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$^{13}C_4$-Secobarbital as the Internal Standard for the Quantitative Determination of Secobarbital—A Critical Evaluation*

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ABSTRACT: In this study, $^{13}C_4$-secobarbital was used as an exemplar compound to illustrate the mechanism based on which the effectiveness of a proposed internal standard (IS) could be evaluated. A deuterated analog, $^2H_5$-secobarbital, was also studied in parallel for comparison purposes. Well-established solid-phase extraction and methylation procedures were used prior to the GC/MS measurement step. The contribution of the intensity of an ion designated for the analyte (secobarbital) by the proposed IS, and similarly, the contribution of the intensity of an ion designated for the IS by the analyte—a phenomenon termed "cross-contribution"—were evaluated based on a "direct measurement" procedure in which equimolar amounts of the analyte and the IS were used to generate intensity data. These intensity data were then used as the basis for the calculation of "cross-contribution" (in percentages) of ions designated for the analyte and the IS. Cross-contribution data were compared with the linearity data resulting from two series of standards containing 25 to 9600 ng/mL secobarbital using two sets of quantitation ion pairs—*m*/*z* 196/200 and 195/199 with $^{13}C_4$-secobarbital as the IS and *m*/*z* 196/201 and 195/200 with $^2H_5$-secobarbital as the IS. $^{13}C_4$-secobarbital was found to be much less problematic and thus can serve as a very effective IS. Cross-contribution data alone cannot fully explain the observed differences resulting from the use of these two ISs; further systematic study is needed to provide better understanding of the underlying interference mechanism.

KEYWORDS: forensic science, toxicology, barbiturates, GC/MS, internal standard, $^{13}C_4$-secobarbital, $^2H_5$-secobarbital, cross-contribution

Contemporary practice of drug analysis in a biological matrix typically involves a set of specimen pretreatment protocol followed by a gas chromatography/mass spectrometry (GC/MS) measurement step (1,2). Recent emphasis (in workplace drug testing programs) in obtaining accurate quantitative results helps promote the development and use of the internal standard (IS) methodology (3–5). ^{2}H-analogs of the analytes are now the most popular choices of ISs. With practically identical chemical properties, isotopic analogs of the analytes can produce the best quantitative result by compensating for condition variations encountered throughout the entire specimen pretreatment and GC/MS analysis processes (6,7).

The use of an isotopic analog as the IS is not without problems. One of the most serious problems derives from the fact that it is rare, if not never, to find an ion designated for the IS that is completely free of contribution by the analyte. It is similarly difficult to find an ion designated for the analyte that is free of contribution by the IS. This phenomenon has been carefully studied and the undesirable contribution of ion intensity between the isotopic analog pair was termed "cross-contribution" (8,9).

Recently, a ^{13}C-analog of secobarbital has become commercially available. This study is designed to conduct a critical evaluation of this newly available IS to determine whether this specific ^{13}C-analog is advantageous over its ^{2}H-analog. Other ^{13}C-analogs will be studied later. It is the authors' long-term goal to characterize specific features of ^{2}H- and ^{13}C-analogs and to determine whether either group holds better potential to serve as ISs in quantitative analysis protocols.

Materials and Methods

Materials

$^{13}C_4$-secobarbital, an IS in 1 mg/mL methanol solution (99% purity) was provided by Isotec (Miamisburg, OH). Secobarbital (the analyte to be studied in this report) and five other barbiturates (amobarbital, butalbital, hexobarbital, pentobarbital, and phenobarbital) were purchased from Sigma (St. Louis, MO). $^2H_5$-secobarbital (99% purity) IS in 1 mg/mL was purchased from Radian Corporation (Austin, TX).

Reagents used for methylation of the analyte (and the ISs), tetramethylammonium hydroxide (TMAH, 25% in methanol), iodomethane, and dimethylsulfoxide (DMSO), were purchased from Aldrich (Milwaukee, WI). Bond Elut Certify™ solid-phase extraction (SPE) columns were obtained from Analytichem International, Varian (Harbor City, CA). Drug-free urine used for the preparation of standard drug solutions was provided by one member of the investigation team.

Standard Solutions

A series of standard solutions containing the following concentrations of six barbiturates were prepared using drug-free urine (pH 7.2) and a single source of stock containing 0.1 mg/mL of six barbiturates: 25, 50, 100, 200, 400, 800, 1200, 1600, 1800, 2400,

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3600, 4800, 6400, 7200, 9600 ng/mL. The stock solution was prepared by dissolving 50 mg of respective barbiturates (free acid) in ethanol (final volume, 50 mL). A single stock was used so that any deviation from perfect linearity, if observed, cannot be attributed to stock difference.

Solid-Phase Extraction and Derivatization

Procedures provided by the Bond Elute Certify™ manufacturer (10) were followed for processing the standard solutions using a specimen size of 2 mL. Each standard solution was spiked with 0.8 mL 0.1 M phosphate buffer (pH = 6) and 40 μL of 10 μg/mL IS resulting in 200 ng/mL IS in each specimen. (It was noted that the capacity of the buffer was exceeded, resulting in pH values ranging from 6.5 to 7.1 for the standard solutions containing the six barbiturates ranging from 25 to 9600 ng/mL. This procedure flaw did not affect the validity of the intended study and will be further discussed in the last paragraph of the Linearity (25 to 9600 ng/mL) Evaluation subsection.)

Conditioned columns were applied the standard solutions, rinsed, then eluted with 4 mL hexane/ethyl acetate into a 5 mL disposable centrifuge glass tube. Extracts were dried, then methylated and cleaned following the exact procedures described in our earlier report (9,11). The final product was dried and reconstituted with 20 μL (or otherwise specified volumes) ethyl acetate prior to GC/MS analysis.

GC/MS Analysis

With some minor variations in GC conditions, the instrumentation and procedures (HP 5890 GC interfaced to a HP 5970 MSD) used in our earlier study (9) were adapted. The initial temperature of 100°C was programmed to 150°C at 10°C/min, then to 270°C at 30°C/min and held for 5 min. These conditions provide adequate separation of six barbiturates (amobarbital, butalbital, pentobarbital, secobarbital, phenobarbital, and hexobarbital).

Standard solutions used for this study contain all six barbiturates; however, only secobarbital data will be discussed in this report. Relevant ions monitored (dwell time 75 ms) for secobarbital, $^{13}C_4$-secobarbital, $^{2}H_5$-secobarbital, and pentobarbital were *m*/*z* 196, 195, 181, 138, 111; 200, 199, 185, 141; 201, 200, 143, 116; and 169, respectively.

Cross-Contribution Evaluation

Full-scan mass spectra of derivatized secobarbital and its isotopic analogs ($^{2}H_5$-secobarbital and $^{13}C_4$-secobarbital) are shown in Fig. 1, based on which corresponding ion pairs with high intensities and apparently no (or insignificant) cross-contribution were selected as candidates for further evaluation.

Cross-contributions of the intensities of the corresponding ions designated for the isotopic analog pair studied were evaluated using a "direct measurement" procedure developed in our early study (9). Briefly, the intensities of ions to be evaluated were

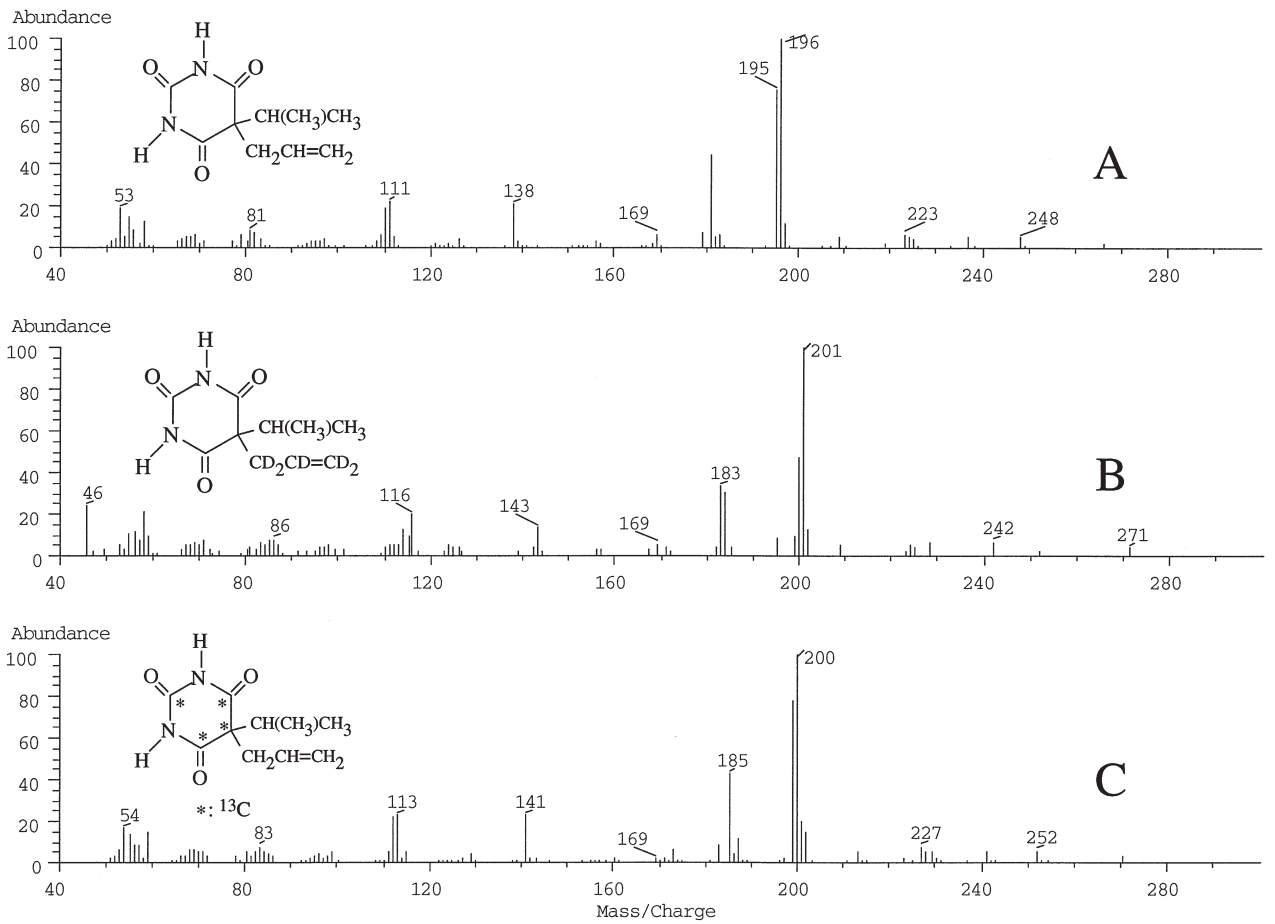

FIG. 1—*Full-scan mass spectra and chemical structures of* (A) *secobarbital,* (B) *$^{2}H_5$-secobarbital, and* (C) *$^{13}C_4$-secobarbital (as methyl derivatives).*

measured by separately injecting the equimolar amounts of the isotopic analogs into the GC/MS system. These ion intensity data were then used directly for the calculation of cross-contribution data.

Ion pairs evaluated were *m/z* 196/201, 195/200, 111/116, and 196/200, 195/199, 181/185 when $^2H_5$-secobarbital and $^{13}C_4$-secobarbital were used as the ISs, respectively. Cross-contribution data resulting from this procedure were further evaluated by their compatibility with parameters derived from a series of calibration standard solutions intended for evaluating the effectiveness of the proposed ISs (see further discussion in a later section).

Comparisons of Effectiveness of $^2H_5$-Secobarbital and $^{13}C_4$-Secobarbital

Standard solutions containing secobarbital ranging from 25 to 9600 ng/mL were used to evaluate the effectiveness (in terms of linearity resulting from a one-point calibration approach) of $^2H_5$-secobarbital and $^{13}C_4$-secobarbital serving as the ISs.

Three ion pairs were evaluated for each proposed IS to determine which ion pair provides the best calibration data for each IS. Results derived from the most effective ion pairs using different ISs were compared. "Inter-internal standard" data were also compared to determine whether there were significant differences between the best results provided by these two ISs studied. The mechanism causing the observed difference was investigated.

Results and Discussion

Linearity (25 to 9600 ng/mL Range) Evaluation

Triplicates of 200 ng/mL standard solutions were used as the one-point calibrator to derive the calibration data shown in Tables 1 and 2 for two series of standard solutions using $^{13}C_4$-secobarbital and $^2H_5$-secobarbital as the ISs, respectively.

Data resulting from the use of the $^{13}C_4$-analog as the IS are shown in Table 1. The observed and the theoretical concentrations show negligible differences when *m/z* 196/200 and 195/199 ion pairs were used as the basis for quantitative determination. Ion pair *m/z* 181/185 demonstrates inferior results (increasing deviation from theoretical values as analyte concentration increases).

Data resulting from the use of the $^2H_5$-analog as the IS are shown in Table 2. None of the best three ion pairs (*m/z* 196/201, 195/200, 111/116) provides results that are comparable with those generated by ion pairs *m/z* 196/200 and 195/199 shown in Table 1.

In the process of evaluating the linearity data shown in Tables 1 and 2, it was noted that, as the analyte concentration increases, the intensity ratios of the designated ions increase as expected; however, absolute intensities of these ions exhibit unexpected decrease when the analyte concentration becomes higher than 3600 ng/mL. pH measurements of repeated experiments concluded that the observed decreases were results of *reduced extraction efficiency* due to pH changes caused by insufficient buffer capacity. Insufficient buffer capacity (and, thus, the reduced extraction efficiency) became apparent in standard solutions in where the barbiturates concentrations were higher than 3600 ng/mL. It is interesting to note that this "protocol flaw" did not affect the validity of the calibration; in fact, this might have been the reason why excellent linearity still holds up to 9600 ng/mL!

Cross-Contribution Evaluation

Cross-contribution data derived from the "direct measurement" procedure are given in Table 3. These data show the following characteristics:

1. Both ISs ($^{13}C_4$-analog and $^2H_5$-analog) appear to make more significant contributions to the intensities of ions designated for the analyte, than the analyte to the ISs.
2. $^2H_5$-secobarbital appears to impose more contribution than $^{13}C_4$-secobarbital toward the intensity of ions designated for the analyte.
3. Ion pairs *m/z* 196/200 and 195/199 resulting from the use of $^{13}C_4$-secobarbital as the IS are the only ion pairs that cause $<0.5\%$ cross-contributions between the analyte and the IS.

TABLE 1—*Comparison of quantitation results using different ion pairs and ISs—$^{13}C_4$-secobarbital: m/z 196/200, 195/199, 181/185.*

Theoretical Conc.	*m/z* 196/200 Int. Ratio	Obs'ed Conc.	Dev. (%)	*m/z* 195/199 Int. Ratio	Obs'ed Conc.	Dev. (%)	*m/z* 181/185 Int. Ratio	Obs'ed Conc.	Dev. (%)
25	0.1694	26.6	+6.3	0.1634	27.0	+8.0	0.1844	30.5	+21.8
50	0.3317	52.0	+4.1	0.3100	51.2	+2.5	0.3401	56.2	+12.3
100	0.6713	105.3	+5.3	0.6342	104.8	+4.8	0.6594	108.9	+8.9
200	1.275*	(Calibrator)		1.210†	(Calibrator)		1.211‡	(Calibrator)	
400	2.578	404.4	+1.1	2.357	389.6	−2.6	2.383	393.6	−1.6
800	5.195	814.9	+1.9	4.895	809.1	+1.1	4.633	765.2	−4.4
1200	7.633	1197	−0.2	7.135	1179	−1.7	6.458	1067	−11.1
1600	10.19	1598	−0.1	9.48	1567	−2.1	8.345	1378	−13.9
1800	11.69	1834	+1.9	10.84	1792	−0.4	9.897	1635	−9.2
2400	15.16	2378	−0.9	14.30	2364	−1.5	12.52	2068	−13.8
3600	22.23	3478	−3.1	20.19	3337	−7.3	15.94	2633	−26.9
4800	30.05	4714	−1.8	27.69	4577	−4.6	20.55	3394	−29.3
6400	41.13	6425	+0.8	37.24	6155	−3.8	25.14	4152	−35.1
7200	44.63	7001	−2.8	40.16	6638	−7.8	25.22	4166	−42.2
9600	60.21	9445	−1.6	52.04	8602	−10.4	43.83	7239	−24.6

* This is the average of nine numbers, resulting from triplicates of the calibrator each injected in triplicates: 1.274, 1.289, 1.395; 1.274, 1.309, 1.280; 1.233, 1.245, 1.265.

† This is the average of nine numbers, resulting from triplicates of the calibrator each injected in triplicates: 1.204, 1.215, 1.210; 1.194, 1.201, 1.180; 1.225, 1.245, 1.218.

‡ This is the average of nine numbers, resulting from triplicates of the calibrator each injected in triplicates: 1.176, 1.207, 1.234; 1.229, 1.238, 1.210; 1.193, 1.189, 1.226.

TABLE 2—*Comparison of quantitation results using different ion pairs and ISs—$^2H_5$-secobarbital: m/z 196/201, 195/200, 111/116.*

Theoretical Conc.	*m/z* 196/201 Int. Ratio	Obs'ed Conc.	Dev. (%)	*m/z* 195/200 Int. Ratio	Obs'ed Conc.	Dev. (%)	*m/z* 111/116 Int. Ratio	Obs'ed Conc.	Dev. (%)
25	0.1743	28.6	+14.4	0.3943	40.3	+61.0	0.2954	45.7	+82.8
50	0.3267	53.6	+7.2	0.6389	65.2	+30.5	0.4557	70.5	+41.0
100	0.6507	106.8	+6.8	1.1171	14.0	+14.0	0.7643	118.2	+18.2
200	1.219*	(Calibrator)		1.959†	(Calibrator)		1.293‡	(Calibrator)	
400	2.413	355.9	−1.0	3.736	381.4	−4.6	2.474	382.7	−4.6
800	4.898	803.6	+0.5	7.440	759.6	−5.1	4.891	756.5	−5.4
1200	5.773	947.2	−21.7	8.718	890.0	−25.8	5.814	899.3	−25.1
1600	7.893	1295	−19.1	11.89	1214	−24.1	7.817	1209	−24.4
1800	9.797	1607	−10.7	14.55	1485	−17.5	9.444	1461	−18.8
2400	11.99	1967	−18.0	18.02	1840	−23.3	11.91	1842	−23.2
3600	15.22	2497	−30.6	23.45	2394	−33.5	16.13	2495	−30.7
4800	19.05	3126	−34.9	27.65	2823	−41.2	19.73	3052	−36.4
6400	27.08	4443	−30.6	41.27	4213	−34.2	27.95	4323	−32.4
7200	31.33	5140	−28.6	46.76	4774	−33.7	34.24	5296	−26.4
9600	52.03	8537	−11.1	77.65	7928	−17.4	47.08	7282	−24.1

* This is the average of nine numbers, resulting from triplicates of the calibrator each injected in triplicates: 1.262, 1.233, 1.233; 1.222, 1.194, 1.190; 1.227, 1.212, 1.197.

† This is the average of nine numbers, resulting from triplicates of the calibrator each injected in triplicates: 1.999, 1.957, 1.963; 1.961, 1.951, 1.938; 1.942, 1.986, 1.937.

‡ This is the average of nine numbers, resulting from triplicates of the calibrator each injected in triplicates: 1.283, 1.308, 1.342; 1.296, 1.301, 1.324; 1.287, 1.247, 1.246.

Thus, the observed inferior linearity (Table 2) resulting from the use of $^2H_5$-analog as the IS is consistent with the cross-contributions data hereby concluded.

The existence and the extent of cross-contribution can also be evaluated by observing the variation of *intra-molecular* ion intensity ratios as the concentrations of the analyte is increased in a series of standard solutions used in calibration runs. Ion intensity data collected for the calibration runs (partially shown in Tables 1 and 2) are used as the basis for this evaluation. Significant intra-molecular ion intensity ratio data are shown in Tables 4 and 5.

It is noted that the *analyte intra-molecular ion pair intensity ratio m/z* 195/196 (Table 4, where $^{13}C_4$-analog is the IS) are not changed as the analyte concentration increases from 25 to 9600 ng/mL. This is an indication that ions *m/z* 195 and 196 (derived from the analyte) receive no or equal contribution from the IS ($^{13}C_4$-analog). On the contrary, ion pair intensity ratios *m/z* 181/195 and 181/196 are obviously decreasing, in the concentration range approximately 25 to 400 ng/mL, as the analyte concentration increases. These are indications that ion *m/z* 181 is contributed by the IS. Thus, the degree of contributions is in the order of $181 > 195 \approx 196$.

When examining the *$^{13}C_4$-secobarbital intra-molecular ion pair intensity ratios*, it is observed that *m/z* 185/199 and 199/200 (Table 4) ratios show obvious increases as the analyte concentration increases. Thus, part of the intensities monitored for ions *m/z* 185 and 199 (and perhaps 200) are contributed by the analyte. The degree of contributions is in the order of m/z $185 > 199 > 200$.

In the case where $^2H_5$-secobarbital is used as the IS, data in Table 5 show decreases in ion pair intensity ratios for *m/z* 195/196 and 111/196 (*analyte intra-molecular ion pair intensity ratios*) in the range where the analyte concentration increases from 25 to 400 ng/mL. These are indications that parts of the intensities monitored for ions (for the analyte) *m/z* 111 and 195 (and perhaps 196) are contributed by $^2H_5$-secobarbital. The degree of contribution is in the order of $111 \approx 195 > 196$.

TABLE 3—*Ion cross-contribution between analyte and internal standard.*

Internal Standard	Ions (*m/z*) Designated for Analyte (% analyte; % contributed by IS)	Ions (*m/z*) Designated for IS (% IS; % contributed by analyte)
$^{13}C_4$-secobarbital	196 (100; 0.34)	200 (100; 0.01)
	195 (69.7; 0.28)	199 (75.1; 0.12)
	181 (40.7; 2.14)	185 (43.7; 0.19)
	138 (19.8; 2.60)	141 (23.5; 1.73)
$^2H_5$-secobarbital	196 (100; 1.38)	201 (100; 0.01)
	195 (70.4; 10.2)	200 (61.8; 0.04)
	138 (20.7; 4.06)	143 (19.2; 3.34)
	111 (19.8; 8.31)	116 (26.6; 0.12)

Similarly, increases in *$^2H_5$-secobarbital intra-molecular ion intensity ratios m/z* 116/201 and 116/200 (Table 5) are indications that part of the intensity monitored for ions *m/z* 116 (and perhaps *m/z* 200 and 201) are contributed by secobarbital. The degree of contribution is in the order of m/z $116 > 200 \approx 201$.

It is worth noting that the degree of cross-contribution data derived from data shown in Tables 4 and 5 are in agreement with those shown in Table 3.

Interference Mechanism

Unexpected ion intensity decreases in where the analyte concentration is higher than 3600 ng/mL (Tables 1 and 2), prompted the investigation for other potential mechanisms that might have been the cause for the observed linearity difference resulting from the use of different ISs ($^{13}C_4$-analog vs. $^2H_5$-analog as shown in Tables 1 and 2). One of the experiments performed involved the reinjection (for SIM GC/MS analysis) of the extraction/derivatization products using different reconstitution volumes. Results shown in Table 6 indicate that, as the reconstitution volume changes, the designated ion pair intensity ratio (*m/z* 196/200) remains constant

TABLE 4—*Comparison of intra-molecular ion pair ratios for evaluating degree of cross-contribution between secobarbital and $^{13}C_4$-secobarbital.*

Theor. Conc.	Ratios of Ions (*m*/*z*) Derived from Analyte			Ratios of Ions (*m*/*z*) Derived from IS		
	195/196	181/196	181/195	199/200	185/200	185/199
25	0.7170	0.5005	0.6980	0.7432	0.4598	0.6184
50	0.7048	0.4789	0.6795	0.7541	0.4671	0.6194
100	0.7147	0.4778	0.6685	0.7566	0.4865	0.6430
200	0.7175	0.4603	0.6416	0.7560	0.4873	0.6446
400	0.6980	0.4280	0.6131	0.7634	0.4630	0.6065
800	0.7120	0.4350	0.6110	0.7557	0.4878	0.6456
1200	0.7143	0.4431	0.6204	0.7641	0.5238	0.6855
1600	0.7053	0.4417	0.6262	0.7582	0.5394	0.7114
1800	0.7132	0.4522	0.6341	0.7685	0.5340	0.6948
2400	0.7261	0.4571	0.6296	0.7700	0.5536	0.7190
3600	0.7081	0.4764	0.6728	0.7797	0.6642	0.8519
4800	0.7235	0.4605	0.6364	0.7852	0.6732	0.8573
6400	0.7217	0.4540	0.6274	0.7993	0.7431	0.9295
7200	0.7117	0.4839	0.6799	0.7907	0.8561	1.083
9600	0.7093	0.4493	0.6334	0.8206	0.6172	0.7521

TABLE 5—*Comparison of intra-molecular ion pair ratios for evaluating degree of cross-contribution between secobarbital and $^{2}H_5$-secobarbital.*

Theor. Conc.	Ratios of Ions (*m*/*z*) Derived from Analyte			Ratios of Ions (*m*/*z*) Derived from IS		
	195/196	111/196	111/195	200/201	116/201	116/200
25	1.039	0.3146	0.2877	0.4823	0.1856	0.3839
50	0.9254	0.2587	0.2796	0.4733	0.1855	0.3920
100	0.8281	0.2245	0.2711	0.4824	0.1911	0.3962
200	0.7756	0.2078	0.2680	0.4816	0.1955	0.4060
400	0.7532	0.1990	0.2642	0.4865	0.1941	0.3989
800	0.7428	0.1979	0.2665	0.4890	0.1982	0.4053
1200	0.7378	0.2048	0.2776	0.4886	0.2034	0.4163
1600	0.7283	0.2047	0.2811	0.4835	0.2067	0.4275
1800	0.7318	0.2088	0.2853	0.4928	0.2166	0.4395
2400	0.7257	0.2154	0.2968	0.4830	0.2169	0.4491
3600	0.7423	0.2329	0.3138	0.4818	0.2198	0.4562
4800	0.6996	0.2389	0.3415	0.4821	0.2307	0.4786
6400	0.7393	0.2311	0.3126	0.4851	0.2239	0.4615
7200	0.7339	0.2307	0.3144	0.4918	0.2111	1.4293
9600	0.7299	0.2059	0.2821	0.4891	0.2276	0.4653

when the $^{13}C_4$-analog is used as the IS; whereas, the corresponding ion intensity ratio (*m*/*z* 196/201) changes when the $^{2}H_5$-analog is used as the IS. Similarly, other analyte/IS ion pair intensity ratios (*m*/*z* 195/199 and 181/185 for secobarbital/$^{13}C_4$-analog; *m*/*z* 195/200 and 111/116 for secobarbital/$^{2}H_5$-analog) exhibit the same trends (data not shown). This same phenomenon was observed for all three sets of standard solutions where the analyte concentrations are 1800, 3600, and 7200 ng/mL (Table 6).

This unexpected phenomenon resulting from the use of the $^{2}H_5$-analog as the IS is further investigated. A standard solution containing 6400 ng/mL secobarbital, 200 ng/mL $^{2}H_5$-secobarbital, and 6400 ng/mL pentobarbital (the third compound) was studied. Data shown in Table 7 demonstrate that ion pair intensity ratio *m*/*z* 196/201 (analyte/IS) increases continuously as the reconstitution volume increases. It is also interesting to note that, as the reconstitution volume is increased, the ion pair intensity ratio *m*/*z* 196/169 (analyte/3rd compound) increases, while the ion pair intensity ratio *m*/*z* 201/169 ($^{2}H_5$-analog/3rd compound) decreases. Thus, as the solution is diluted prior to its injection into the GC/MS system, relative intensities of the ions designated for the analyte increase, while that designated for the IS decrease.

The implications of the hereby observed phenomenon are alarming. While the exact mechanism underlying this phenomenon is still under investigation, it is sufficient to note here that an unknown interference mechanism plays a significant role when the $^{2}H_5$-analog is used as the IS.

Data hereby presented clearly demonstrated that the $^{13}C_4$-analog is superior over its $^{2}H_5$-analog in serving as the IS for the quantitation of secobarbital. Other series of isotopic analogs are currently being studied in the authors' laboratories to determine whether the observed advantages of the ^{13}C-analog also hold for other compounds.

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TABLE 6—*Analyte/IS ion pair intensity ratio as a function of molecular abundance—$^{13}C_4$-secobarbital versus $^{2}H_5$-secobarbital as IS.*

Theo. Conc.	Reconstitute Volume*	Ion Pair: *m/z* 196/200 ($^{13}C_4$-analog as IS)			Ion Pair: *m/z* 196/201 ($^{2}H_5$-analog as IS)		
		Analyte	IS	Ratio	Analyte	IS	Ratio
1800	20 μL†	14,878,363	1,273,227	11.69	33,747,776	3,444,629	9.797
	20 μL	26,506,997	2,252,966	11.77	30,481,113	3,062,372	9.953
	40 μL	5,630,347	484,704	11.62	11,536,986	1,037,331	11.12
3600	20 μL†	62,571,014	2,815,062	22.23	61,015,175	4,009,233	15.22
	20 μL	84,945,046	3,567,002	23.81	56,959,620	3,876,715	14.69
	40 μL	31,926,593	1,359,238	23.49	21,413,378	1,011,270	21.17
7200	20 μL†	50,536,998	1,132,435	44.63	53,211,356	1,698,361	31.33
	20 μL	76,406,911	1,727,121	44.24	56,753,079	1,785,856	31.78
	40 μL	24,741,936	554,001	44.66	23,058,590	567,319	40.64

* Reuse of the samples left after one injection by first evaporating to dryness, then reconstituting with 20 μL, followed by another aliquot of 20 μL.
† These data were from previous injections for establishing the calibration curve.

TABLE 7—*Analyte/IS ion pair intensity ratio as a function of molecular abundance—$^{2}H_5$-secobarbital (secobarbital: 6400 ng/mL; $^{2}H_5$-secobarbital: 200 ng/mL).*

Reconstitute Volume	Intensity Ratios of Ions (*m/z*) Monitored		
	196/201	196/169	201/169
20 μL	27.08*	0.6300	0.02327
10 μL†,‡	25.16	0.7267	0.02890
20 μL‡	25.69	0.7511	0.02932
40 μL‡	34.19	0.9688	0.02845
60 μL‡	37.18	1.034	0.02797
80 μL‡	39.42	1.053	0.02682
120 μL‡	41.97	1.103	0.02642
140 μL‡	44.10	1.107	0.02520

* This ratio is the same as that shown in Table 2.
† Reuse of the samples left after one injection by first evaporating to dryness, then reconstituting with 10 μL, followed by addition of more ethyl acetate.
‡ Three injections were made; data shown are means of three injections.

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