FORENSIC SCIENCE JOURNAL SINCE 2002

Forensic Science Journal
2012;11(1):25-32
Available online at:fsjournal.cpu.edu.tw

Optimal Parameters for the GC/MS Quantitation of Barbiturates in Urine at Lower Concentration Levels

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Abstract

This study is placed on evaluating optimal factors for the quantitative determination of barbiturates in urine at lower concentration levels. An automatically well-established solid phase extraction (SPE)is used for the urine samples, and then the extract is methylated prior to the GC/MS measurement steps. The optimization of GC/MS quantitative effectiveness is evaluated based on four specific parameters including the use of different internal standards (ISs), the added magnitude of ISs, reconstitute volume, and temperature programming conditions. The resulting data indicate the following conditions can be recommended for the routine quantitation: the added magnitude of 50 ng/mL IS, and 90μL reconstitution volume and 20℃ ramp rate in temperature programming conditions. The quantitation using all evaluated conditions results in the following observations: 10 ng/mL LOD and 40 ng/mL LOQ for butalbital, 20 ng/mL LOD and 80 ng/mL LOQ for amobarbital, 20 ng/mL LOD and 20 ng/mL LOQ for pentobarbital, 10 ng/mL LOD and 20 ng/mL LOQ for secobarbital, 20 ng/mL LOD and 40 ng/mL LOQ for phenobarbital.

Keywords: GC/MS quantitation, butalbital, amobarbital, pentobarbital, secobarbital, phenobarbital, LOD, LOQ

Introduction

Accurate quantitation of abused drugs and their metabolites in biological specimens for the forensic laboratory often involves determinations at a very low concentration level and interpretation of quantitative data with small interspecimen concentration differences [1]. Internal standard (IS) method has long been established as one of the most effective approaches for the quantitations of analytes in specimens with complex matrix [2, 3]. The ion cross-contribution between the analyte and its label analog that is the isotope-labeled IS has been regarded as a major factor affecting the level of accuracy achievable in a routine quantitative determination protocol [4, 5]. "The non-proportional overall changes in ionization efficiencies" phenomenon between the analyte and its ^{2}H-analogs has also been proved to influence the calibration characteristic [6]. Furthermore, interference factors involving molecular abundance and retention time difference have been studied for quantitative GC/MS analysis in our earlier study [7, 8].

This study is carried out to evaluate the quantitative effectiveness based on selecting optimal parameters to generate the lower limit of detection (LOD) and the lower limit of quantitation (LOQ) for the determination of barbiturates in urine. The following parameters including (1) the use of different internal standards (ISs), (2) the added magnitude of ISs, (3) the reconstitute volume, and (4) temperature programming conditions are used for this evaluation purpose.

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Experimental

Reagents

Analytes (butalbital, amobarbital, pentobarbital, secobarbital, phenobarbital) and four isotope-label analogs ($^2H_5$-butalbital, $^2H_5$-pentobarbital, $^2H_5$-secobarbital, $^2H_5$-phenobarbital) of 1 mg/mL methanol solution with 99% purity were purchased from Radian Corp. (Austin, TX). Reagents used for the derivatization of the analytes and the ISs, tetramethylammonium hydroxide (TMAH) were provided by Acros Organics (USA) and iodomethane were obtained from Wako Pure Chemical Industries, Ltd. (Japan). Drug-free urine specimen, used in the preparation of standard drug solutions, was provided by a member of the research group.

Solid-Phase Extraction and Derivatization

The solid-phase extraction (SPE) procedures specified by the Varian Bond Elut Certificate II were followed for processing the standard solutions of 10, 20, 40, 80, 150, 200, 250, 500, 1000 and 2000 ng/mL using a urine specimen size of 2 mL. Extracts were derivatized as methyl-derivatives prior to GC/MS analysis following the same procedures adapted in our earlier studies [3, 6, 8].

GC/MS Analysis

A Hewlett-Packard (Palo Alto, CA) HP 6890 gas chromatograph interfaced to a HP 5973 mass selective detector (MSD) equipped with a DB-5MS column(30-M, 0.25-mm ID, 0.25-μm film thickness) was used to acquire full-scan and SIM mass spectrometric data. For quantitative determination, ion pairs monitored for butalbital/$^2H_5$-analog, amobarbital/pentobarbital, pentobarbital/$^2H_5$-analog, secobarbital/$^2H_5$-analog, and phenobarbital/$^2H_5$-analog were m/z 196/201, 169/189, m/z 184/189; 196/201 and 232/237, respectively. To select ion pairs for the quantitation, “direct measurement” and “improved direct measurement" approaches under SIM mode were used to evaluate the extents of “cross-contribution” of the ion pairs [5].

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

In theory, two methods including statistical method and empirical method can be adopted for establishing LOD and LOQ as applied to the quantitation of abused drugs while using GC-MS analysis. The empirical method for determining LOD and LOQ herein is performed by analyzing a series of standard solutions of 10, 20, 40, 80, 150, 200, 250, 500, 1000 and 2000 ng/mL concentration levels for five analytes. The LOD is defined as the lowest concentration at which the ion ratios meet within ±20% relative to those obtained for a calibrating standard assayed in the same batch. The LOQ is defined as the lowest concentration at which the ion ratios meet acceptance criteria and the assayed and target concentrations meet within ±20% as well [9].

Selection of Optimal Parameters for the Quantitation at Low Concentration Levels

To generate the better quantitative effectiveness at low concentration levels, the following parameters including (1) the use of different internal standards (ISs), (2) the added magnitude of ISs, (3) the reconstitute volume, and (4) temperature programming conditions were respectively evaluated. Linear calibration approach was adopted to elucidate the quantitative effectiveness of standard solutions based on selecting optimal parameters by comparing results of LOD and LOQ.

Results and Discussions

Effects of the IS magnitude to the Quantitative Effectiveness

The results in **Table 1** show that different barbiturates have different LOD and LOQ values. Compared with LOD values for all analytes, the larger IS magnitude spiked to the standards solutions will generate the more ion cross-contribution to the analyte. Therefore, the lager LOD values are found when 200 ng/mL IS magnitude was used for all analytes. However, the lager LOQ values observed using smaller IS magnitudes may derive from variation of peak area integration. The degree of difference between LOD and LOQ values when using smaller IS magnitudes are generally larger than those using larger IS magnitudes. The LOD and LOQ values resulting from quantitation of amobarbital when using pentobarbital as IS are similar to those of pentobarbital when using $^2H_5$-analog as IS.

Table 1. Comparison of LOD and LOQ results using different IS magnitudes (unit: ng/mL)

IS magnitude	25 ng/mL		50 ng/mL		200 ng/mL	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Butalbital	10	150	20	80	40	80
Amobarbital	150	150	80	80	200	200
Pentobarbital	150	150	150	150	200	200
Secobarbital	20	150	40	80	80	80
Phenobarbital	40	150	40	150	150	150

Effects of the Reconstitute Solvent Volume to the Quantitative Effectiveness

The LOD and LOQ results in **Table 2** using different reconstitute solvent volumes show that the reconstitute solvent volumes is not underlying cause for quantitation of barbiturates at lower concentration levels. The lager amount of the reconstitute volume may practically be used for the routine quantitative determination when the recheck determination is required.

Table 2. Comparison of LOD and LOQ results using different reconstitute solvent volume (unit: ng/mL)different IS magnitudes (unit: ng/mL)

Reconstitute Volume	30 µL		60 µL		90 µL	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Butalbital	20	80	40	40	40	40
Amobarbital	80	80	80	80	80	80
Pentobarbital	80	80	80	80	80	80
Secobarbital	40	80	40	40	40	40
Phenobarbital	40	150	40	40	40	40

Based on the "non-proportional overall change in ionization efficiencies" phenomenon, reconstitution with smaller solvent volume will result in smaller ion-pair intensity ratios for barbiturates/isotopic ^{2}H-analog shown as **Figure 1**. This phenomenon clearly indicates 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 [10]. Increases in ion-pair intensity ratios appear to be more significant at the beginning and gradually reduced. However, ion-pair intensity ratios of amobarbital/pentobarbital show different changes because the "non-proportional overall change in ionization efficiencies" phenomenon can't be found when two peaks are not overlapping.

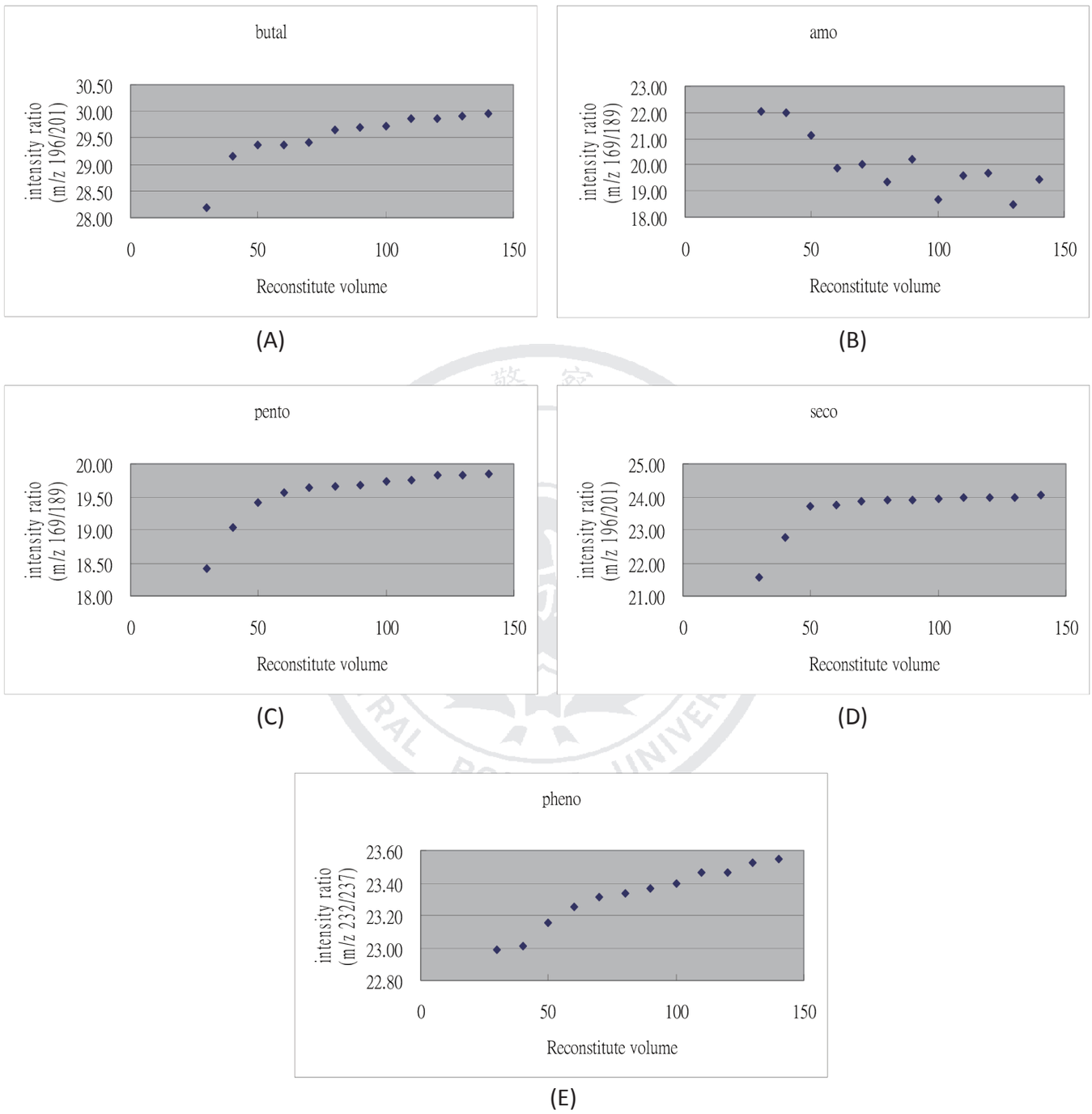

Fig 1. Changes in ion-pair intensity ratios result from (A)butalbital/$^2H_5$-analog, (B) amobarbital/pentobarbital, (C)pentobarbital/$^2H_5$-analog, (D)secobarbital/$^2H_5$-analog:, (E)phenobarbital/$^2H_5$-analog when their methylated products of a 2000 ng/mL solution (IS:50 ng/mL) are reconstituted with 30 to 140 μL.

A series of solutions were prepared to further investigate the relationship between the ion-pair intensity ratio changes and the phenobarbital/^2H-analog concentration levels. The first series of solutions include a constant amount of $^2H_5$-phenobarbital (50 ng/mL), with the concentration of phenobarbital ranging from 250 to 2000 ng/mL. Data derived from this series of solutions in 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. The second series of solutions include a constant amount of phenobarbital (50 ng/mL), with the concentration of $^2H_5$-phenobarbital ranging from 250 to 2000 ng/mL. Ion-pair intensity ratios for analyte with low concentration levels and IS with high concentration levels in the second series of solutions decrease as the reconstitute volumes increase. The third series of solutions include the same concentration levels for phenobarbital and $^2H_5$-phenobarbital at 250, 500, 1000 and 2000 ng/mL. Changes of ion-pair intensity ratios for analyte and IS with same concentration levels in the third series of solutions remain constant as the reconstitute volumes increase. This changes of the ion-pair intensity ratios resulting from increasing the reconstitute volumes also involve the "non-proportional overall change in ionization efficiencies" phenomenon. To select an ideal molecular abundance, an appropriate reconstitute volume is needed to evaluate for the quantitation purpose, especially for the determination of specimens at high concentration levels.

Table 3. Ion intensity ratios of phenobarbital/isotopic IS as a function of molecular abundance under different concentration levels between analyte and IS.

Analyte conc.(ng/mL)	IS conc. (ng/mL)	Intensity ratio (30µL)	Intensity ratio (60µL) Ratio change (%)	Intensity ratio (90µL) Ratio change (%)
250	50	5.18	5.22; 0.77	5.23; 0.97
500	50	9.17	9.33; 1.74	9.40; 2.51
1000	50	15.97	16.47; 3.13	16.67; 4.38
2000	50	37.17	38.66; 4.01	40.10; 7.88
50	250	0.198	0.197; -0.51	0.196; -1.01
50	500	0.114	0.113; -0.88	0.112; -1.75
50	1000	0.068	0.0662; -2.65	0.0656; -3.53
50	2000	0.032	0.0309; -3.44	0.0306; -4.38
250	250	0.972	0.956; -1.65	0.981; 0.93
500	500	0.961	0.988; 2.81	0.973; 1.25
1000	1000	0.988	1.002; 1.42	0.980; -0.81
2000	2000	0.990	1.005; 1.52	0.981; -0.91

Effects of the Programming in GC Column Temperature to the Quantitative Effectiveness

Because of variation in the degree of peak-overlap, the retention time difference between the analyte and the IS will generate different percentage of ion cross-contribution and different degree of "proportional overall change in ionization efficiencies" phenomenon. The former factor will significantly influence the quantitative determination at low concentration levels. In comparison, the later factor will significantly influence the calibration curve at high concentration levels that the theoretical ratio of analyte/IS will be lower than the determined ratio of analyte/IS. That is, when two chromatographically closely-eluted compounds (such as analytes and their ^{2}H-analogs) with their overlapping portions appearing at the ion source at the same time, the nonoverlapping portions will have a higher ionization efficiency; thus, overall ionization efficiency of the major component will be lower than that of the minor one. The major component herein is the analyte and the minor one is the ^{2}H-analog IS. This difference in ionization efficiency between the major and the minor compound becomes more significant when the molecular population at the ion source is higher, i.e., with smaller reconstitution volume. This phenomenon found in our earlier study [12] indicates that an appropriate reconstitution volume should be selected to generate an ideal calibration curve.

This section is focused on evaluating an interference factor from the ion cross-contribution to the quantitative determination at low concentration levels. A series of experiments were performed, in which GC column temperature programming conditions were varied to modify the separation between the analyte and the IS. The narrow separation between the analyte and the IS under the higher ramp rate will increase ion cross-contribution. Thus, the LOD and LOQ data in **Table 4** under 40℃ ramp rate are larger than those using 10℃ ramp rate. The results indicate that GC programming conditions will influence the quantitative results, especially at low concentration levels.

Table 4. Comparison of LOD and LOQ results using different temperature ramp in GC column programming. (unit: ng/mL)

Temperature Ramp	**10℃**		**20℃**		**40℃**	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Butalbital	10	40	10	40	40	40
Amobarbital	10	80	20	80	80	80
Pentobarbital	10	40	20	20	80	80
Secobarbital	10	20	10	20	40	40
Phenobarbital	10	10	20	40	40	40

Effects of the Adapted Various IS to the Quantitative Effectiveness

^{2}H-analogs of the analytes are now the most popular choices of ISs for the quantitation by GC/MS approach. 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. The LOD and LOQ data obtained from using isotope-label analogs as ISs under above optimal parameters in **Table 5**.are lower than those using other analytes, $^{2}H_5$-pentobarbital and Tolybarbiturate, as ISs. These resulting data indicate that the best quantitation effectiveness of a specific analyte in urine is performed by using its isotopic analog as IS. Tolybarbiturate is not an appropriate choice for the quantitative determination of barbiturates in urine.

Table 5. Comparison of LOD and LOQ results using different IS (unit: ng/mL)

IS	Isotopic analog		$^{2}H_5$-Pentobarbital		Tolybarbiturate	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Butalbital	10	40	40	40	40	1000
Amobarbital	--	--	20	80	40	1000
Pentobarbital	20	20	20	20	40	1000
Secobarbital	10	20	40	80	40	150
Phenobarbital	20	40	10	150	10	80

Conclusions

Based on methylation and linear calibration, four barbiturates, butalbital, pentobarbital, secobarbital, and phenobarbital, using the isotope-labeled analogs as ISs could generate the better quantitative results. The optimal conditions were selected for the routine quantitation of barbiturates in urine as following: the added magnitude of 50 ng/mL IS, 90μL reconstitution volume and 20℃ ramp rate in temperature programming conditions. The quantitative determinations of barbiturates in urine using all optimal conditions result in the following observations: 10 ng/mL LOD and 40 ng/mL LOQ for butalbital, 20 ng/mL LOD and 80 ng/mL LOQ for amobarbital, 20 ng/mL LOD and 20 ng/mL LOQ for pentobarbital, 10 ng/mL LOD and 20 ng/mL LOQ for secobarbital, 20 ng/mL LOD and 40 ng/mL LOQ for phenobarbital.

Acknowledgement

This work has finished with financial supports granted by the National Bureau of Controlled Drugs (DOH93-NNB-1002).

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