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Characteristic of Calibration Curve Resulting from the Use of ^{2}H-analogs of the Analyte as Internal Standards——Methamphetamine Example

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ABSTRACT

Methamphetamine and its isotopic ^{2}H-analogs serving as internal standards (ISs) were used to investigate the characteristics of calibration curves for the quantitation purpose. Specific parameters studied include (a) reconstitution and injection volume that may affect the molecular abundance of the analyte and the IS in the ion source; and (b) column temperature programming conditions and the number of the ^{2}H-atom incorporated in the IS that may affect the separation of the analyte and the IS. $^2H_5$-, $^2H_8$-, $^2H_9$-, $^2H_{11}$- and $^2H_{14}$-analogs of methamphetamine are adopted as the ISs for the calibration curve evaluation. The well-established solid-phase extraction and pentafluoropropionyl (PFP) derivatization procedures were used to pretreat standard solutions prepared in urine matrix. A series of standard solutions containing 100-9,600 ng/mL methamphetamine and 500 ng/mL IS are used to establish the observed concentrations using one point calibration approach.

Resulting data indicate that ion-pair intensity ratios increase as the injection volume decreases. Calibration lines clearly indicate significant difference when the calibration line is established with different injection volumes. We believe this is caused by the non-proportional decrease (in relative to the IS) in the ionization efficiency of the analyte as its molecular abundance at the ion source increases. To further study the molecular abundance issue, a second series of experiments is performed, in which 50, 150, and 450 μL ethyl acetate were used to reconstitute the extraction-derivatization residue. The resulting data clearly demonstrate that the ion intensity of the designated ion-pair at the higher concentration increases as the reconstitution volume increases (i.e., the molecular abundance at the ion source decreases),

We have hypothesized that the retention time difference between the analytes and their ^{2}H-analogs is the other underlying cause for the observed non-proportional decreases in ionization efficiency in the analyte/^{2}H-analog systems. Empirical data clearly indicate that, as the analyte and its ^{2}H-analog IS are further overlapped (by programming at a higher ramp rate), the designated ion-pair intensity ratios for the standards with higher analyte concentrations become closer to their "expected" values, i.e., the "linearity" of the calibration can be extended to a higher concentration level. To further study the retention time difference parameter, another series of experiments are performed, in which $^2H_5$-, $^2H_9$, and $^2H_{14}$-methamphetamine (showing increasing retention time difference with the analyte) are used as the ISs. The results indicate that, compared to the methamphetamine/$^2H_9$-analog and the methamphetamine/$^2H_{14}$-analog systems, the deviation data at concentration ranges from 100 to 9,600 ng/mL for methamphetamine/$^2H_5$-analog demonstrate the values close to the random error.

To establish an ideal calibration curve within a desired concentration range, specific parameters such as cross-contribution, the extraction-derivatization reconstitution volume, injection volume, and temperature programming should be evaluated prior to the GC/MS quantitative analysis.

Keywords: Internal Standard, Quantitation, Methamphetamine, Cross-contribution, Non-proportional Over-all Change in Ionization Efficiency

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Introduction

With the implement of 10 ng/mL cutoff for 6-acetylmorphine assay and the required inclusion of a "40% of cutoff" control in the workplace drug testing programs, accurate quantitation is now required at a much lower concentration level than previously mandated [1]. Isotopic analogs serving as internal standards (ISs) in conjunction with selected ion monitoring (SIM) GC/MS procedures are currently the state-of-the-art approach for the quantitative analyses of drugs and their metabolites. Fuller understanding on the performance characteristics of the internal standard (IS) and the calibration approaches plans an important role in achieving the now focused analytical goal.

We have long been interested and working on various issues that may impact the accuracy in quantitation and have published a series of articles in these areas, including (a) performance characteristics of ^{13}C- and ^{2}H-labeled IS [2,3]; (b) methods for selecting most suitable ion-pairs for designating the analyte and the IS [4-6]; and (c) characteristics of linear and non-linear calibration approaches [7]. During the course of these studies, we have made a very important discovery which, to the best of our knowledge, has been reported previously. Specifically, the intensity-ratio of an ion-pair selected to designate the analyte-to-^{2}H-IS concentration ratio in a sample changes as any of the following operating parameters are changed: (a) the volume of the constitution solvent; (b) the injection volume; or (c) the column temperature program rate. Molecular abundance (intensity) in ion source and different retention time between analyte and its IS are the two major underlying causes of the interfering phenomenon. This was called "non-proportional over-all change in ionization efficiency" [8,9].

Barbiturates and their corresponding $^{2}H_5$-analogs have been used for the study and the discovery mentioned above. With the availability of ^{2}H-methamphetamine incorporating 5, 8, 9, 11, and 14 ^{2}H-atoms, these compounds lend a much greater opportunity to understand the mechanism accounted for the observed phenomena: variations in column temperature programming rates affect the intensity-ratio of the ion-pair intended to designate and solely affected by the analyte-to-^{2}H-IS concentration ratio.

Experimental

Reagents

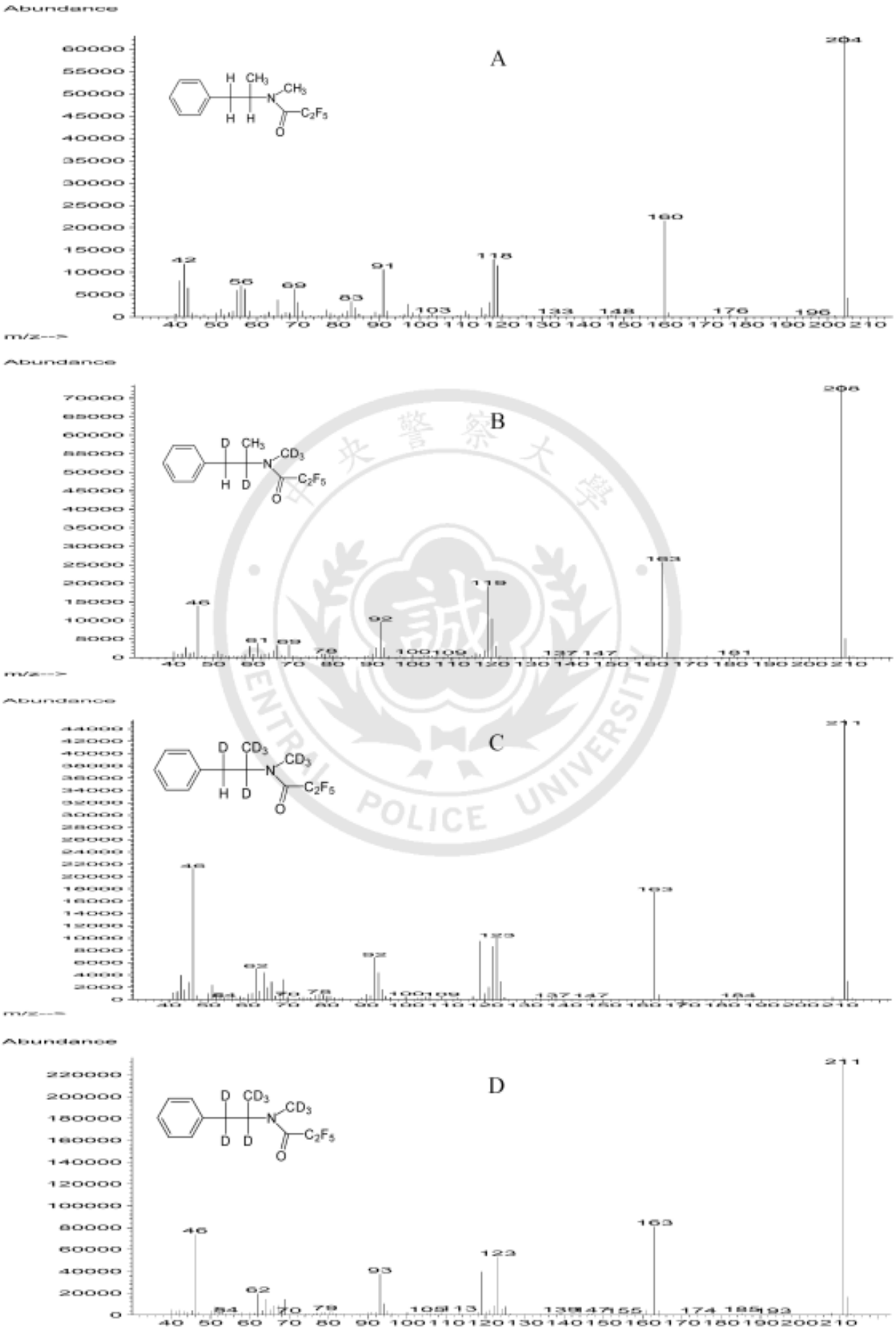
Methamphetamine, $^{2}H_5$-, $^{2}H_8$-, $^{2}H_9$-, $^{2}H_{11}$-, and $^{2}H_{14}$-analogs (1 mg/mL methanol solution; 99% purity) were purchased from Radian Corp. (Austin, TX). Pentafluoropropionic anhydride (PFPA) was purchased from Ardrich Chemical Corp. (Milwaukee, WI). Methanol, ethyl acetate, hydrogen chloride, and di-sodiumhydrogenphosphate were purchased from Merck Corp. (Germany). Drug-free urine used for the preparation of standard drug solutions was provided by one member of our investigation team.

Solid-Phase Extraction and Derivatization

Procedures of solid-phase extraction (SPE) provided by the International Sorbent Technology Ltd [10] were followed for processing the standard solutions using a specimen size of 2 mL. Each standard solution was spiked with 500 μ L of 2 μ g/mL IS resulting in 500 ng/mL IS in each sample. Extracts were dried (40 ºC), added 50 μ L PFPA and 50 μ L ethyl acetate, incubated (60 ºC) for 20 mins, and then dried under a steam of nitrogen (40 ºC). The final product was reconstituted with ethyl acetate prior to GC/MS analysis.

GC/MS Analysis

A Hewlett-Parkard (Palo Alto, CA) HP 6890 Gas chromatograph interfaced to a HP 5972 mass selective detector (MSD) was used for collecting full-scan and SIM mass spectrometric data. Full-scan spectra (m/z 45 to 320 amu) of the analyte and the analog s (all derivatized) are shown in Fig. 1 along with their chemical structures. These data were used to preliminarily select the following analogous ion pairs for the quantitative analyses, that are apparently free (or with minimal) cross-contribution between the analyte and the isotopic analog: m/z 204/208 and 160/163 for methamphetamine/$^{2}H5$-analog; m/z 204/211 and 160/163 for methamphetamine/$^{2}H_8$-, /$^{2}H_9$-, and $^{2}H_{14}$-analogs; m/z 204/210 and 160/163 for methamphetamine/$^{2}H_{11}$-analog. The ion cross-contribution data were determined by "direct measurement" and the "normalized direct measurement", respectively [4].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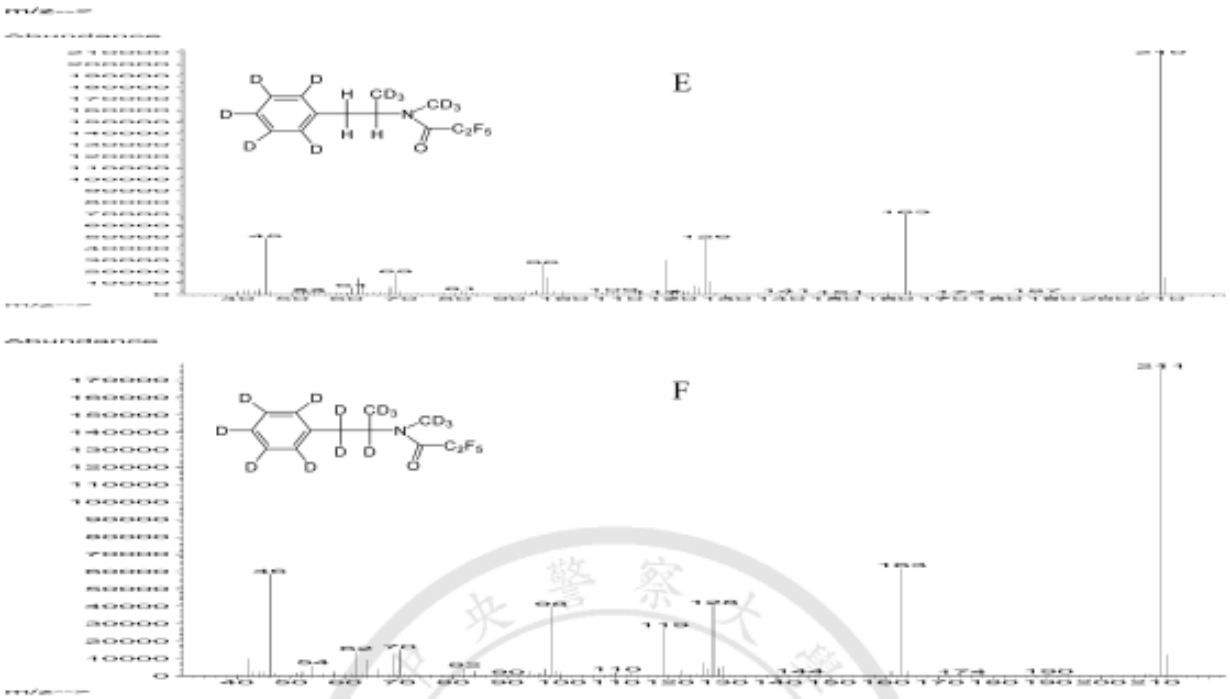

Fig.1 Full-scan mass spectra of analyte/isotope-labeled analogs (all as acyl-derivatives): methamphetamine (A); $^{2}H_{5}$-methamphetamine (B); $^{2}H_{8}$-methamphetamine (C); $^{2}H_{9}$-methamphetamine (D); $^{2}H_{11}$-methamphetamine (E); $^{2}H_{14}$-methamphetamine (F).

To investigate the mechanistic factors affecting the quantitative effectiveness resulting from the use of a ^{2}H-analog of the analyte as the internal standard (IS), the varying reconstitution volumes (50, 150, and 450 μL) were studied along with the observed concentrations in the standard solutions of methamphetamine/$^{2}H_{14}$-analog under one-point calibration approach. Ion intensity ratios of the methamphetamine/$^{2}H_{11}$-analog solutions containing different magnitude of analyte and its IS were further investigated their ratio changes when reconstituting different solvents. The concentrations of standard methamphetamine/$^{2}H_{14}$-analog solutions containing 100 to 9,600 ng/mL were evaluated when injecting different injection volumes (1, 2, 3, and 5 μL). To investigate the "non-proportional over-all change in ionization efficiency' phenomenon associated with the retention time difference between analyte and its IS, changes of ion-pair intensity ratios and the observed concentrations in standard solutions deriving from the varying peak overlapping were studied under three temperature programming in GC as follows:

1. High ramp rate: 100 ℃ initial temperature for 1 min, 25 ℃/min ramp to 220 ℃ end temperature for 5 min.
2. Middle ramp rate: 100 ℃ initial temperature for 1 min, 10 ℃/min ramp to 220 ℃ end temperature for 5 min.
3. Low ramp rate: 60 ℃ initial temperature for 1 min, 5 ℃/min ramp to 220 ℃ end temperature for 5 min.

The quantitative effectiveness in which the $^{2}H_{5}$-, $^{2}H_{9}$-, and $^{2}H_{14}$-analogs were served as ISs was evaluated by comparing the observed concentrations of standard solutions from 100 to 9,600 ng/mL.

Results and Discussion

Cross-contribution data leading to the characteristics of calibration curves

To describe the characteristics of the calibration curve deriving from the cross-contribution, the direct measurement and the normalized direct measurement were used for the comparison purpose. Resulting data shown in Table 1 present that ^{2}H-analogs appear to make more contribution to the intensity of ions designated for methamphetamine than methamphetamine to ^{2}H-analogs. The low mass ions make more contribution to the corresponding ions than the high mass ions do. Specifically, the contribution of methamphetamine to the intensity of ions m/z 211 and 163 (designated for $^{2}H_{9}$-analog) are negligible, while the contribution of $^{2}H_{9}$-analog to the intensities of ions m/z 204 and 160

(designated for $^{2}H_{9}$-analog) are approximately 8-9% via the direct approach and approximately 2-3% via the normalized approach. The contribution of $^{2}H_{11}$- and $^{2}H_{14}$-analogs to the intensity of ion m/z 160 (designated for methamphetamine) are also approximately 2-3% via the direct measurement. Thus, analytes at low concentration levels may be serious contributed from the IS as the IS makes more ion contribution, and may appear higher positive observed concentrations in the standard solutions.

The cross-contribution data derived from the direct and the normalized approaches are significantly different. Parts of data obtaining from the direct approach are greater than those from the latter method. In converse, some data resulting from the direct approach are smaller than the normalized method. This indicates that the most accurate determination should be evaluated by multiple approaches for the reconfirmation such as direct or/and normalized direct measurement, internal standard or/and standard addition methods [4].

Table 1 Cross-contribution of ions designated for MA and its deuterated analogs under PFP derivatization – "direct measurement" (left in parentheses in %) and "normalized direct measurement" (right in parentheses in %).

Isotopic analogs	Ions (m/z) suitable for monitoring (% Cross-contribution by analog)					
	Methamphetamine			Internal standard		
$^{2}H_{5}$-MA	204 (0.13; 0.06)	160 (0.85; 0.39)	118 (18.02; 8.03)	208 (0.00; 0.00)	163 (0.00; 0.00)	120 (1.99; 4.32)
$^{2}H_{8}$-MA	204 (0.05; 0.15)	160 (0.13; 0.38)	118 (0.95; 5.76)	211 (0.00; 0.00)	163 (0.15; 0.05)	123 (0.65; 0.22)
$^{2}H_{9}$-MA	204 (8.13; 2.46)	160 (9.74; 2.88)	118 (22.7; 6.72)	211 (0.01; 0.03)	163 (0.02; 0.08)	123 (0.05; 0.18)
$^{2}H_{11}$-MA	204 (0.93; 0.40)	160 (2.41; 1.04)	118 (2.84; 1.23)	210 (0.00; 0.01)	163 (0.07; 0.16)	126 (0.81; 1.87)
$^{2}H_{14}$-MA	204 (0.79; 0.40)	160 (2.03; 1.03)	118 (1.93; 0.98)	211 (0.01; 0.02)	163 (0.06; 0.13)	128 (0.71; 1.39)

Effects of molecular abundance-Reconstitution volume and injection volume

Intensity factor was observed to associate with the changes of ion intensity ratios for secobarbital and butalbital in our earlier studies [2,3,8]. One main objective herein is to further investigate the intensity dependency of analyte/labeled-analog ion-pair intensity ratios observed in the methamphetamine/^{2}H-analog systems. Resulting data in Table 2 show ion intensity ratios at varying concentration levels increase as the reconstituted volumes are increased from 50 to 450 μL except those with 150 μL volume from 100 to 1800 ng/mL levels. The ratio changes were presented by the percentage levels that ion intensity ratios obtained from 50 μL reconstitute were divided by those obtained from other reconstitutes. Meanwhile, ratio change increase is more significant when the concentration of analyte is at higher levels. This trend illustrates the following characteristics:

1. The amount of ion intensity ratio is increased along with the difference of concentration levels between methamphetamine and $^{2}H_{14}$-analog when the reconstitute volume is increased. This phenomenon implies that ion-pair intensity ratios at higher concentration levels showing non-representative ratios result in a non-linear calibration curve.
2. Ion intensity ratios are changed by intensities being detected while the ratio of concentration level between methamphetamine/ $^{2}H_{14}$-analog keeps constant. The higher intensities are determined, the larger changes of ion intensity ratios are derived.
3. Deviation data using 450 μL reconstitute volume are smaller than those of using 50 μL reconstitute volume. An appropriate reconstitute volume resulting in stable and representative ratios should be considered. Obviously, the less reconstitute volume generating the higher intensity is not the suitable choice.
4. The intensity (which is unkown) in a test sample can be accurately determined only if the test sample is injected the same magnitude as calibration standard at the representative range.

Table 2 Quantitation of methamphetamine using $^{2}H_{14}$-analog as IS under various reconstitution volumes—methamphetamine/$^{2}H_{14}$-analog (500 ng/mL): m/z 204/211.

Theor. Conc.	50 μL reconstitute volume*		150 μL reconstitute volume*		450 μL reconstitute volume*	
(ng/mL)	Ion ratio	Dev. (%)	Ion ratio: Ratio change (%)†	Dev. (%)	Ion ratio: Ratio change (%)†	Dev. (%)
100	0.247	17.5	0.239: -3.23	16.4	0.250: 1.21	14.9
200	0.429	2.14	0.413: -3.73	0.59	0.434: 1.17	-0.17
400	0.841	Calibrator	0.822: -2.26	Calibrator	0.870: 3.45	Calibrator
600	1.246	-1.25	1.224: -1.77	-0.69	1.299: 4.25	-0.45
900	1.870	-1.16	1.828: -2.25	-1.14	1.967: 5.19	0.50
1,200	2.403	-4.77	2.379: -1.00	-3.53	2.546: 5.95	-2.46
1,800	3.629	-4.12	3.606: -0.63	-2.51	3.906: 7.63	-0.22
2,400	5.119	1.44	5.140: 0.41	4.22	5.585: 9.10	6.99
3,600	7.082	-6.44	7.135: 0.75	-3.55	7.825: 10.49	-0.06
4,800	9.123	-9.60	9.373: 2.74	-4.98	10.278: 12.66	-1.55
6,400	11.657	-13.37	11.974: 2.72	-8.96	13.385: 14.82	-3.84
7,200	13.328	-11.96	13.601: 2.05	-8.08	15.197: 14.02	-2.96
9,600	17.216	-14.70	17.757: 3.22	-9.99	19.633: 14.04	-5.97

* The extraction-derivatization residue is first reconstituted with 50 μL of ethyl acetate, then diluted with 150, and 450 μL step-wise. Measurements are performed following initial reconstitution and between each dilution.

† Ratio changes are calculated by dividing the ratio observed with the reconstitution volume of 50 μL by the ratio observed with the reconstitution volume of interest.

Changes of ion intensity ratios designated for varying amounts between methamphetamine and $^{2}H_{11}$-analog IS were further investigated using different reconstitute volumes. Resulting data in Table 3 shows the following characteristics:

1. Ion intensity ratios increase when the concentration of analyte is greater than that of IS. On the contrary, the ratios decrease when the concentration of analyte is smaller than that of IS. The ratio remains constant when the concentrations of analyte and its IS are same.
2. This trend shows the reason why the linear range in the calibration curve is close to the concentration of the IS.

Table 3 Methamphetamine/$^{2}H_{11}$-analog IS ion intensity ratio as a function of molecular abundance — m/z 204/210.

Analyte (ng/mL)	IS (ng/mL)	150 μL recons. Vol.* Ion intensity ratio	300 μL recons. Vol.* Ion intensity ratio	450 μL recons. Vol.* Ion intensity ratio	Ratio Change† (%)
2,400	1,000	4.749	4.795	4.803	1.14
4,800	1,000	7.688	7.900	7.983	3.84
19,200	1,000	32.618	33.657	34.627	6.16
28,800	1,000	44.000	45.373	47.839	8.73
1,000	4,000	0.298	0.296	0.293	-1.68
1,000	8,000	0.145	0.142	0.141	-2.76
1,000	16,000	0.063	0.062	0.061	-3.17
1,000	24,000	0.036	0.035	0.034	-5.56
480	480	1.779	1.752	1.796	0.96
960	960	1.502	1.522	1.516	0.93
4,800	4,800	1.397	1.394	1.391	0.43
9,600	9,600	1.336	1.326	1.338	0.20

* The extraction-derivatization residue is first reconstituted with 150 μL of ethyl acetate, then diluted with 300, and 450 μL step-wise. Measurements are performed following initial reconstitution and between each dilution.

† Ratio changes are calculated by dividing the ratio observed with the reconstitution volume of 150 μL by the ratio observed with the reconstitution volume of interest.

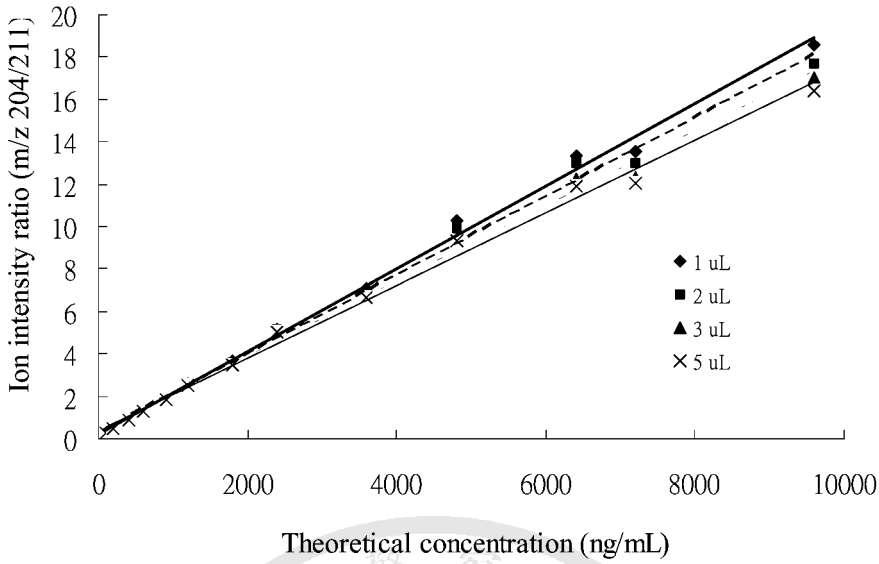

Fig. 2 Changes of ion intensity ratios (methamphetamine/$^2H_9$-analog) from 100 to 9,600 ng/mL standard solutions containing 500 ng/mL IS when solutions were injected with 1 to 5 μL.

To further investigate the characteristic of the calibration curve associated with the intensity factor, standard solutions of methamphetamine/$^2H_9$-analog (containing concentrations of methamphetamine from 100 to 9600 ng/mL and 500 ng/mL IS) were established different calibration curves when using 1, 2, 3, and 5 μL of injection volumes. The results shown in Table 4 and Fig. 2 indicate that (a) the monitored ion-pair intensity ratio decreases as the injection volume increases; (b) the observed decrease is more significant when the concentrations of the standards are higher; (c) the deviation at a higher concentration level increases as the injection volume increases. Apparently, the intensity ratios of the designated ion-pair at the higher concentration end derived from smaller injection volume are relatively higher. The ion intensity of the designated ion-pair at the higher concentration end stays closer to its "expected" value, i.e., the "linearity" of the calibration curve can be extended to a higher concentration level.

This study demonstrates that molecular abundance at the ion source (therefore the ion intensity) appears to be the underlying factor causing the observed variation in the monitored ion-pair intensity ratio. The reconstitute volume and injection volume should be evaluated prior to the GC/MS determination. The ion-pair intensity ratio monitored for a specific analyte/^{2}H-analog system appears to be lower than the true value when the analyte concentration is at the higher end; thus, the calibration line will inherently be non-linear if the same reconstitution and injection volumes are used for all standards. The non-linear approaches may be adapted to establish the calibration curve for most accurate quantitation applications.

Table 4 Quantitation of methamphetamine using $^{2}H_{9}$-analog as IS under various injection volumes – methamphetamine/$^{2}H_{9}$-analog (500 ng/mL): m/z 204/211.

Theor. Conc.	Injection vol.* (1 μL)		Injection vol.* (2 μL)		Injection vol.* (3 μL)		Injection vol.* (5 μL)	
(ng/mL)	Ion ratio	Dev. (%)	Ion ratio; Ratio change (%)†	Dev. (%)	Ion ratio; Ratio change (%)†	Dev. (%)	Ion ratio; Ratio change (%)†	Dev. (%)
100	0.275	24.15	0.269; -2.18	22.97	0.268; -3.27	22.79	0.266; -3.27	22.02
200	0.467	5.42	0.454; -2.78	3.77	0.454; -2.78	4.01	0.454; -2.78	4.13
400	0.886	Calib.	0.875; -1.24	Calib.	0.873; -1.24	Calib.	0.872; -1.58	Calib.
600	1.301	-2.11	1.288; -1.00	-1.87	1.204; -7.46	-8.06	1.291; -0.77	-1.30
900	1.857	-6.85	1.840; -1.70	-6.54	1.829; -2.80	-6.89	1.826; -1.67	-6.93
1,200	2.583	-2.82	2.571; -0.46	-2.06	2.562; -0.81	-2.18	2.498; -3.29	-4.51
1,800	3.670	-7.95	3.606; -1.74	-8.42	3.597; -1.99	-8.44	3.479; -5.20	-11.34
2,400	5.185	-2.46	5.138; -0.91	-2.13	5.080; -2.03	-3.02	5.022; -3.14	-4.01
3,600	7.106	-10.88	6.949; -2.21	-11.76	6.808; -4.19	-12.43	6.649; -6.43	-15.28
4,800	10.296	-3.16	9.942; -3.44	-5.31	9.635; -6.42	-8.03	9.314; -9.54	-10.99
6,400	13.308	-6.12	13.014; -2.21	-7.04	12.275; -7.76	-12.12	11.918; -10.44	-14.58
7,200	13.565	-14.94	12.981; -4.31	-17.58	12.324; -9.15	-21.57	12.022; -11.37	-23.41
9,600	18.560	-12.72	17.711; -4.57	-15.66	17.000; -8.41	-18.86	16.428; -11.49	-21.50

* All samples were reconstituted with 150 μL ethyl acetate.

† Ratio changes are calculated by dividing the ratio observed with the 1 μL injection volume by the ratio observed with the intention volume of interest.

Effects of temperature programming

$^{2}H_{14}$-analogs are found to have a slightly shorter retention time than methamphetamine shown as Fig. 3. This condition may result in ion intensity ratio change to be the "unexpected" value (lower than the expected value), since two different intensities of peaks with non-corresponding retention time have different ionization efficiencies. It is much more serious when there are large difference of concentration levels between analyte and ^{2}H-analog. It was called "non-proportional over-all change in ionization efficiency" phenomenon. However, $^{13}C_{4}$-secobarbital [2] and $^{13}C_{4}$-butalbital [3] could almost match the retention time of secobarbital and butalbital. This unexpected phenomenon, therefore, were not found by ion intensity ratios of barbiturates/$^{13}C_{4}$-analogs because their different ionization efficiencies can be normalized by the same peak shapes. Thus it follows from what have been said that "non-proportional over-all change in ionization efficiency" phenomenon is a more important factor resulting in a non-linear calibration curve using ^{2}H-analogs as ISs for wider and higher concentration range.

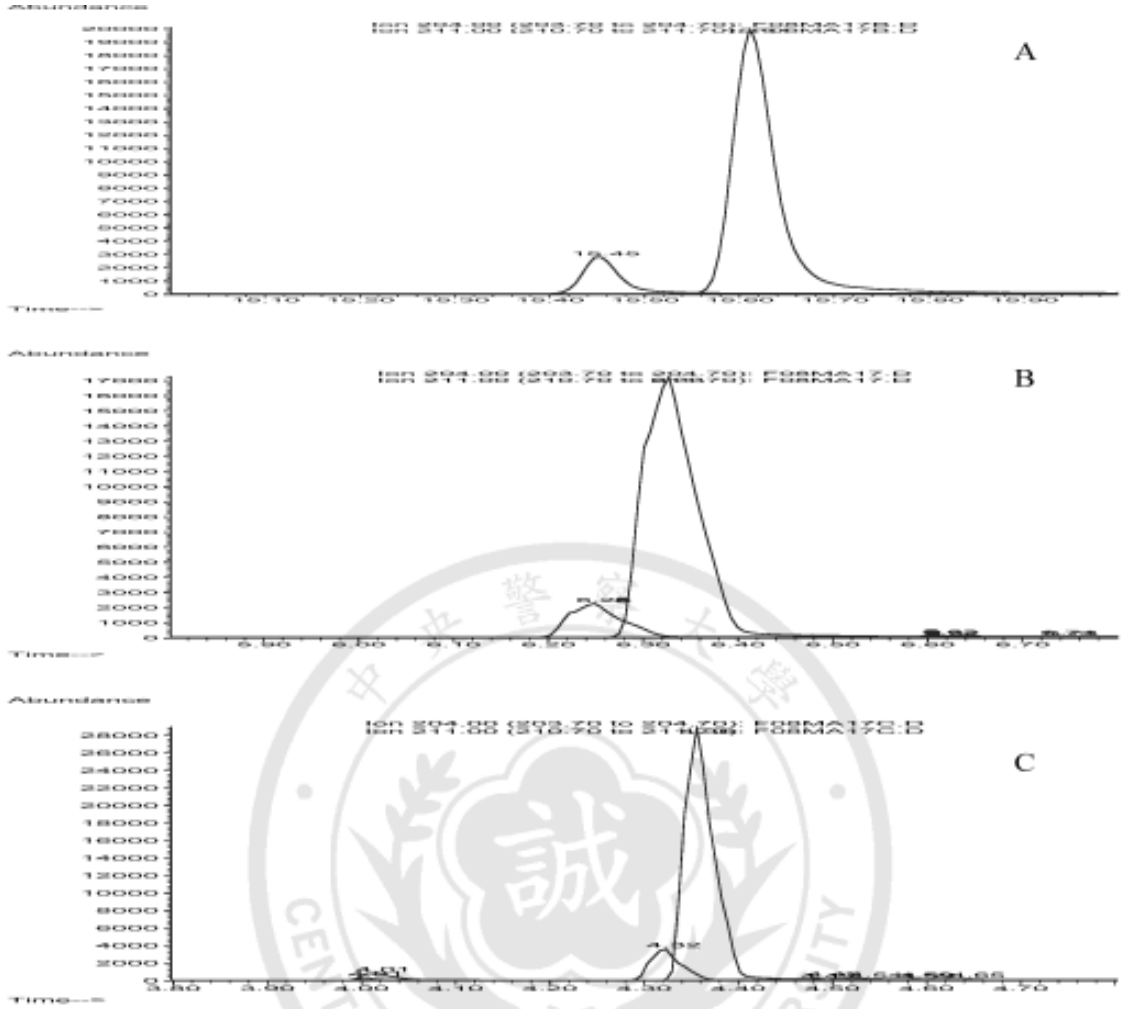

Fig. 3 SIM chromatograms of methamphetamine (3,600 ng/mL) and $^{2}H_{14}$-analog (m/z 204/211) (500 ng/mL) under different temperature programming — 5 ℃ temperature ramp rate (A); 10 ℃ temperature ramp rate (B); 25 ℃ temperature ramp rate (C).

Table 5 Quantitation of methamphetamine using $^{2}H_{14}$-analog as IS under various temperature programming at GC — methamphetamine/$^{2}H_{14}$-analog (500 ng/mL): m/z 204/211 IS.

Theor. Conc	5 ℃ temperature ramp rate			10 ℃ temperature ramp rate			25 ℃ temperature ramp rate		
(ng/mL)	Ion ratio	Obs'ed conc.	Dev. (%)	Ion ratio	Obs'ed conc.	Dev. (%)	Ion ratio	Obs'ed conc.	Dev. (%)
100	0.243	107.5	7.52	0.271	118.0	18.0	0.283	122.8	22.8
200	0.449	198.7	-0.66	0.459	200.1	0.04	0.473	205.4	2.70
400	0.904	Calibrator	--	0.918	Calibrator	--	0.921	Calibrator	--
600	1.374	608.0	1.33	1.382	602.3	0.38	1.387	602.4	0.40
900	1.954	864.6	-3.94	1.951	850.2	-5.53	1.952	847.6	-5.82
1,200	2.727	1,207	0.56	2.740	1,194	-0.49	2.701	1,173	-2.24
1,800	3.901	1,726	-4.10	3.849	1,677	-6.83	3.824	1,661	-7.73
2,400	5.462	2,417	0.70	5.456	2,378	-0.94	5.353	2,325	-3.13
3,600	8.094	3,581	-0.52	7.960	3,468	-3.66	7.747	3,364	-6.54
4,800	10.96	4,849	1.02	10.94	4,768	-0.66	10.15	4,406	-8.21
6,400	14.78	6,540	2.19	14.49	6,315	-1.32	14.20	6,166	-3.66
7,200	16.81	7,437	3.29	16.80	7,319	1.66	16.03	6,960	-3.33
9,600	25.32	11,203	16.7	24.58	10,712	11.6	23.84	10,353	7.85

To further investigate the interference to the quantitative effectiveness from "non-proportional over-all change in ionization efficiency" phenomenon, methamphetamine/$^{2}H_{14}$-analog was used as an example to illustrate the calibration data associated with different GC temperature programming conditions. The difference degree of the retention time between analyte and the IS under three temperature programming in Fig. 3 are as following: 0.16 min for low ramp rate; 0.08 min for middle ramp rate; 0.04 min for high ramp rate. Analyte and its IS almost completely separate each other under low ramp rate. The quantitation data shown in Table 5 indicate the following characteristics:

1. Ion intensity ratios and percent deviation at lower concentration levels increase along with temperature programming from low to high ramp rate. This trend indicates that the increase of ion intensity ratios resulting from ion cross-contribution increases as the peak-overlapping increases. Thus, we have confirmed that the methamphetamine/ $^{2}H_{14}$-methamphetamine system exhibits the same characteristics as the barbiturate/ $^{2}H_{5}$-analog systems.
2. Ion intensity ratios along with the increase of concentration levels under low ramp rate become larger than those of concentration levels under high ramp rate. This trend involves two factors, cross-contribution and the "non-proportional over-all change in ionization efficiency" phenomenon. At the higher concentration levels, the analyte contributes much more ions to the IS as the peak-overlapping increases resulting in decreasing the ion intensity ratio. In converse, the intensity of the IS decreases when its peak more overlaps with analyte, which has a lower ionization efficiency than the IS. With the compensation effect deriving from the partial "proportional over-all change in ionization efficiency" phenomenon, the ion intensity ratio then results in increasing. The former factor, herein, demonstrates more significant interference to the change of the ion ratio for the smaller different retention time between analyte and the IS at higher concentration levels. Based on the larger ratio at calibrator and the smaller ratios at higher concentration levels, calibration standards at higher concentrations remain smaller observed deviations under high ramp rate.
3. It is helpful to determine specimen at the lower concentrations by using temperature programming of low ramp rate. Meanwhile, the smaller amount of the IS used could decrease the cross-contribution problem for the determination at the low concentration levels.

Comparison of calibration data-Evaluation by cross-contribution and different 2H-atom number

To further study the retention time difference parameter, another series of experiments are performed, in which $^{2}H_{5}$-, $^{2}H_{9}$, and $^{2}H_{14}$-methamphetamine (showing increasing retention time difference with the analyte shown in Fig. 4) are used as the ISs. Resulting data shown in Table 6 indicate that, compared to the methamphetamine/ $^{2}H_{9}$-analog and the methamphetamine/ $^{2}H_{14}$-analog systems, the intensity ratio of the ion-pair designated for methamphetamine/ $^{2}H_{5}$-methamphetamine at the higher concentration end is closer to their "expected" values. Again, this will allow the extension of the linearity of the calibration curve to a higher concentration level. Compared with the cross-contribution data using direct measurement in Table 1, 2H9-analog contributes 8.13 % and $^{2}H_{14}$-analog only contributes 0.79 % ion to methamphetamine. However, these two ion-pairs show the similar quantitative effectiveness. Especially, the deviation of the 100 ng/mL concentration level for the methamphetamine/ $^{2}H_{9}$-analog is smaller than that of methamphetamine/ $^{2}H_{14}$-analog. This phenomenon indicates that cross-contribution is not the only factor affecting the calibration curve. The retention time difference and ionization efficiency difference between analyte and its IS may be other factors. Thus, the quantitative effectiveness should be evaluated for every isotopic analog based on showing an excellent calibration curve.

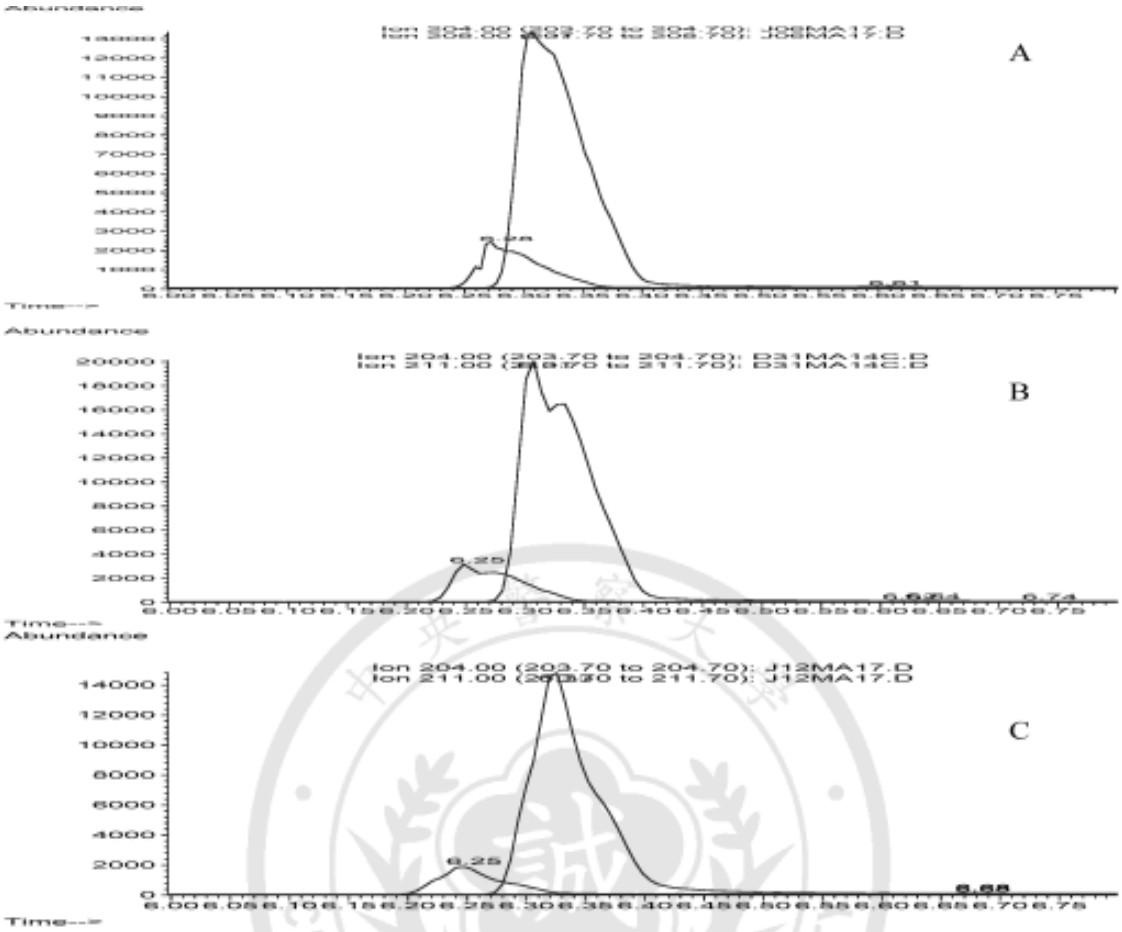

Fig. 4 SIM chromatograms of methamphetamine (3,600 ng/mL) and isotopic ISs (500 ng/mL) (as PFP-derivatives) — methamphetamine/$^{2}H_{5}$-analog (m/z 204/208) (A); methamphetamine/$^{2}H_{9}$-analog (m/z 204/211) (B); methamphetamine/$^{2}H_{14}$-analog (m/z 204/211) (C).

Table 6 Effect of variation in analyte/^{2}H-analog IS retention difference (resulting from the use of the ^{2}H-analogs with different number of ^{2}H-atoms) on the linearity of the calibration line—IS: 500 ng/mL.

Theor. Conc.	Methamphetamine/$^{2}H_{5}$-analog (m/z 204/208)		Methamphetamine/$^{2}H_{9}$-analog (m/z 204/211)		Methamphetamine/$^{2}H_{14}$-analog (m/z 204/211)	
	Ion int. ratio	Deviation (%)	Ion int. ratio	Deviation (%)	Ion int. ratio	Deviation (%)
100	0.226	-1.79	0.307	10.02	0.263	11.24
200	0.467	1.51	0.572	2.43	0.475	0.61
400	0.921	Calibrator	1.117	Calibrator	0.945	Calibrator
600	1.398	1.17	1.703	1.64	1.378	-2.79
900	2.047	-1.23	2.613	3.96	1.993	-6.26
1200	2.782	0.69	3.324	-0.82	2.844	0.32
1800	4.215	1.69	5.035	0.16	3.988	-6.22
2400	5.646	2.17	6.540	-2.42	5.514	-2.76
3600	8.487	2.39	9.891	-1.62	7.979	-6.18
4800	11.23	1.59	12.69	-5.30	10.82	-4.56
6400	14.86	0.84	17.79	-0.45	14.64	-3.19
7200	16.81	1.37	20.00	-0.55	16.14	-5.11
9600	22.63	2.36	26.15	-2.46	22.16	-2.32

Conclusions

Difference in the retention time and in the intensity between the analye and the IS appears to be the underlying cause for the observed interference in the calibration curve. The analyte/^{2}H-analog system when reconstituting with different solvent volumes, injecting different volumes or varying in temperature programming conditions in the GC column causes variations in the percentage of the analyte/IS appearing at the ion source; thus causing "non-proportional over-all changes in ionization efficiencies" of the analyte and the IS. This interference mechanism will cause the non-linear calibration curve for the quantitative analysis when ^{2}H-analogs are used as ISs. We have hereby demonstrated that establishing a calibration line within a desired concentration range, in addition to careful selection of the internal standard, requires thoughtful considerations in the extraction-derivatization reconstitution volume, injection volume, temperature programming parameters and cross-contribution data.

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