J Forensic Sci, July 2002, Vol. 47, No. 4
Paper ID JFS2001286_474
Available online at: www.astm.org

Wei-Tun Chang,[1] *Ph.D.; Jaimie Smith,*[2] *B.S.; and Ray H. Liu,*[2] *Ph.D.*

Isotopic Analogs as Internal Standards for Quantitative GC/MS Analysis—Molecular Abundance and Retention Time Difference as Interference Factors*

ABSTRACT: The following analyte/isotope-labeled internal standard (IS) systems are adapted to further study the interference phenomenon previously reported from our laboratory—the intensity ratio of the ion-pair designated for a specific analyte/^{2}H-analog system increases as the solvent used to reconstitute the extraction/derivatization residue is increased: (a) Three analyte/^{2}H-analog pairs with ^{2}H-atoms positioned at allylic sites (butalbital, secobarbital, methohexital); (b) Two analyte/^{2}H-analog pairs without these structural features (pentobarbital, phenobarbital); and (c) Two analyte/^{13}C-analog pairs (butalbital, secobarbital). Major experimental parameters adapted in this study include: (a) Varying reconstitution solvent volume while keeping a constant analyte/IS concentration ratio; (b) Varying analyte/IS concentration ratio; (c) Varying gas chromatograph (GC) injection port temperature; and (d) Varying GC column temperature programming conditions, rendering difference in the degree of overlap of the peaks derived from the analyte and the ^{2}H-analog. This study results in the following observations: (a) Changes in the intensity ratio of the ion-pair designated for a specific analyte/^{2}H-analog system depend on molecular abundance, regardless of whether the ^{2}H-atoms are positioned at active allylic positions or not—thus, ruling out hydrogen/deuterium exchange as the cause of the observed interference phenomenon; (b) Variations in GC injection port temperature do not alter the observed interference phenomenon—thus, ruling out chemical reactions at the injection port as the underlying cause; (c) Variations in peak-overlapping between the analyte and the ^{2}H-analog, facilitated by changing GC column programming conditions, alter the observed interference phenomenon. Abundance of the analyte and the ^{2}H-analog and their overlapping characteristic in the mass spectrometer ion source are believed to be the underlying cause of the observed interference phenomenon. The interference phenomenon observed for a specific analyte/^{2}H-analog system has significant consequences on the linearity of the thereby generated calibration curves. Nonlinear approaches can better describe the calibration data and are needed more in comparison to systems in which ^{13}C-analogs are used as the ISs.

KEYWORDS: forensic science, GC/MS, quantitation, internal standard, barbiturate, cross-contribution

Internal standard (IS) method has long been established (1,2) as one of the most effective approaches for the quantitations of analytes in specimens with complex matrix. ^{2}H-Analogs of the analytes are now preferred ISs and routinely adapted for the analysis of abused drugs and their metabolites (analytes) in biological matrices (3). In this application, accurate quantitation of an analyte relies on measuring the intensity ratio of a selected ion-pair (designated for the analyte and the IS) that precisely reflects the analyte/IS concentration ratio in the test specimen.

While evaluating the effectiveness of the $^{13}C_4$- and $^2H_5$-analogs of secobarbital (4) and butalbital (5) in serving as the ISs, we have observed an interference phenomenon in cases where $^2H_5$-analogs of these two barbiturates were adapted. Specifically, the intensity ratios of the ion-pairs designated for these two analytes and their respective $^2H_5$-analogs increase as the volume of the solvent (ethyl acetate) used to reconstitute the extraction/derivatization residue increases. This phenomenon was not observed when the respective $^{13}C_4$-analogs were used as the ISs in parallel experiments.

This current study is carried out to determine whether any of the following parameters is the underlying cause of the observed phenomenon: (a) Chemical reaction at the GC injection port; (b) ^{2}H-atoms at the active allylic sites in the ^{2}H-analogs; (c) Analytes/^{2}H-analogs molecular abundance at the mass spectrometer ion source; and (d) Overlap of the peaks derived from the analytes and their ISs.

Three barbiturates (butalbital, secobarbital, methohexital) and their ^{2}H-analogs with ^{2}H-atoms positioned at the allylic sites are studied along with two barbiturates (pentobarbital, phenobarbital) and their ^{2}H-analogs without this structural feature. Butalbital/$^{13}C_4$-butalbital and secobarbital/$^{13}C_4$-secobarbital systems are also included in this study for comparison purpose.

Materials and Methods

Butalbital, methohexital, pentobarbital, secobarbital, and phenobarbital (five analytes in 1 mg/mL methanol solution, 99% purity) were purchased from Radian (Austin, TX). $^2H_5$-butalbital, $^2H_5$-methohexital, $^2H_5$-pentobarbital, $^2H_5$-secobarbital, and $^2H_5$-phenobarbital (five ISs in 0.1 mg/mL methanol solution, 99% purity), were also purchased from Radian. $^{13}C_4$-butalbital and $^{13}C_4$-secobarbital (two ISs in 1 mg/mL methanol solution, 99% purity) were provided by Isotec (Miamisburg, OH). Reagents used for the derivatization of the analytes (and the ISs), tetramethylammonium

[1] Professor, Department of Forensic Science, Central Police University, Taoyuan, Taiwan.
[2] Graduate student and professor and program director, respectively, Graduate Program in Forensic Science, University of Alabama at Birmingham, Birmingham, AL.

* This study was presented in part at the 53rd Annual Meeting of the American Academy of Forensic Sciences held in Seattle, WA, February 2001.

Received 20 Aug. 2001; and in revised form 14 Jan. 2002; accepted 14 Jan. 2002; published 7 June 2002.

hydroxide (TMAH, 25% in methanol), iodomethane, and dimethylsulfoxide (DMSO), were purchased from Aldrich (Milwaukee, WI).

All analytes and ISs were methylated prior to GC/MS analysis following the same procedures adapted in our earlier studies (4–6). A Hewlett-Packard (Palo Alto, CA) HP 5890 gas chromatograph interfaced to a HP 5970 mass selective detector (MSD) was used to acquire full-scan and SIM mass spectrometric data. Full-scan mass spectra of the derivatized analytes and ISs were obtained by scanning from *m/z* 45 to 320. The resulting mass spectra and the chemical structures of these compounds are shown in Fig. 1.

These data were then used to preliminarily select analogous ion

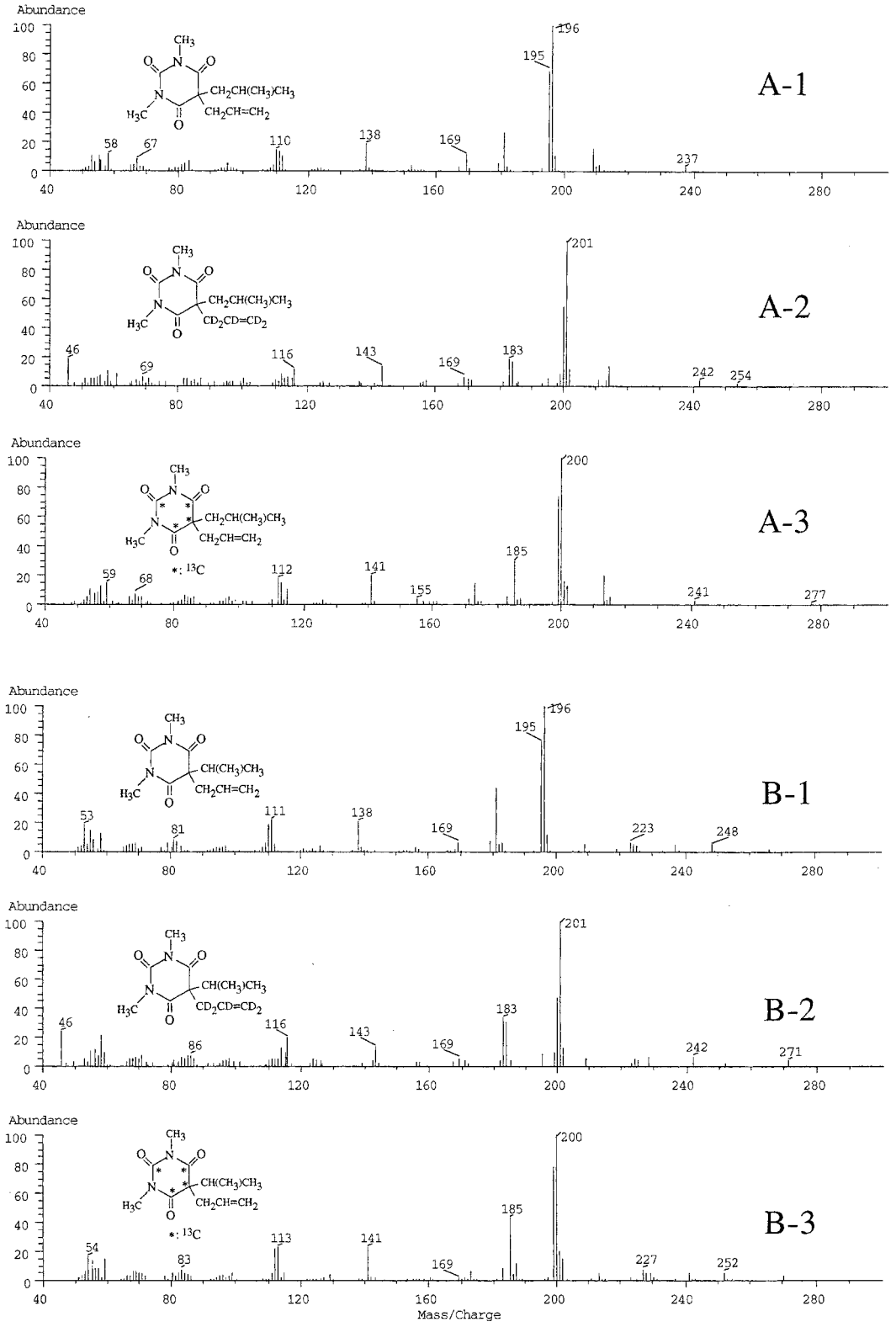

FIG. 1—*Full-scan mass spectra of analytes/isotope-labeled analogs (all as methyl-derivatives): Secobarbital/$^{2}H_5$-secobarbital/$^{13}C_4$-secobarbital* (A)*; Butalbital/$^{2}H_5$-butalbital/$^{13}C_4$-butalbital* (B)*; Methohexital/$^{2}H_5$-methohexital* (C)*; Pentobarbital/$^{2}H_5$-pentobarbital* (D)*; Phenobarbital/$^{2}H_5$-phenobarbital* (E)*.*

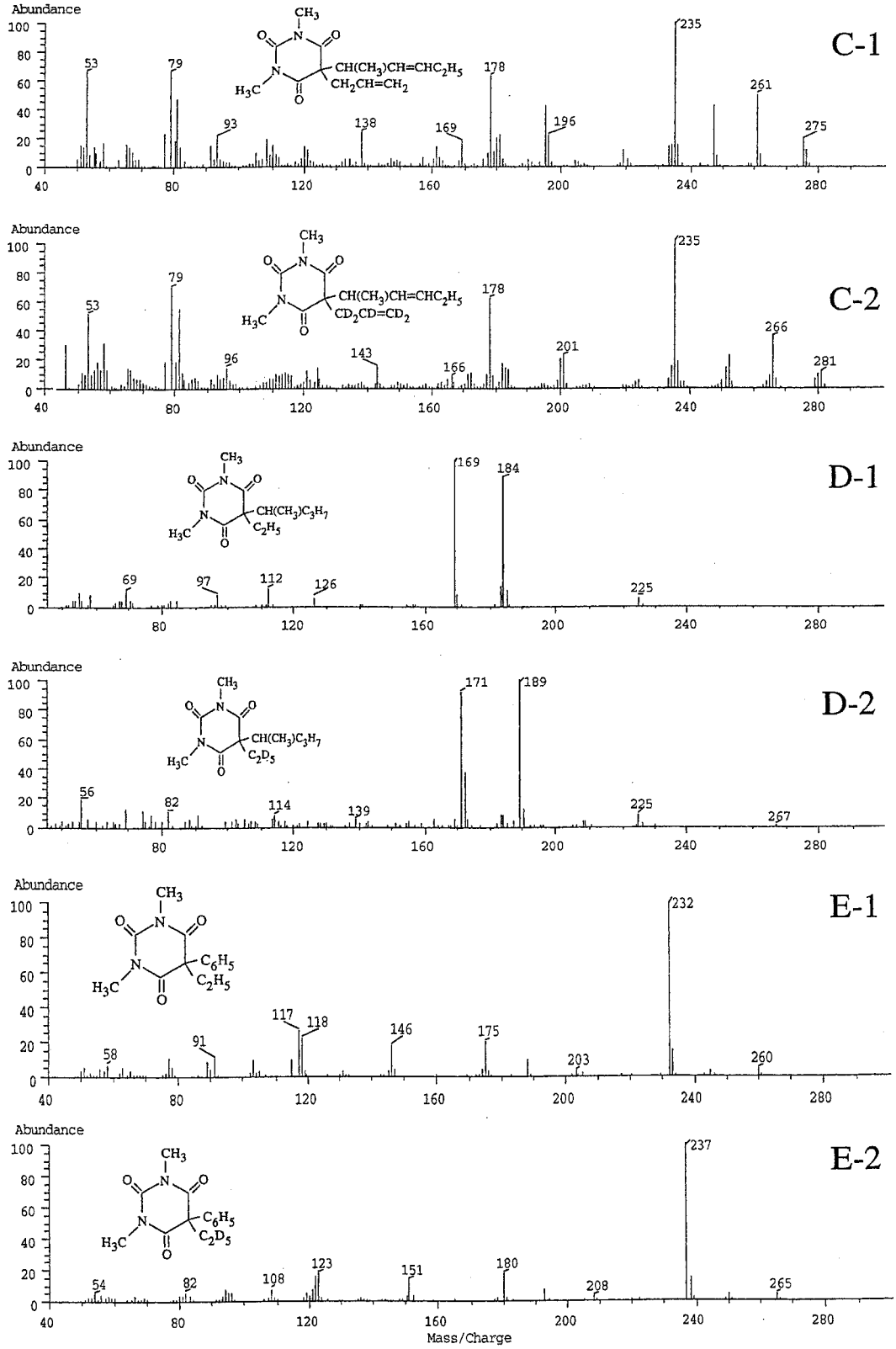

FIG. 1—*(continued)*

pairs that were apparently free (or with minimal) cross-contribution between the analytes and their respective isotopic analogs. For quantitative determination, ion pairs monitored (dwell time 30 ms) for butalbital/$^{2}H_5$-butalbital/$^{13}C_4$-butalbital, methohexital/$^{2}H_5$-methohexital, pentobarbital/$^{2}H_5$-pentobarbital, secobarbital/$^{2}H_5$-secobarbital/$^{13}C_4$-secobarbital, and phenobarbital/$^{2}H_5$-phenobarbital were *m/z* 196/201/200, 261/266, 184/189, 196/201/200, and 232/237, respectively.

Results and Discussion

The main objective of this study is to identify the cause underlying the concentration dependency characteristics of analyte/la-

beled-analog ion-pair intensity ratios observed in the barbiturate/$^2H_5$-analog, but not in barbiturates/$^{13}C_4$-analog systems (4,5). Parameters studied include (a) GC injector temperature; (b) variation in analyte/^{2}H-analog molecular abundance with a constant concentration ratio; (c) variation in relative analyte/^{2}H-analog molecular abundance; (d) position of ^{2}H-atoms at the molecular framework; and (e) variation in the degree of overlap for peaks derived from the analytes and their ^{2}H-analogs.

Effect of GC Injector Temperature

Data derived from the variations of GC injection port temperature (from 175 to 275°C) are shown in Table 1. Ion-pair intensity ratio characteristics for the butalbital/$^2H_5$-analog and the butalbital/$^{13}C_4$-analog remain the same at various injection port temperature settings. Specifically, for a set reconstitution volume (20 or 60 μL), the monitored ion-pair intensity ratios stay the same regardless of the injection port temperature. On the other hand, as the reconstitution volume is changed from 20 to 60 μL, the monitored ion-pair intensity ratios for the butalbital/$^2H_5$-analog system changed, while the corresponding ratios for the butalbital/$^{13}C_4$-analog system stay the same. Thus, chemical reaction at the injector is ruled out as the underlying cause for the phenomenon observed for the barbiturate/$^2H_5$-analog systems.

Position of ^{2}H-Atoms in the Molecular Structure

Molecular structures included in Fig. 1 indicate that ^{2}H-atoms in $^2H_5$-secobarbital (A-2), $^2H_5$-butalbital (B-2), and $^2H_5$-methohexital (C-2) are positioned at active allylic positions, while ^{2}H-atoms in $^2H_5$-pentobarbital (D-2) and $^2H_5$-phenobarbital (E-2) are positioned at less active sites.

Data shown in Table 2 indicate that the monitored ion-pair intensity ratios for all five barbiturate/$^2H_5$-analog systems increase as the reconstitution volume is increased—regardless of whether the ^{2}H-atoms are placed in active sites (butalbital, secobarbital and methohexital), or non-active sites (pentobarbital and phenobarbital). This phenomenon is not observed in the two barbiturate/$^{13}C_4$-analog systems.

This phenomenon is further studied by three sets of extended dilution experiments (methohexital/$^2H_5$-analog, butalbital/$^2H_5$-analog, and butalbital/$^{13}C_4$-analog), using 2500 ng/mL of each analyte and 400 ng/mL of the corresponding IS. Each sample is reconstituted with 10-μL ethyl acetate, followed by one 1-μL injection; and then, the addition of another 10-μL ethyl acetate and 1-μL injection. This addition-and-injection process is continued until a total of 150 μL of the reconstitution solvent is added and then injected.

Increases in ion-pair intensity ratios (as shown in Fig. 2*A* and 2*B*) appear to be more significant at the beginning and gradually reduced. Thus, the magnitude of the ratio increase for the analyte/$^2H_5$-analog systems appears to associate with the ion intensities of the concerned analytes, i.e., when the ion intensity of the analyte is significantly higher, increases in the monitored ion-pair intensity ratios following the addition of the reconstitution solvent are more significant. As shown in Fig. 2*C,* following the addition of reconstitution solvent, the ion-pair intensity ratio monitored for the butalbital/$^{13}C_4$-butalbital system remains the same.

Variation in Analyte/^{2}H-Analog Relative Molecular Abundance

Three series of solutions were prepared to further investigate the relationship between the ion-pair intensity ratio change and the analyte/^{2}H-analog concentration level. The first series of solutions include a constant amount of ^{2}H-butalbital (200 ng/mL), with the concentration of butalbital ranging from 200 to 2500 ng/mL. Data derived from this series of solutions (Table 3) clearly indicate that, as the volume of the reconstitution solvent is increased, the observed ion-pair intensity ratio increase is more significant when the concentration of the analyte is at a higher level. Again, this phenomenon is not observed when the ^{13}C-analog is used as the IS.

TABLE 1—*Butalbital/isotopic analog ion-pair intensity ratio as a function of molecular abundance under different injector temperatures—Butalbital: 2500 ng/mL; $^2H_5$- and $^{13}C_4$-analog: 200 ng/mL.*

	Butalbital/$^2H_5$-analog Ion Intensity Ratio				Butalbital/$^{13}C_4$-analog Ion Intensity Ratio			
	(*m/z* 196/201)		(*m/z* 181/184)		(*m/z* 196/200)		(*m/z* 181/185)	
Injector Temp.	20 μL	60 μL	20 μL	60 μL	20 μL	60 μL	20 μL	60 μL
175°C	13.63	15.03	19.79	21.17	15.13	15.30	14.66	14.66
200°C	13.12	15.28	19.21	21.79	15.31	15.28	14.55	14.88
225°C	13.15	15.48	19.43	21.78	15.30	15.33	14.85	14.87
250°C	13.20	15.45	18.90	21.64	15.17	15.34	14.57	14.63
275°C	13.43	15.49	19.31	21.82	15.10	15.63	14.50	14.80

TABLE 2—*Analyte/isotopic analog ion-pair intensity ratio as a function of molecular abundance—Analyte: 2500 ng/mL; Isotopic analog: 400 ng/mL.*

	Analyte/$^2H_5$-analog (*m/z*)					Analyte/$^{13}C_4$-analog (*m/z*)	
Reconstitute Volume (μL)	Butalbital (196/201)	Secobarbital (196/201)	Methohexital (261/266)	Pentobarbital (184/189)	Phenobarbital (232/237)	Butalbital (196/200)	Secobarbital (196/200)
10	5.429	5.997	8.814	11.87	6.831	7.586	7.018
30	6.697	7.167	9.110	12.58	7.726	7.548	7.051
60	6.790	7.435	9.715	12.93	7.878	7.549	7.022
100	7.134	7.644	10.56	13.34	7.878	7.547	6.926
150	7.132	7.703	10.84	13.44	7.955	7.518	6.867

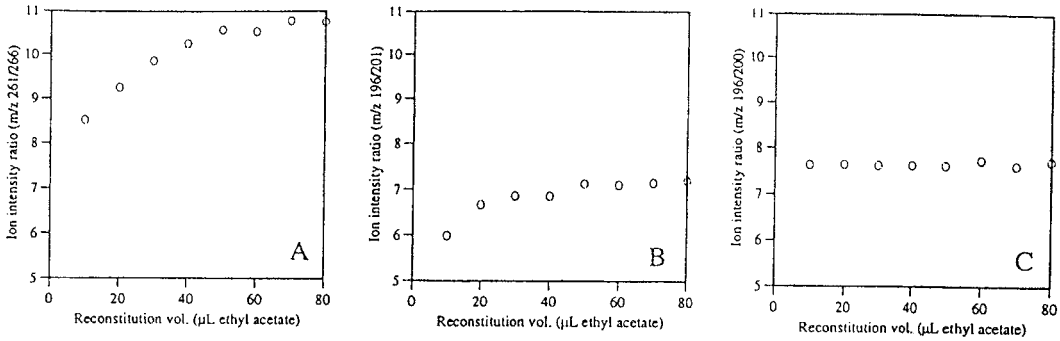

FIG. 2—*Changes of ion intensity ratios (analyte: 2500 ng/mL; isotopic IS: 200 ng/mL) when solutions are reconstituted with 10 to 80 μL of ethyl acetate: Methohexital/$^2H_5$-methohexital* (A)*; Butalbital/$^2H_5$-butalbital* (B)*; and Butalbital/$^{13}C_4$-butalbital* (C)*. (All as methyl-derivatives.)*

TABLE 3—*Comparison of changes in ion-pair intensity ratios in butalbital/$^2H_5$-analog and butalbital/$^{13}C_4$-analog system using 20 and 60 μL of ethyl acetate for constitution.*

	Butalbital/$^2H_5$-analog (*m/z* 196/201)			Butalbital/$^{13}C_4$-analog (*m/z* 196/200)		
Concn* (ng/mL)	Intensity Ratio (20 μL)	Intensity Ratio (60 μL)	Ratio Change (%)	Intensity Ratio (20 μL)	Intensity Ratio (60 μL)	Ratio Change (%)
200	1.199	1.201	0.25	1.104	1.107	0.25
400	2.358	2.387	1.20	2.395	2.448	2.19
600	3.049	3.158	3.57	3.484	3.456	−0.79
800	3.805	4.119	8.20	4.545	4.579	0.75
1,000	5.617	6.040	7.50	5.738	5.928	3.30
1,250	6.281	6.782	8.00	7.253	7.148	−1.44
1,600	7.320	8.367	14.3	9.366	9.319	−0.50
2,000	9.133	10.68	16.9	11.79	11.88	0.73
2,500	11.53	13.45	16.7	15.10	15.05	−0.34

* Internal standard concentrations: 200 ng/mL.

The second series of solutions include a constant amount of the analyte, while the amount of the IS is varied. Data shown in Table 4 indicate a larger increase in the monitored ion intensity ratio when the analyte/^{2}H-analog concentration ratio is larger.

The third series of solutions involve secobarbital and pentobarbital—two compounds that are chromatographically resolved. Again, the monitored ion-pair intensity ratio is increased as the reconstitution volume is increased—until a certain amount of the reconstitution volume is reached (Table 5).

Data derived from these three series of solutions demonstrate one common phenomenon, i.e., the *magnitude* and the *difference* in the intensities of the ions derived from the analyte and the ^{2}H-analog IS are the deciding factors for the observed increase of the monitored ion-pair intensity ratios. To emphasize again, the monitored ion-pair intensity ratio remains constant when the $^{13}C_4$-analogs are used as the ISs, as exemplified by the secobarbital and the butalbital systems.

Programming in GC Column Temperature (Variation in the Degree of Peak-Overlap)

Analyte/$^{13}C_4$-analog systems differ from the corresponding analyte/$^2H_5$-analog systems in displaying an identical retention time for the analytes and the ISs (Figs. 3*F* and 3*G*). Thus, *retention time difference between the analyte and the IS* is hypothesized as the underlying factor causing the increase in the ion-pair intensity ratio observed for the analyte/$^2H_5$-analog systems (but not for the analyte/$^{13}C_4$-analog systems). To prove this hypothesis, a series of experiments were performed, in which GC column temperature pro-

TABLE 4—*Secobarbital/$^2H_5$-analog ion-pair intensity ratio (m/z 196/201) as a function of molecular abundance—Concentrations of secobarbital and $^2H_5$-analog: 10 μg/mL.*

Reconstitute Volume (μL)	Secobarbital/$^2H_5$-analog (μL/μL)				
	500/40	500/80	500/120	500/240	500/500
10	12.65	5.997	4.377	2.091	0.9753
30	14.53	7.167	4.814	2.276	1.048
60	15.23	7.435	5.010	2.346	1.049
100	15.35	7.644	5.129	2.382	1.060
150	16.12	7.703	5.126	2.402	1.064
Ratio change*	27.4%	28.4%	17.1%	14.9%	9.1%

* Ratio changes are calculated based on the values observed with reconstitute volumes of 10 μL and 150 μL.

TABLE 5—*Secobarbital/pentobarbital ion-pair intensity ratio as a function of molecular abundance—Secobarbital: 2500 ng/mL; pentobarbital: 500 ng/mL.*

	Ion Intensity		
Reconstitute Volume (μL)	Secobarbital (*m/z* 196)	Pentobarbital (*m/z* 169)	Ratio
10	23,659,688	5,964,237	3.967
30	12,012,431	2,849,291	4.216
60	4,299,982	943,654	4.557
100	2,792,676	630,932	4.426
150	2,138,446	486,560	4.397

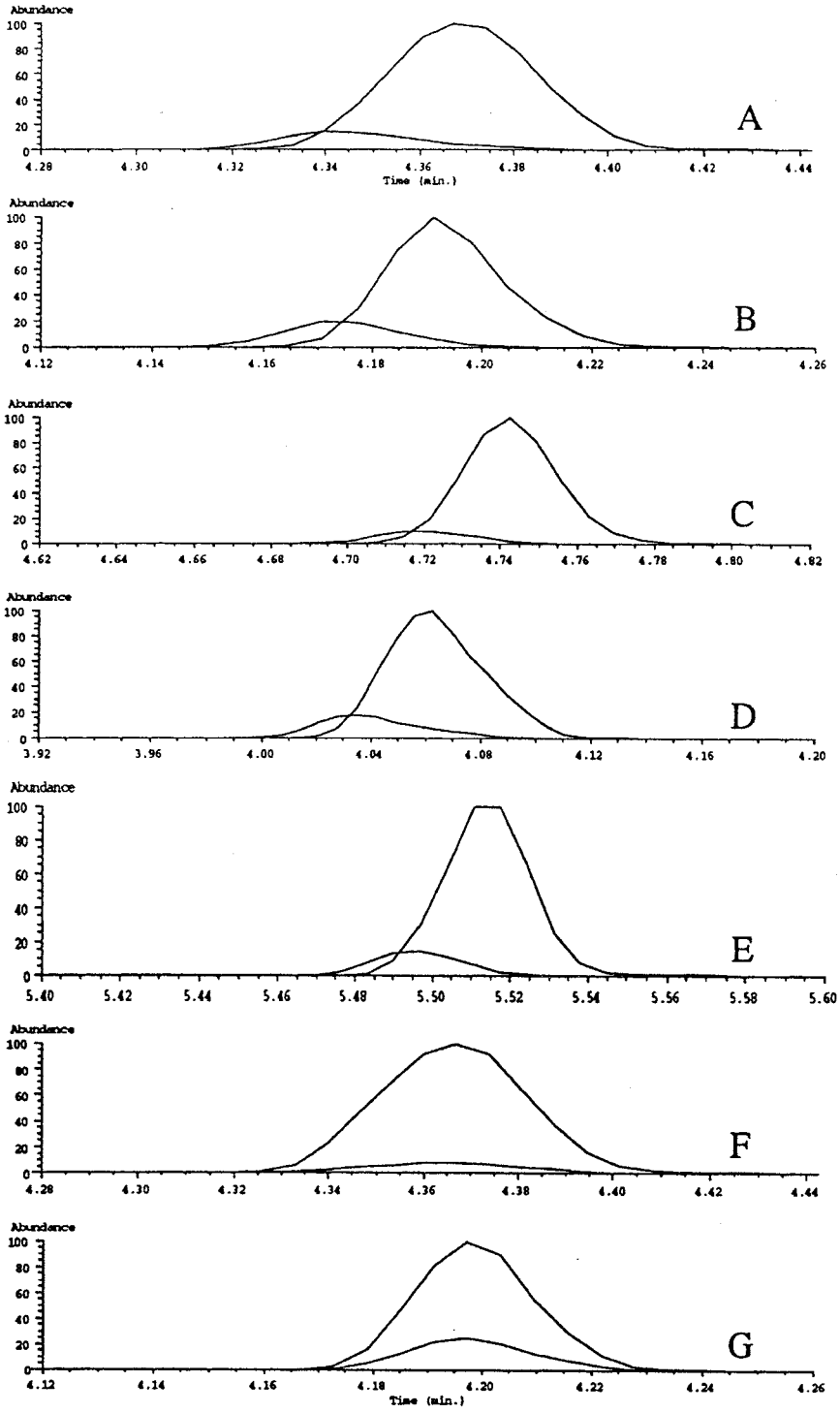

FIG. 3—*Retention time characteristics of analytes/^{2}H-analogs and analytes/^{13}C-analogs (all as methyl-derivatives): Secobarbital/$^{2}H_5$-secobarbital* (A)*; Butalbital/$^{2}H_5$-butalbital* (B)*; Methohexital/$^{2}H_5$-methohexital* (C)*; Pentobarbital/$^{2}H_5$-pentobarbital* (D)*; Phenobarbital/$^{2}H_5$-phenobarbital* (E)*; Secobarbital/$^{13}C_4$-secobarbital* (F)*; Butalbital/$^{13}C_4$-butalbital* (G)*.*

gramming conditions are varied to modify the separation between the analyte and the IS. The resulting analyte/IS ion-pair intensity ratio changes are characterized and evaluated.

Data shown in Table 6 (analyte as the major component and the ^{13}C-analog as the minor component) demonstrate that the monitored ion-pair intensity ratio for the secobarbital/$^{13}C_4$-secobarbital system remains constant as the reconstitution volume is increased. This ideal characteristic remains the same as the temperature programming rate is changed from 30 to 15, and then to 5°C/min. This is expected because the retention times of the analyte and the IS remain the same (no separation) regardless of the programming rate. It is believed that the monitored ion-pair intensity ratios will remain the same in parallel experiments in which the analyte is the minor component, while the ^{13}C-analog is the major component.

TABLE 6—*Secobarbital/$^{13}C_4$-analog ion-pair intensity ratio (m/z 196/200) as a function of molecular abundance under three temperature programming resulting in different peak overlapping between analyte and IS—Secobarbital: 4800 ng/mL; $^{13}C_4$-analog: 400 ng/mL.*

	Ion-pair Intensity Ratio (*m/z* 196/200)		
Reconstitute Volume (μL)	30°C Temperature Ramp	15°C Temperature Ramp	5°C Temperature Ramp
20	11.09	12.28	11.05
30	11.06	12.15	11.29
40	11.10	12.11	11.30
60	11.15	12.00	11.17
80	11.18	12.24	11.23
120	10.99	12.11	11.37
160	11.02	12.29	11.41
200	11.30	12.20	11.36

Data resulting from a series of parallel experiments for the secobarbital/$^2H_5$-secobarbital system are shown in Table 7. Here, as the programming rate is reduced from 30 to 15, and then to 5°C/min, the separation between the analyte and the IS increases, with the percentage of *m/z* 196 overlapped (by *m/z* 201) reducing from 89.5 to 77.7, and then to 70.2%. Under these three temperature programming conditions and when the reconstitution volume is changed from 20 to 200 μL, the monitored ion-pair intensity ratio for the secobarbital/$^2H_5$-secobarbital system changed 11.92%, 15.71%, and 18.35%, respectively.

Another series of experiments for the secobarbital/$^2H_5$-secobarbital system were performed and the resulting data are shown in Table 8. This series of experiments differ from that shown in Table 7 in the relative concentrations of the analyte and the IS, i.e., analyte is the major component in Table 7, while the IS is the major one in Table 8. In this latter case, as the programming rate is reduced from 30 to 15, and then to 5°C/min, the separation between the analyte and the IS similarly increases, with the percentage of *m/z* 201 overlapped (by *m/z* 196) reducing from 100 to 94.3, and

TABLE 7—*Secobarbital/$^2H_5$-analog ion-pair intensity ratio (m/z 196/201) as a function of molecular abundance under three temperature programming resulting in different peak overlapping between analyte and IS—Secobarbital: 4800 ng/mL; $^2H_5$-analog: 400 ng/mL.*

	30°C Temperature Ramp			15°C Temperature Ramp			5°C Temperature Ramp		
Reconstitute Volume (μL)	Ion Int. Ratio	Ratio Change (%)	Overlap* (%)	Ion Int. Ratio	Ratio Change (%)	Overlap* (%)	Ion Int. Ratio	Ratio Change (%)	Overlap* (%)
20	10.49		…†	10.82		…	11.50		…
30	10.69	1.91	97.3	11.32	4.62	80.5	11.89	3.39	70.0
40	11.03	5.15	…	11.67	7.86	…	12.35	7.39	…
60	10.99	4.77	83.4	11.90	9.98	78.0	12.50	8.70	71.8
80	11.41	8.77	…	11.89	9.89	…	12.87	11.91	…
120	11.39	8.58	89.6	12.35	14.14	79.1	12.99	12.96	73.2
160	11.62	10.77	…	12.38	14.42	…	13.36	16.17	…
200	11.74	11.92	87.6	12.52	15.71	73.2	13.61	18.35	66.0
Average			89.5			77.7			70.2

* Percentage of overlaps are calculated by dividing the area of *m/z* 196 that is overlapped with *m/z* 201 by the total peak area of *m/z* 196. Percentages of overlap with 30, 15, and 5°C temperature ramps are approximately 89.5 ([97.3 + 83.4 + 89.6 + 87.6]/4), 77.7 ([80.5 + 78.0 + 79.1+ 73.2]/4), and 70.2 ([70.0 + 71.7 + 73.2 + 66.0]/4), respectively. Area calculations were done by rectangular summation method (7).

† Data not calculated.

TABLE 8—*Secobarbital/$^2H_5$-analog ion-pair intensity ratio (m/z 196/201) as a function of molecular abundance under three temperature programming resulting in different peak overlapping between analyte and IS—Secobarbital: 400 ng/mL; $^2H_5$-analog: 4800 ng/mL.*

	30°C Temperature Ramp			15°C Temperature Ramp			5°C Temperature Ramp		
Recons. Volume (μL)	Ion Int. Ratio	Ratio Change (%)	Overlap (%)	Ion Int. Ratio	Ratio Change (%)	Overlap (%)	Ion Int. Ratio	Ratio Change (%)	Overlap (%)
20	0.1163			0.1250			0.1203		
30	0.1128	−3.01	100*	0.1148	−8.16	94.3*	0.1209	0.499	62.4
40	0.1137	−2.24	…†	0.1095	−12.4	…	0.1149	−4.49	…
60	0.1103	−5.16	…	0.1058	−15.4	…	0.1101	−8.48	…
80	0.1093	−6.02	…	0.1056	−15.5	…	0.1040	−13.6	…
120	0.1077	−7.39	…	0.1062	−15.0	…	0.0969	−20.2	…
160	0.1074	−7.65	…	0.1072	−14.2	…	0.0924	−23.2	…

* Percentage of overlaps are calculated by dividing the area of *m/z* 201 that is overlapped with *m/z* 196 by the total peak area of *m/z* 201. Area calculations were done by rectangular summation method (7).

† Data not calculated.

then to 62.4%. Under these three temperature programming conditions and when the reconstitution volume is changed from 20 to 200 μL, the monitored ion-pair intensity ratio for the secobarbital/$^2H_5$-secobarbital system changed from −7.65%, −14.2%, and −23.2%, respectively.

The observed phenomena shown in Tables 6–8 are rationalized as follows:

- When two chromatographically closely-eluted compounds (such as analytes and their ^2H-analogs) with their overlapping portions appearing at the ion source at the same time, the non-overlapping portions will have a higher ionization efficiency; thus, overall ionization efficiency of the major component will be lower than that of the minor one.
- This difference in ionization efficiency between the major and the minor compounds becomes more significant when the *molecular population at the ion source* is higher, i.e., with smaller reconstitution volume. This explains why, as the reconstitution volume is increased from 20 to 200 μL, the monitored ion-pair intensity ratios increase in Table 7 (secobarbital as the major component), while decrease in Table 8 ($^2H_5$-secobarbital as the major component).
- As the analyte and the IS are *more closely eluted,* larger portions of these two compounds will appear at the ion source at the same time. Since these portions are proportionally affected by the decrease in their ionization efficiencies, the difference in the overall ionization efficiency of these two compounds will decrease as they are more closely eluted. This explains why the rate of the changes (as the reconstitution volume is increased from 20 to 200 μL) in the monitored ion-pair intensity ratio is much higher when the temperature programming rate is lowered (analyte and IS are better resolved).

The above reasonings are consistent with the observed peak overlapping data and ion-pair intensity ratio change (or no change) characteristics shown in Tables 6–8. They may also account for the reported interference on the quantitation of benzoylecgonine caused by the coelution of fluconazole (8). The authors attributed the observed "coeluting interference" to "saturation of the ionization chamber", but did not mention non-proportional variations in benzoylecgonine/d_3-benzoylecgonine ionization efficiencies.

Conclusions

GC injection port temperature and the positions of the ^2H-atoms at the active sites of the molecular framework have been ruled out as the cause of the ion-pair intensity ratio changes observed for the analyte/^2H-analog systems. (Thus, neither chemical reaction in the injection port nor deuterium/hydrogen exchange at the ion source is the interference factor.) Difference in the retention time between the analytes and the ^2H-analog ISs appears to be the underlying cause for the observed interference phenomenon. For these systems, *variation in temperature programming condition for the GC column* causes variations in the percentages of the analyte/IS appearing at the ion source at the same time; thereby resulting in different degree of "non-proportional overall changes in ionization efficiencies" of the analytes and their ^2H-analog ISs. Since the ^{13}C-analog ISs display the same retention time as the analytes, these systems are free of the interference phenomenon displayed by the corresponding analyte/^2H-analog systems.

The exact intensity ratio of the selected ion-pair (that is used to represent the concentration of a test specimen or standard) depends on: (a) the molecular population of the analyte/^2H-analog IS in the ion source and (b) the overlapping characteristics of the analyte and the ^2H-analog IS. The first parameter (molecular population) is affected by: (a) the volume of the solvent used to reconstitute the dried extraction/derivatization residue and (b) the volume injected into the GC/MS system for analysis. Thus, the following two common practices may introduce error and should be avoided: (a) using less solvent to reconstitute the dried extraction/derivatization residue derived from a sample with lower concentration; (b) injecting larger volume into the GC/MS system for a sample with lower concentration.

The second parameter (overlapping characteristics) varies when: (a) the number of ^2H atoms used in the IS is different or (b) the GC oven temperature operation condition is changed. The implications of the above observation and interpretation include:

- The calibration curves for any analyte/^2H-analog systems are inherently nonlinear.
- As a result of its peak overlapping characteristics with the analyte, each ^2H-analog will display a different calibration characteristics.
- The calibration curve for any analyte/^2H-analog system will display different characteristics under different temperature programming conditions.

Thus, in addition to the now well known ion cross-contribution issue (9), calibration data generated through the use of ^2H-analogs as ISs are inherently nonlinear. For most accurate quantitations, nonlinear approaches (10) should be seriously considered when calibration curves are established.

Acknowledgments

The preparation of the manuscript was facilitated by a visiting appointment granted to Ray H. Liu by the Taiwanese National Science Council (NSC 90-2811-B-043A-001). Wei-Tun Chang was benefited from: (a) financial supports provided by the Taiwanese National Science Council (NSC 87-2418-H-015-010-Q6), Taiwanese Ministry of Justice Institute of Forensic Medicine (IFM-89-C006), and Rotary International Foundation; and (b) visiting appointment arrangement provided by the Department of Justice Sciences, University of Alabama at Birmingham, Birmingham, AL. The authors are thankful to Isotec (Miamisburg, OH) for providing $^{13}C_4$-butalbital and $^{13}C_4$-secobarbital used in this study at no cost.

References

1. Willard HH, Merritt LL Jr, Dean JA, Settle FA. Instrumental Methods of Analysis, 7th ed. Belmont (CA): Wadsworth Publishing, 1988;32.
2. Krull I, Swartz M. Quantitation in method validation. LC GC 1998;16: 1984–90.
3. A comprehensive list of quantitative analysis references has been compiled, many of which adapted deuterated analogs of the analytes as the internal standards. Karch SB, Ed. Drug abuse handbook. Boca Raton (FL): CRC Press, 1998;1066–109.
4. Chang W-T, Lin D-L, Low I-A, Liu RH: $^{13}C_4$-Secobarbital as the internal standard for the quantitative determination of secobarbital—A critical evaluation. J Forensic Sci 2000;45:659–64.
5. Chang W-T, Liu RH. Mechanistic studies on the use of ^2H- and ^{13}C-analogs as internal standards in selected ion monitoring GC/MS quantitative determination—Butalbital example. J Anal Toxicol 2001;25:659–69.
6. Liu RH, McKeehan AM, Edwards C, Foster G, Bensley WD, Langner JG, et al. 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 1994;39:1502–14.

7. Hinney RT, Thomas GB Jr. Calculus. Reading (MA): Addison-Wesley, Chap 5, 1990.
8. Wu AHB, Ostheimer D, Cremese M, Forte E, Hill D. Characterization of drug interferences caused by coelution of substances in gas chromatography/mass spectrometry confirmation of targeted drugs in full-scan and selected-ion monitoring modes. Clin Chem 1994;40:216–20.
9. Liu RH, Foster GF, Cone EJ, Kumar SD. Selecting an appropriate isotopic internal standard for gas chromatography/mass spectrometry analysis of drugs of abuse—Pentobarbital example. J Forensic Sci 1995;40: 983–9.
10. Whiting TC, Bodapati MR, Liu RH, Chang W-T. Isotopic analogs as internal standards for quantitative analyses of drug/metabolites by GC/MS—Non-linear calibration approaches. J Anal Toxicol 2001;25:179–89.

Additional information and reprints requests:
Ray H. Liu, Ph.D.
Department of Justice Sciences
University of Alabama at Birmingham
Birmingham, AL 35294-2060
E-mail: rayliu@uab.edu