) fused silica capillary column (0.25-mm ID; 0.25-μm film thickness). The injection port was equipped with a split silanized glass insert packed with OV-101 (80/100 mesh). Helium was used as the carrier gas with a flow rate of 1.0 mL/min and a split ratio of 10:1. The injector, oven, and interface temperatures were maintained at 270, 160, and 270°C, respectively.

The MSD was used under SIM mode (dwell time 50 ms) with the monitoring of the following ions: m/z 181, 195, 196; 184, 185, 169; 169, 185, 184; 181, 195, 196; and 171, 189 for the methylated butalbital; methylated amobarbital; methylated pentobarbital; methylated secobarbital; and methylated d_5-pentobarbital internal standard, respectively. The last ion listed for each compound was used for quantitation.

A typical quantitative GC/MS protocol includes SIM of the selected ions for the analyte and the isotopic analog, followed by comparing a selected analyte-to-isotopic analog ion intensity ratio observed from the *test sample* and the same ratio observed from the *calibration standard.* The calibration standard contains the same amount of the internal standard and a known amount of the analyte and is processed in parallel with the test sample. The analyte concentration in the test sample is then calculated using the formula shown in Fig. 2.

Results and Discussion

Selection of d_5-Pentobarbital as the Potential Internal Standard

The use of a deuterated analog of the analyte provides distinct characteristics otherwise not available [*6,11*]. The mass spectra of pentobarbital and d_5-pentobarbital are compared in Fig. 3. Earlier evaluation of d_5-pentobarbital [*6*] has concluded that it is an acceptable internal standard for the quantitation of pentobarbital. It is adopted in this protocol as the sole internal standard for all four analytes for the following reasons: (a) a single internal standard simplifies the analytical process and reduces the reagent cost; and (b) compared to the deuterated analogs of other three analytes, a deuterated pentobarbital analog has the least retention time differences from all analytes of interest, thus potential GC-related variations can be minimized.

Calibration Model—One-Point, Linear, and Hyperbolic

Standard solutions containing barbiturates targeted at 0 to 3200 ng/mL were used for calibration studies. With the same amount of d_5-pentobarbital used in all samples, the above mentioned one-point calibration methodology was used to derive the observed analyte concentrations using the following quantitation ion intensity ratios: m/z 196/189 for butalbital, 169/189 for amobarbital, 184/189 for pentobarbital, and 196/189 for secobarbital. Results

$$\frac{\left[\frac{(\text{Selected Ion Intensity})_{\text{Analyte}}}{(\text{Selected Ion Intensity})_{\text{Internal Std}}}\right]_{\text{Test Sample}}}{\left[\frac{(\text{Selected Ion Intensity})_{\text{Analyte}}}{(\text{Selected Ion Intensity})_{\text{Internal Std}}}\right]_{\text{Calibration Standard}}} \times \left[\text{Standard Conc.}\right]_{\text{Calibration Standard}} = \left[\text{Analyte Conc.}\right]_{\text{Text Sample}}$$

FIG. 2—*Formula for the calculation of the analyte concentration using an one-point calibration protocol.*

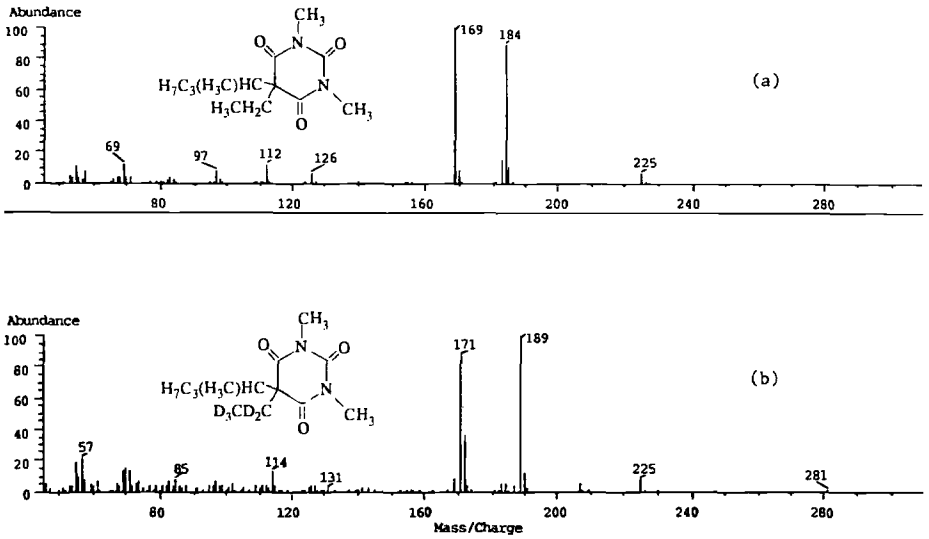

FIG. 3—*Mass spectra of methylated d_0-pentobarbital* (a) *and d_5-pentobarbital* (b).

summarized in Table 1 demonstrate two important points. First, d_5-pentobarbital can be used as an effective internal standard for the quantitation of *butalbital, amobarbital,* and *secobarbital.* Secondly, there appears to be a systematic error in the quantitation of *pentobarbital.* The observed concentrations deviate positively or negatively from the theoretical ones when the analyte concentration is lower or higher, respectively, than the analyte concentration in the calibration standard. Furthermore, the extent of the deviation appears to be in proportion to the magnitude of the analyte concentration difference between the *calibration standard* and the *standard solution* under examination.

This apparent systematic error is consistent with the observation [*6*] that d_5-pentobarbital contributes (cross-contribution) approximately 1.9% to the intensity of the m/z 184 ion—the ion used for the quantitation of pentobarbital.

This phenomenon is further examined by evaluating the quantitation results derived from three different calibration methodologies, that is, one-point calibration and linear and hyperbolic curve regression analysis. Hyperbolic curve model, which takes into account the above mentioned cross-contribution effect [*12*], is considered an appropriate model for relating the monitored response ratio (y) to the quantity of the analyte (x) in an analytical protocol using an isotopic analog as the internal standard:

$$y = R + Px - Qxy$$

The m/z 185 ion (instead of the routinely used m/z 184 ion) is selected as the pentobarbital quantitation ion for the comparison of calibration models. This selection is based on our earlier observation [*6*] that d_5-pentobarbital contributed approximately 2.8% to the intensity of the m/z 185 ion (comparing to 1.9% for the m/z 184 ion) to be used for the quantitation of pentobarbital; thus, differences (of the three models) caused by the cross-contribution interference from the internal standard will be more apparent.

Using the intercept model for linear regression [*13*] and weighted least-squares regression method [*14*] for both the linear and the hyperbolic curve fitting models, results obtained by these three models are summarized in Table 2. The hyperbolic curve model, which takes into account the cross-contribution phenomenon, produces the best result.

TABLE 1—*Quantitation using one-point calibration methodology.*

Butalbital				Amobarbital				Secobarbital				Pentobarbital			
Theor. Conc.	Ion Int. Ratio	Observed Conc.	Dev. (%)	Theor. Conc.	Ion Int. Ratio	Observed Conc.	Dev. (%)	Theor. Conc.	Ion Int. Ratio	Observed Conc.	Dev. (%)	Theor. Conc.	Ion Int. Ratio	Observed Conc.	Dev. (%)
0	—	—	—	0	—	—	—	0	—	—	—	0	—	—	—
13	0.029	13.8	6.2	13	0.048	12.1	−6.9	13	0.039	13.0	0	13	—	—	—
25	0.059	27.6	10	26	0.097	24.6	−5.4	27	0.081	27.0	0	25	0.0168	32.6	30
54	0.107	50.3	−6.9	51	0.199	50.4	−1.2	53	0.162	54.0	1.9	50	0.0288	56.2	12
108	0.189	89.4	−17	102	0.386	98.1	−3.8	106	0.340	114	7.5	100	0.0534	104	4.0
208	0.454	215	3.4	204	0.801	204	0	216	0.633	212	−1.9	199	0.104	202	1.5
430	0.878	416	−3.3	408	1.56	395	−3.2	424	1.28	427	0.7	398	0.203	394	−1.0
860	1.78	879	2.2	816	3.07	777	−4.8	848	2.58	864	1.9	796	−0.382	741	−6.9
1720	3.82	1805	4.9	1632	6.40	1626	−0.4	1697	5.01	1679	−1.1	1592	0.765	1485	−6.5
3440	7.62	3608	4.9	3264	10.9	3075	−5.8	3393	9.48	3173	−6.5	3184	1.47	2853	−10

TABLE 2—*Comparison of pentobarbital quantitation results using different calibration methodologies.*

Theoretical Concentration	Observed Quantitation, Ions Int. Ratio	One-point Calibration		Linear Regression		Hyperbolic Curve	
		Observed Conc.	Dev. (%)	Observed Conc.	Dev. (%)	Observed Conc.	Dev (%)
0	—	—	—	—	—	—	—
13	0.0112	22.5	80	10.5	−16	11.5	−8.3
25	0.0176	35.4	42	27.5	10	27.6	10
50	0.0259	52.1	4.2	49.6	−0.79	48.5	−3.1
100	0.0452	90.9	−9.1	100.9	0.90	97.2	−2.9
200	0.0888	179	−11	216.8	8.39	207.7	3.9
400	0.1660	334	−17	422.0	5.50	405.4	1.3
800	0.3090	621	−22	802.1	0.26	778.0	−2.8
1600	0.6055	1217	−24	1590	−0.61	1578	−1.3
3200	1.162	2336	−27	3069	−4.1	3192	−0.26
6400	2.144	4310	−33	5680	−11	6451	0.80

The one-point calibration model, which does not make any correction for the mentioned ion interference effect, generates acceptable results only when the analyte concentration in the test sample falls within a very limited range centered at the concentration of the calibrator. However, the one-point calibration model is not without merit; in addition to its simplicity, it may also provide more accurate results for samples containing the analyte in the immediate vicinity of the concentration of the single calibrator. In light of the heavy emphasis on adopting a "cutoff" concentration for reporting a sample as legally positive, the merit of using a one-point calibrator at the cutoff concentration should not be overlooked.

Method Reproducibility and Precision of Day-to-Day Data—Merit of Methylated Protocol

Method reproducibility was evaluated at three levels by comparing: (a) results obtained from repeated injections of the same extraction-derivatization product into the GC/MS system; (b) results obtained from replicates of a control in the same operation batch; and (c) day-to-day data of control replicates that were tested along with unknown samples over an approximately 2-month period (13 analytical batches). The ruggedness of the methodology is demonstrated by the observed precision data shown in Table 3.

Extraction Recovery

With the development of various sorbent materials, solid-phase extraction approaches have become effective analytical procedures and are widely adopted [*15,16*] for the isolation and concentration of analyte in biological samples. The solid-phase extraction approach offers the following advantages over the conventional liquid-liquid procedures: (a) less organic solvent usage; (b) shorter sample preparation time; and (c) easier incorporation into automatic operation protocols.

The recovery efficiency of the above mentioned solid-phase extraction protocol is studied by comparing results obtained from a series of two sets of three replicates containing the same amount of barbiturates (400 or 1200 ng of each barbiturate) prepared from a 10 μg/mL methanol stock solution. The first set of replicates was diluted to 2 mL with drug-free urine and processed with the solid-phase extraction protocol; the second set of replicates was not diluted with drug-free urine and was not extracted. After completing the extraction

TABLE 3—*Reproducibility for GC/MS injection, over-all procedure, and day-to-day.*

No.	GC/MS Injection				Over-all Procedure				Day-to-Day			
	Butalb	Amobarb	Pentobarb	Secobarb	Butalb	Amobarb	Pentobarb	Secobarb	Butalb	Amobarb	Pentobarb	Secobarb
1	369.5	421.0	417.6	477.4	624.9	606.3	573.3	649.9	225	221	229	241
2	379.0	428.0	424.3	487.0	579.4	559.4	529.9	576.6	226	213	207	222
3	383.4	428.5	433.1	489.8	608.8	589.6	544.3	610.2	228	244	228	249
4	379.3	424.6	413.0	487.1					260	247	242	254
5	367.7	417.5	412.7	472.6					265	236	223	237
									254	212	210	241
									213	214	202	210
									253	228	218	227
									284	231	216	208
									250	234	226	236
									248	234	224	244
									240	229	219	243
									255	217	213	225
Mean	375.8	423.9	420.1	482.8	604.4	594.8	549.2	612.2	246	228	220	234
Std Dev	6.8	4.7	8.6	7.4	23.1	23.2	22.1	36.7	19.4	11.6	10.6	14.3
CV (%)	1.8	1.1	2.0	1.5	3.8	3.9	4.0	6.0	7.9	5.1	4.8	6.1

process for the first set of replicates, both sets are processed identically—spiked with the internal standard, treated with the derivatization procedure, and analyzed by the GC/MS protocol. Recovery data (Table 4) for the four barbiturates observed in four different batches (each performed at a different date) indicate a recovery of 80% or better.

Summary

The over-all protocol dramatically improves analyte chromatographic characteristics with effective extraction recovery (80% or better, Table 4), detection limits (25 ng/mL or better, Table 1) and good quantitation precision (1.1–2.0%, 3.8–6.0%, and 4.8–7.9% for GC/*MS analysis, overall procedure,* and *day-to-day,* respectively, Table 3).

Data shown in Table 2 illustrate a very interesting and important point, i.e., the use of a deuterated analog of the analyte does not guarantee the generation of the best possible quantitation result. These data indicate that one-point calibration quantitation results for pentobarbital were actually inferior when compared to those for the other three barbiturates—even though d_5-pentobarbital was used as the single internal standard for all analytes. Factors that should be carefully evaluated include: (a) cross-contribution of quantitation ions selected for the analyte and the internal standard; and (b) selection of an appropriate calibration model (Table 2) to take into accounts of the cross-contribution interference—a common phenomenon.

TABLE 4—*Extraction recovery efficiency.*

Batch	Butalbital			Amobarbital			Pentobarbital			Secobarbital		
	Unex'ted	Ex'ted	%	Unex'ted	Ex'ted	%	Unex'ted	Ex'ted	%	Unex'ted	Ex'ted	%
B-373[a]	240	210	87.5	218	195	89.4	205	180	87.8	201	182	90.5
B-374[a]	181	130	71.8	192	150	78.1	191	169	88.5	217	196	90.3
B-380[b]	478	418	87.4	481	437	90.9	467	425	91.0	547	477	87.2
B-383[b]	646	558	86.4	513	468	91.2	528	480	90.9	556	499	89.7
Ave.			83.3			87.4			89.6			89.4

[a]Targeted concentrations for butalbital, amobarbital, pentobarbital, and secobarbital are 200 ng/mL. The certified values for these four barbiturates used in these two extraction batches are 200, 215, 208, and 198 ng/mL, respectively.

[b]Targeted concentrations for butalbital, amobarbital, pentobarbital, and secobarbital are 600 ng/mL. Test samples in these two extraction batches were separately prepared from the same concentrated stocks for recovery comparison purpose. The resulting preparation have not been certified and their true concentrations were not known.

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