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Short communication

Validation of a simple and sensitive gas chromatographic method for the analysis of tri-*n*-butyl phosphate from virally inactivated human immunoglobulin

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Abstract

Tri-*n*-butyl phosphate (T*n*BP), a solvent used in combination with Triton X-100 to inactivate lipid-enveloped viruses from immunoglobulin purified from human plasma is routinely measured in our laboratories by gas chromatography–flame ionization dectection (GC–FID) after extraction with C-18. We modified our present assay by extracting the analyte into hexane prior to measurement by GC–FID. We also found that the addition of a small volume of ethanol to the organic layer facilitates the extraction process by breaking the resulting emulsion formation caused by the hexane addition. The sample preparation and subsequent assay were fully validated in our laboratory. The process time for each sample is less than 2 min, a 15-fold improvement over solid-phase extraction techniques that were previously used in our laboratories. The recovery of T*n*BP in immunoglobulin using this newer method approximates 100%. The limit of quantitation (LOQ) was found to be 2 μg/ml or 2 ng per injection. The linear dynamic range of the assay is reported to be from the LOQ up to 50 μg/ml. The method is simple, relatively inexpensive and rapid. In addition, validation of the method demonstrates that it is accurate, precise, rugged and robust as demonstrated by reproducibility between analysts, instruments, laboratories, and columns. Finally, no problems were observed with regard to sample carryover.

Keywords: Tri-*n*-butyl phosphate; Immunoglobulins

1. Introduction

Human immunoglobulins are widely used therapeutically as passive immunization agents to treat diseases caused by cytomegalovirus (CMV), respiratory synsitial virus (RSV) and varicella-zoster virus [1–4]. Broad-spectrum human immunoglobulins are also used prophylatically against a variety of infectious disease [5]. During the purification of immunoglobulin from human plasma, lipid-enveloped viruses such as HIV, hepatitis B (HBV) and hepatitis C (HCV) can be inactivated by using a combination of solvent-detergent procedures developed by Horowitz et al. [6,7]. After viral inactivation, the solvent and detergent can be removed by a variety of procedures such as diafiltration [8], solid-phase extraction using a reversed-phase support [9], ion-exchange chromatography on Q-Sepharose [10], adsorption chromatography on Amberlite XAD-7 [11] or extraction with castor oil [12]. Regulations from the United

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States Food and Drug Administration require that the levels of residual solvent and detergent in the product be determined. The method traditionally used in our laboratory to measure tri-*n*-butyl phosphate (T*n*BP) levels in purified immunoglobulin has involved solid-phase extraction of sample using C-18 supports followed by measurement using gas chromatography with flame ionization detection (GC–FID). In our laboratory, this particular sample preparation is relatively time consuming, and the results have varied when different lots of solid support (C-18 columns) and different suppliers are used. Confounding the analysis, hydrophobic proteins also compete with C-18 thus making the T*n*BP elution less precise. One previous study using liquid–liquid extraction of T*n*BP after protein precipitation by perchloric acid showed poor recovery of T*n*BP [13]. In our present method, T*n*BP from human immunoglobulin was extracted directly without protein precipitation by vigorous vortexing in presence of hexane. The consequent emulsion formation was clarified with ethanol, facilitating the removal of the hexane phase containing T*n*BP and internal standard tri-*n*-amyl phosphate (T*n*AP). Extraction efficiencies of analyte approached 100%. No major impurities were observed in the chromatographic analyses.

2. Experimental

2.1. Plasma products and chemicals

Tri-*n*-butyl phosphate (T*n*BP) was purchased from Sigma (St. Louis, MO, USA). Tri-*n*-propyl phosphate (T*n*PP) and *n*-hexane (capillary GC grade) were purchased from Aldrich (Milwaukee, WI, USA). Tri-*n*-amyl phosphate (T*n*AP) was purchased from TCI (Tokyo, Japan). Triton X-100 was from Spectrum (New Brunswick, NJ, USA). Purified immunoglobulin (IVG-9) and immunoglobulin containing 0.3% T*n*BP and 1% Triton X-100 were obtained from the Plasma Fractionation Department at the Massachusetts Public Health Biologic Laboratories.

2.2. Equipment

A Hewlett-Packard GC System (model 6890) equipped with an injector (model 7673) and auto sampler for a model 6890 GC and equipped with a flame ionization detector (Hewlett-Packard, Wilmington, DE, USA) was used as the primary system for the validation of the method. Ruggedness studies were performed on a Hewlett-Packard GC system located in the Drug Laboratory at the Massachusetts Department of Public Health, Boston, MA, USA. Both systems were similar except the primary chromatograph was equipped with a Packard Hydrogen Generator model 9200 (Deerfield, IL, USA) and used helium (ultra high purity) as the carrier gas. The secondary chromatograph was equipped with a hydrogen tank (ultra high purity) and used nitrogen (ultra high purity) as the carrier gas.

3. Validation of the T*n*BP assay by gas chromatography

3.1. Extraction efficiency and accuracy

Efficiency of sample preparation was determined by comparison of direct injection data derived from T*n*BP and T*n*AP prepared in hexane to data derived from samples of T*n*BP and T*n*AP in immunoglobulin that were taken through the liquid–liquid extraction process before GC analysis. Samples spanned the linear dynamic range of the assay.

Accuracy was measured by percent recovery of five different concentrations of T*n*BP in immunoglobulin that spanned the low, middle, and high range of the standard curve. All samples were taken through the hexane liquid–liquid extraction process as described.

3.2. Precision

Intra-assay precision (assay repeatability, single analyst) was evaluated by testing immunoglobulin-containing T*n*BP at three different concentrations tested in a single assay. Inter-assay precision (defined as assay ruggedness among different analysts) was also evaluated by testing T*n*BP in immunoglobulin at three different concentrations. Three assays were run by each analyst.

3.3. Additional ruggedness studies

Instrument to instrument ruggedness was validated by performing the assay side by side on the two GC instruments.

3.4. Selectivity

The selectivity of the assay was tested by studying the effects of Triton X-100 and immunoglobulin on the assay performance. Typical samples analyzed in our laboratories contain 60–70 mg immunoglobulin/ml. In order to test the effect of immunoglobulin, samples containing 167 μg/ml Triton X-100 were spiked with additional immunoglobulin (15–120 mg). These samples were then spiked with 0–50 μg of T*n*BP. A reference condition was also run which contained approximately 35 mg of immunoglobulin to which 0–50 μg T*n*BP was spiked. Performance of the assay was assessed by comparing the recovery of T*n*BP against the reference condition.

The effects of Triton X-100 on the assay were studied by testing the detergent concentrations from 50 to 400 μg/ml in the assay. Similarly to the immunoglobulin studies, the different concentrations of Triton X-100 were compared to a reference condition; the reference condition contained 50 μg/ml Triton X-100.

Selectivity was further validated by adding tri-*n*-propyl phosphate (T*n*PP) to the chromatographic mix and demonstrating the resolution of this particular compound.

3.5. Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by performing four different statistical analyses on data obtained from five different assays. The first method measured the mean standard deviation (MSD) of the assay response (T*n*BP/T*n*AP ratio). Assay noise was measured by dividing the MSD of the response in the lowest quarter of the assay range by the mean slope of the calibration curve for the five assays [14]. LOD was determined by multiplying the noise by 3.3. LOQ was determined by multiplying the noise by 10.

In the second method, assay noise was measured by taking the standard deviation of the *y*-intercepts derived from the calibration curves and dividing by the mean slope of the same curves. LOD and LOQ were determined as above.

The third method calculated assay noise by dividing the standard deviation of the residuals of the mean regression line by the mean slope of the calibration curves. LOD and LOQ were determined as above.

The fourth method measured assay noise based on the peak area. A blank sample containing immunoglobulin, Triton X-100, and T*n*AP was processed and analyzed by the GC method. Noise was measured near the typical T*n*BP retention time (6.2–6.3 min). The average area from 20 injections was used to determine noise. The corresponding concentration was converted to LOD and LOQ as above.

3.6. Linearity and range

The linear dynamic range for the assay was determined by analysis of calibration curve data. Residuals determined from calibration curve data were evaluated for trending, non-randomness and deviation from the *X*-axis. Linearity was also evaluated by calculating the response/concentration ratio across the range of standards. The percent C.V. was determined across this range. Range of the assay is defined as the inclusive interval between the upper and lower levels of analyte that can be determined with precision, linearity, and accuracy [15].

3.7. Assay robustness

In order to ensure assay robustness, the method was evaluated by performing it on two different capillary columns. The columns were the same type (HP-5) but from different batches purchased from the manufacturer.

3.8. Carryover

Sample carryover was measured by injecting a sample containing 50 μg/ml T*n*BP and 25 μg/ml T*n*AP five times followed by three neat hexane injections.

4. Conditions

4.1. Chromatographic conditions

4.1.1. Columns, flow-rates, carrier gases

Both chromatographic systems were equipped with High Performance Capillary GC columns, model HP-5 crosslinked with 5% PH ME Siloxane, Film thickness: 0.25 μm Length: 30 m (Hewlett-Packard, part number 19091J-413). Linear flow-rate: 148 cm/s. The primary chromatograph used helium (ultra high purity grade) as a carrier gas; the secondary chromatograph used nitrogen (ultra high purity) as the carrier gas.

4.1.2. Injector conditions

Splitless mode, initial temperature: 215°C, pressure: 45.80 p.s.i., purge flow: 18.7 ml/min, purge time 1.00 min, total flow: 40.2 ml/min, gas type: helium (primary GC), nitrogen (backup GC).

4.1.3. Temperature program

The oven temperature: 40°C for 2 min and increased to 210°C at the rate of 35°C/min then held isothermally for 1.14 min at 210°C.

4.1.4. Detector

The detector temperature was 240°C. The hydrogen flow-rate was 35.0 ml/min. Air flow was 400.0 ml/min. Make up gas (helium, ultra high purity) flow was 30.0 ml/min. (Nitrogen was the makeup gas on the backup GC).

4.2. Sample preparation

Immunoglobulin samples containing T*n*BP (0.3% w/v) were diluted 60-fold with deionized water before subsequent preparation and analysis. However, samples of immunoglobulin containing residual T*n*BP and Triton X-100 were processed as follows: The samples were taken (0.5 ml) and 50 μl of internal standard solution (500 μg/ml T*n*AP in ethanol v/v) was added to each. Hexane (1 ml) was added and each sample was vortexed at high speed until an emulsion was observed. The emulsion was clarified by the addition of 0.1 ml of ethanol followed by gentle vortexing. A 200–300 μl aliquot from the upper organic phase was removed for analysis by GC; 1 μl of each standard or sample was injected for each GC analysis.

5. Results and discussion

5.1. Extraction efficiency–accuracy

Recoveries of T*n*BP from immunoglobulin, when compared to T*n*BP measured by direct injection are presented in Table 1. The recoveries of T*n*BP from immunoglobulin ranged from 94 to 101%. Accuracy data as tested by spiking five different concentrations of T*n*BP into neat immunoglobulin and measuring the respective recoveries is summarized in Table 2. Recoveries ranged from 91 to 108%.

Table 1
Extraction efficiency of T*n*BP from immunoglobulin[a]

T*n*BP (μg/ml)	Direct[b] injection (peak area)	IVG-9[b] spiked (peak area)	Recovery[b] (%)	Direct injection T*n*BP/T*n*AP ratio[c]	IVG-9 spiked T*n*BP/T*n*AP ratio[c]	Recovery[c] (%)
0	0	0	NA	0	0	NA
2	22.67	21.82	96	0.078	0.076	97
5	52.00	52.52	101	0.179	0.180	101
10	109.00	106.75	98	0.380	0.384	101
25	264.22	261.47	99	0.925	0.922	100
50	551.43	517.82	94	1.930	1.852	96

[a] Each data point represents the mean of triplicate injections.

[b] Peak areas are peak areas of T*n*BP. No normalization to internal standard was performed in order to demonstrate the absolute amount of analyte that was extracted (i.e. extraction efficiency).

[c] Ratios are calculated as Peak area T*n*BP/Peak area T*n*AP. Ratios are compared to peak areas to show that T*n*AP extracts similarly to T*n*BP.

Table 2
Accuracy: spiking and recovery (addition of various amounts of immunoglobulin containing T*n*BP to immunoglobulin without T*n*BP[a]

T*n*BP (μg/ml) after addition to immunoglobulin	Recovery (%) using # 9I10[b]	Recovery (%) using # 9I19[b]	Recovery (%) using G-4[c]
2	108	106	97
5	104	102	95
10	102	102	99
25	95	100	100
50	96	99	91

[a] Concentrations were determined from a standard curve that plotted the ratio of T*n*BP/T*n*AP peak areas against concentration (μg/ml).
[b] 9I10 and 9I19 are preparations received from the Plasma Fractionation Department at Massachusetts Public Health Biologic Laboratories. They contain approximately 3000 μg/ml T*n*BP and 10 000 μg/ml Triton X-100 in a 7% human immunoglobulin matrix.
[c] G-4 was prepared by our Quality Control Assay Development Laboratory at MPHBL. It contains the same components as 9I10 and 9I19.

Table 3
Assay repeatability (intra-assay response precision, single analyst)[a]

T*n*BP in sample (after dilution) (μg/ml)	Mean response (T*n*BP/T*n*AP) (peak area)	Standard deviation	C.V. (%)
2	0.079	0.0048	6.00
25	0.880	0.0469	5.33
50	1.840	0.0200	1.09

[a] Each sample was prepared in replicates of three. Each sample in turn was taken through the hexane extraction process and analyzed in replicates of three by GC in a single assay.

5.2. Precision, ruggedness, and robustness studies

The repeatability of the assay was tested by measuring T*n*BP (in immunoglobulin) at three different concentrations in a single assay. The data are summarized in Table 3. Intermediate precision or ruggedness among analysts was measured by taking samples at similar T*n*BP concentrations and testing them in three separate assays each performed by three analysts. The data are summarized in Table 4. Both levels of precision are acceptable as demonstrated by relatively low coefficients of variation.

Table 4
Intermediate response precision (assay ruggedness, three analysts)[a]

Analyst	Assay number	Response at 2 μg/ml	Response at 25 μg/ml	Response at 45 μg/ml
Number 1	1	0.084	1.07	1.89
	2	0.087	1.07	1.91
	3	0.082	0.92	1.58
Number 2	1	0.082	0.90	1.65
	2	0.083	0.87	1.64
	3	0.079	0.91	1.68
Number 3	1	0.078	0.98	1.72
	2	0.088	0.95	1.69
	3	0.078	0.94	1.71
Mean response		0.082	0.96	1.72
SD		0.0037	0.0718	0.1118
(%) C.V.		4.5	7.5	6.5

[a] Data represent a total of nine assays. Three different analysts performed the assay three times. Data represent samples analyzed by GC in replicates of three. Response represents the ratio of T*n*BP/T*n*AP peak areas.

Table 5
Instrument and column reproducibility[a]

Sample[b]	9I10-VI-1 Concentration (T*n*BP μg/ml)	9I19-VI-2 Concentration (T*n*BP μg/ml)	G-4 Concentration (T*n*BP μg/ml)	TC-2 Concentration (T*n*BP μg/ml)
Laboratory 1	2769	2919	3045	3184
Laboratory 2	2672	2636	2950	2946
Mean	2720	2778	2997	3066
SD	67	200	68	169
(%) C.V.	2.5	7.2	2.3	5.5
Column #1	2449	2614	2978	2890
Column #2	2504	2552	2989	2821
Mean	2477	2583	2984	2855
SD	38.9	43.8	7.8	48.1
(%) C.V.	1.6	1.7	0.3	1.7

[a] Data represent samples analyzed by three injections.
[b] Laboratory 1: Quality control assay development and validation laboratory, Massachusetts Public Health Biologic Laboratories. Laboratory 2: Drug Laboratory, Massachusetts Department of Public Health. Column #1: Batch #58249817; Column #2: Batch #59307822. 9I10 and 9I19 are samples received from the Plasma Fractionation Department, Massachusetts Biologic Laboratories; Samples G-4 and TC-2 contain 3000 μg/ml TNBP and 10 000 μg/ml Triton X-100 in 7% human immunoglobulin.

Additional ruggedness/robustness studies (instrument to instrument and column to column reproducibility) are summarized in Table 5.

5.3. Selectivity

Quantification of T*n*BP is not affected by immunoglobulin or by Triton X-100 as summarized by the data in Table 6. The chromatographic method appears to be specific for T*n*BP in our products as shown by complete separation of structurally similar compounds T*n*AP (used as our internal standard) and T*n*PP (tri-*n*-propyl phosphate) as shown in Fig. 1A. The blank chromatogram (immunoglobulin extracted with hexane) shows that the noise is very low after 6 min, the time of elution of both T*n*BP and T*n*AP (Fig. 1B).

5.4. Limit of detection and limit of quantitation

Determination of the residual T*n*BP concentration in the immunoglobulin product is critical to ensure that human-derived pharmaceutical products are safe and that our manufacturing process is consistent. Thus the determination of the LOD and the LOQ is essential to our particular application. These particular assay parameters were determined by performing four different statistical analyses on data

Table 6
Selectivity: effect of protein and Triton X-100 on TNBP recovery[a]

Concentration of TNBP (μg/ml)	% TNBP recovery (15–17.5 mg protein)[b]	% TNBP recovery (60–70 mg protein)[b]	% TNBP recovery (120–140 mg protein)[b]	% TNBP recovery (100 μg/ml Triton X-100)	% TNBP recovery (200 μg/ml Triton X-100)	% TNBP recovery (400 μg/ml Triton X-100)
2	100	101	100	97	100	99
5	97	101	102	102	102	106
10	99	101	101	98	97	98
25	97	100	101	101	101	99
50	100	103	105	103	103	102

[a] Each data point represents sample prepared once and analyzed by GC analysis in triplicate. All data are compared to a reference condition (35 mg immunoglobulin/ml, 50 μg Triton X-100/ml).
[b] Different volumes of 60–70 mg/ml immunoglobulin were added to achieve different amounts of protein before hexane extraction.

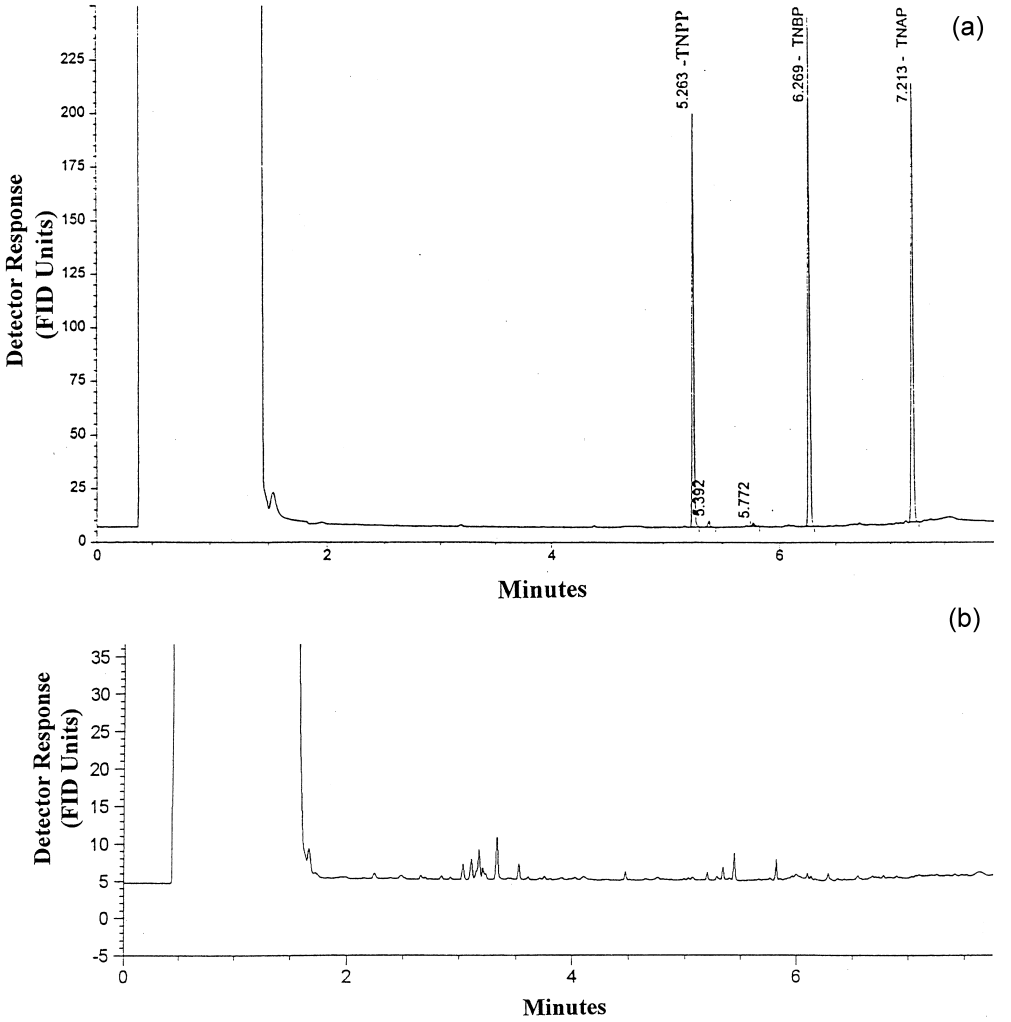

Fig. 1. (A) Gas chromatogram demonstrating resolution of T*n*PP, T*n*BP and T*n*AP. A preparation containing 25 μg/ml of each component in human immunoglobulin was extracted with hexane as described. One microliter injected into GC. (See Experimental for details). (B) Blank chromatogram. Five hundred microliters of human immunoglobulin was extracted with 1 ml of hexane. One microliter was injected into GC. The *Y*-axis scale is expanded to show noise of the baseline. Note the noise is very low after 6 min when T*n*BP and T*n*AP elute.

obtained from five different assays. The LOD and LOQ of the T*n*BP assay determined from different statistical methods are summarized in Table 7. Because noise measurements vary from method to method, conservative estimates are a LOQ of 2 μg/ml and a LOD of 0.65 μg/ml.

5.5. Carryover

The assay did not demonstrate any sample-to-sample carryover even after repeated injections of

Table 7
Limit of detection, limit of quantitation and assay noise

Method	Noise (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
MSD of response	0.197	0.649	1.966
SD *Y*-intercept	0.160	0.528	1.599
Residual SD	0.194	0.640	1.940
Blank peak area	0.117	0.385	1.168

TNBP at 50 μg/ml and TNAP at 25 μg/ml. Injections of hexane that followed did not contain any detectable quantities of analyte or internal standard.

5.6. Linearity and range

The linearity of the GC assay of T*n*BP was determined by testing T*n*BP standards ranging from 0 to 50 μg/ml spiked into 0.5 ml. The least squares linear regression analysis from an average of five different assays of a typical standard curve with concentrations ranging from 0 to 50 μg/ml T*n*BP reveals that the standard curve is linear with the coefficient of determination (R^2) 0.9999±0.0005, slope 0.0348±0.0008 and intercept of 0.004±0.003 (Table 8). The data also demonstrates that the assay response is proportional to concentration across the range of standards. The insignificant small residuals (Table 8) indicates that there is no significant deviation from the predicted variable line or concentration, again suggesting good linearity of the assay. Additionally, the residuals do not show any trending or non-randomness, consistent with linearity of the assay. Thus, the linear range of this assay is from 2 to 50 μg/ml TNBP.

Liquid–liquid extraction method is potentially troublesome because of emulsion formation and difficulties in separating the organic phase from the aqueous phase. Emulsion formation could be avoided by adding sodium chloride [16]. However, we observed that addition of salt results in the precipitation of immunoglobulin and low recovery of T*n*BP. In the present investigation we overcame the emulsion formation by layering with a small volume of ethanol. Ethanol breaks the emulsion resulting in a clear hexane upper layer, which can be easily removed for GC analysis. Acetone also can be used to break the emulsion (unpublished observation). The only precaution to be taken is that there may be slight increase in the concentration of acetone or ethanol in the aqueous phase. However, in the present study, a slight increase of ethanol in the aqueous phase did not affect the extraction efficiency of TNBP or TNAP into the hexane organic phase. The present method of assaying T*n*BP is rapid, sensitive, accurate, precise, robust, rugged, economic and time saving.

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Table 8
Linearity of assay response to concentration[a]

Standard concentration (μg/ml)	Response	Standard deviation	Standard error	Residuals	Response/ concentration
2	0.071	0.002	0.001	−0.0014	0.036
5	0.167	0.013	0.006	0.0004	0.033
10	0.353	0.017	0.008	−0.0083	0.035
25	0.881	0.05	0.022	0.0040	0.035
50	1.773	0.034	0.015	0.0106	0.035
				Standard deviation	0.001
				Mean	0.035
				% C.V.	3.1

[a] Slope=0.034; Standard deviation of slope=0.0008; Intercept=0.004; Standard deviation of intercept=0.003; R^2=0.9999; Standard deviation of R^2=0.0005. Data was compiled from an average of five different standard curves, each performed by triplicate injections.

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