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Rational approach to quantitative sodium dodecyl sulfate capillary gel electrophoresis of monoclonal antibodies

Huey G. Lee, Steve Chang, Edward Fritsche*

Analytical Research Laboratory, Bayer Diagnostics, 511 Benedict Avenue, Tarrytown, NY 10191, USA

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Abstract

Sodium dodecyl sulfate capillary gel electrophoresis has been used to separate and quantify murine monoclonal antibodies. The method uses a murine IgG, whose subclass differs from the analyte antibody, as an internal reference. The internal reference is chosen based on knowing that mouse IgG_1 can be separated from mouse IgG_{2a} or IgG_{2b}. Good intra- and inter-day reproducibility [relative standard deviation (RSD)<2%] of peak-area ratio has been obtained. A calibration curve also demonstrates high linearity ($R^2=0.9999$) of response for the analyte. The described method is highly suitable for accurate determination of the antibody concentration even if a capillary electrophoresis apparatus is unable to provide good injection reproducibility. © 2002 Published by Elsevier Science B.V.

Keywords: Monoclonal antibodies; Immunoglobulins; Proteins

1. Introduction

Monoclonal antibodies have been widely used in the immunodiagnostics industry. Their specificity for target analytes makes them highly valuable in analytical and clinical immunoassays. When tagged with either a fluorescence or chemiluminescence agent, the antibody conjugates provide a means for superior selectivity and sensitivity for analyte detection and with minimal sample manipulation. In the biopharmaceutical industry, monoclonal antibodies have been used either as therapeutic agents or in drug delivery systems. Their overall importance has generated analytical interests in the area of separation not only to characterize these antibodies but to accurately quantify them [1]. The latter is the focus of the present study.

Sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE) is a high-performance version of the conventional SDS–polyacrylamide gel electrophoresis (PAGE), which has been widely used for purity assessment and for molecular mass (M_r) estimation of proteins. Electrophoretic separation of a SDS–protein complex according to its size or M_r is achieved in a sieving medium [2,3]. The main advantages offered by the capillary technique include on-column direct UV or fluorescence detection, better quantitative analysis, resolution, reproducibility, computerized data handling, fast single sample analysis, and automation [4–6]. There are, however, disadvantages that include poor mass sensitivity with UV detection and inability to simultaneously separate multiple samples. Nevertheless, the benefits outweigh its limitations.

Historically, performing SDS-CGE has evolved from the use of cross-linked polyacrylamide gels

*Corresponding author. Fax: +1-914-524-3155.

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[7,8] to the use of more UV-transparent replaceable polymer solutions of polyethylene glycol and dextran [4,5,9–16]. It is important to note that the expression SDS-CGE continues to be used by the authors and many others to describe the technique, though the later sieving medium clearly is not gel but replaceable polymer solution. In many cases, separation is further simplified by using uncoated fused-silica capillaries [17–20]. Replaceable dextran polymer solution is commercially available and has been used for antibody separation in uncoated capillaries [19,20]. Several research groups have also reported the use of SDS-CGE for quantitative analyses of immunoglobulin Gs (IgGs) [19,21,22]. Bennett et al. [21] used the technique to quantify bovine colostral and serum IgGs and reported RSD (relative standard deviation) values $\geq$10% for the assay. Hunt et al. [22] reported better RSD values (3–8%) for normalized peak-areas of several peaks of a ''recombinant humanized'' monoclonal antibody.

Although capillary electrophoresis (CE) instruments have been around for more than ten years, injection imprecision remains to be one of the weakest points of CE. A recent review by Mayer [23] gives an excellent summary on this topic. In general, quantitative reproducibility of CE is worse than that of high-performance liquid chromatography (HPLC). Reported RSD values for peak-area precision can be quite high ($>$10%). The main difficulty lies in a CE apparatus capable of precisely injecting and reproducibly delivering nanoliter sample volumes into the capillary. Hydrodynamic injection generally provides better precision than electrokinetic injection. The former often uses a built-in pressure or vacuum capability of a CE apparatus, but many factors such as temperature, buffer viscosity, sample carryover, evaporation, and diffusion, etc. as well as differences in pressure or vacuum level can all play roles in affecting injection reproducibility.

One way, perhaps the most effective way, to circumvent the problem of injection imprecision is to use a proper internal reference plus a hydrodynamic sample-delivery mode. An ideal internal reference is one that is nearly identical to the analyte of interest and migrates near it. Those requirements help to ensure that both exhibit the same assay precision so that their peak-area ratio (analyte:internal reference) will be consistent. In this study, we use a murine IgG, whose subclass differs from the analyte antibody, as an internal reference. The reference IgG is chosen simply based on the fact that mouse IgG_1 can be separated from mouse IgG_{2a} or IgG_{2b}. With this rational approach, we have been able to improve intra- and inter-day RSD values from as high as $>$10% to $<$2% for peak-area ratio reproducibility.

2. Experimental

2.1. Reagent and materials

A CE-SDS protein kit (Bio-Rad, Hercules, CA, USA) that provides separation for proteins of molecular masses 10 000–200 000 was used. Sodium phosphate, sodium chloride, hydrochloric acid (1 *M*) and sodium hydroxide (1 *M*) were from Fisher (Fair Lawn, NJ, USA). Fused-silica capillaries of 75 μm I.D.$\times$360 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Murine monoclonal antibodies (IgG_1, IgG_{2a} and IgG_{2b}) were purified by protein A affinity chromatography at Bayer, Diagnostics Division (East Walpole, MA, USA). Carbonic anhydrase, lysozyme, cytochrome *c*, and trypsinogen were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin was from Pentex Division (Bayer, Kankakee, IL, USA).

2.2. Instrumentation

SDS-CGE was performed on an Agilent Technologies ^{3D}CE CHEMSTATION (Waldbronn, Germany) equipped with a Hewlett-Packard Vectra VE pentium II computer. Signals were detected at 220 nm using the electropherograph's built-in diode array detection system. Temperature was regulated to 20 °C by the instrument's air-circulating heating and cooling system. Operation of the instrument and data collection/analysis were controlled by Agilent Technologies CHEMSTATION system software revision A.06.03 (509).

2.3. Methods

2.3.1. Sample preparation

Antibody samples (0.07–1.07 mg/ml) were in phosphate-buffered saline (PBS; 10–20 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4). Sample was then

diluted 1:1 with the SDS sample buffer (1% SDS in 100 m*M* Tris–HCl, pH 9.2) available from the Bio-Rad kit, and then heated at 70 °C for 5 min. Appropriate internal reference would be added to the SDS sample buffer prior to its use. A Reacti-Therm III heating module (Pierce, Rockford, IL, USA) was used to heat SDS–antibody mixtures at 70 °C.

2.3.2. SDS-CGE

SDS-CGE was performed according to the previously described procedure [20]. Injection was carried out by applying pressure (50 mbar) for 160 s, and a constant voltage of −17.5 kV was applied for electrophoresis. The Agilent Technologies CHEMSTATION software corrected the peak area for migration time. In the text, the term ''peak area ratio'' equals peak area (analyte) divided by peak area (internal reference).

3. Results and discussion

An important aspect of quantitative analysis by any separation technique is to obtain a homogeneous analyte peak that is easy to integrate. Even under non-reduced SDS-CGE conditions, IgG tends to fragment significantly if the SDS–antibody mixture is first subject to prolonged heat treatment (>5 min) at 100 °C [19,20]. Heat treatment is necessary to obtain ''stable'' and uniform SDS–protein complexes; however, excessive heating can be detrimental. Hunt and Nashabeh [19] described the observation for antibody as ''thermally-induced fragmentation'' and minimized it by essentially reducing temperature and time of exposure. Lee [20] observed that high ionic strength and/or high pH conditions plus heat treatment also enhanced the fragmentation of SDS–IgG complexes. Accordingly, we optimized the sample preparation conditions for the formation of the SDS–antibody complexes at a temperature (70 °C) and an exposure time (5 min). A good quantitative IgG peak was obtained (Fig. 1). Fragments were nearly absent.

To qualify the separation in Fig. 1 for quantitative analysis, RSD values <5% for peak-area reproducibility were expected. Peak-area precision (RSD = 11–16%, $n = 6$) for the antibody turned out to be inadequate (Table 1). Benzoic acid was better (RSD = 6–7%, $n = 6$) and using it as internal reference improved the RSD values for the peak area ratio (antibody:benzoic acid) precision to 8–9%. It was clear then that injection reproducibility was poor under the experimental conditions and that quantitative precision suffered. The separation buffer contained modified dextran of high-molecular-mass (viscosity = 43 cP) [24], and relatively long injection time (>1 min) was required to inject sufficient amount of sample into capillary to achieve sufficient UV sensitivity. A combination of the prolonged time of sample loading plus potential temperature variation to affect viscosity of the dextran solution could produce the undesirable outcome. Furthermore, in-

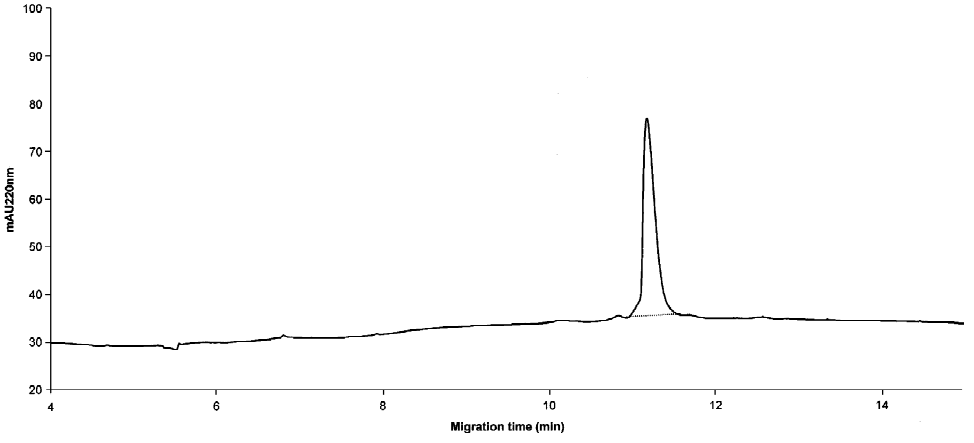

Fig. 1. Electropherogram of a monoclonal antibody (IgG_{2a}).

Table 1
Peak area and peak area ratio reproducibility for an antibody and an internal reference (benzoic acid)

Experiment[a]		Peak area (mAU) and ratio		RSD (%)
		Average ($n=6$)	SD	
1	Benzoic acid (Ba)	0.5564	0.0338	6.1
	Antibody (Ab)	0.5795	0.0611	10.5
	Ab:Ba	1.0416	0.0936	9.0
2	Benzoic acid (Ba)	0.5566	0.0343	6.2
	Antibody (Ab)	0.2905	0.031	10.7
	Ab:Ba	0.5216	0.0393	7.5
3	Benzoic acid (Ba)	0.3058	0.0226	7.4
	Antibody (Ab)	0.3251	0.0503	15.5
	Ab:Ba	1.0579	0.0982	9.3

[a] In experiment 2, initial concentration of the antibody was halved; whereas in experiment 3, initial concentrations of benzoic acid and antibody were both halved.

consistent capillary regeneration due to poor reproducibility of the performance of the apparatus could also affect migration time and sample recovery. The use of the small molecule internal reference was insufficient to improve quantitative precision to acceptable level (RSD$<$5%). However, a capillary electrophoresis apparatus equipped with better temperature control and pressure output consistency might improve the quantitative precision both with and without the internal standard.

Commercially available proteins that include bovine serum albumin, carbonic anhydrase, lysozyme, cytochrome *c*, and trypsinogen were investigated as internal references for quantitative SDS-CGE analysis of monoclonal antibody. Reproducibility for peak area ratio was in the range 1–8% (RSD, $n=6$), but consistent inter- and intra-day precision ($<$5%) could not be achieved. It was this latter observation that precluded us from performing more experiments using these unrelated proteins. Run-to-run variation in migration time and possibly in mass recovery across the capillary between each of these proteins and the murine IgG, due to the factors discussed above, could account for the observation. None of the proteins tested was therefore considered ''good'' enough to function as an internal reference for quantifying murine IgG on SDS-CGE.

Table 2
Peak area and peak area ratio reproducibility for an antibody and an internal reference (IgG)

	Peak area (mAU) and ratio		RSD (%)
	Average ($n=6$)	SD	
IgG(2b)	0.2773	0.0362	13.1
IgG(1)	0.2768	0.0349	12.6
IgG(2b):IgG(1)	1.0013	0.0107	1.1
IgG(1):IgG(2b)	0.9988	0.0106	1.1

Fig. 2 shows that monoclonal antibodies subclasses 1 and 2b are well-separated under the SDS-CGE conditions. Each of the two antibodies gives an equally poor RSD value 13% ($n=6$) for their peak area precision (Table 2). However, when peak areas of one antibody are normalized against those of the

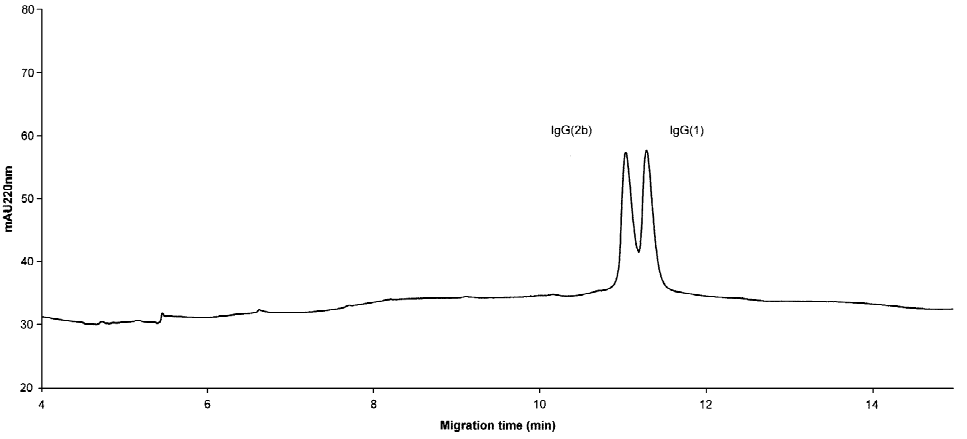

Fig. 2. Electropherogram of monoclonal antibodies (IgG_1 and IgG_{2b}).

other, the resulting peak area ratios in both cases have excellent RSD values (1.1%, $n=6$). Clearly, we have a working system in which two monoclonal antibodies can be separated due to differences in their subclasses yet they are so closely-related that their peak area precision is essentially identical within a separation. The use of one IgG subclass as an internal standard for another IgG subclass circumvents the difficulty of maintaining ''equivalent'' electrophoretic behavior between the standard and analyte. Any random decrease or increase in UV signal of an IgG peak is well compensated by an ''equivalent'' decreased or increased signal of another IgG in a separation. These properties qualify them as internal references for each other and ensure consistent peak-area ratios necessary for good quantitative reproducibility for the analyte antibody.

SDS-CGE separates proteins according to their size. Immunoglobulin G has an apparent molecular mass 150 000, but there are subclasses of IgG. These subclasses are defined by their variation in heavy chain sequences [25]. In addition to the distinctive immunochemical properties among them, different IgG subclasses (e.g. IgG_1 and IgG_2) exhibit different electrophoretic and ultracentrifugal behavior, and IgG_{2a} and IgG_{2b} are similar in those behavior [26,27]. Their physicochemical properties may be most directly responsible for the SDS-CGE separation of IgGs even though they have the same molecular mass. However, under SDS-CGE conditions, free-zone electrophoretic mobility of an IgG molecule become insignificant when the protein is bound by excess level of the negatively-charged SDS and migrates in a sieving medium. Fahey et al. [26] reported the sedimentation coefficients of 6.9 S and 6.7 S for IgG subclasses 1 and 2, respectively. Although the sedimentation coefficient is also related to frictional coefficient and partial specific volume of an macromolecule [28], the difference might not be significant enough to account for the observed separation. Therefore, any SDS-CGE separation of murine IgG_1 and IgG_2 is most likely due to their difference in interaction with SDS leading to any difference in their effective sizes as the complexes. Accordingly, a proper IgG can always be selected as an internal reference for quantitative analysis of another IgG on SDS-CGE.

Fig. 3A–E shows a series of electropherograms with varying IgG_{2a} concentrations from 0.07 to 1.07 mg/ml. The internal reference is an IgG_1. Like IgG_{2b} in Fig. 1, IgG_{2a} also migrates earlier than IgG_1. RSD values ($n=3$) at the four concentrations (0.13, 0.27, 0.54 and 1.07 mg/ml) of the analyte antibody are 0.8, 1.2, 1.3 and 0.6%, respectively. The lowest concentration (0.07 mg/ml) has the worst RSD value—3.8%, indicating that limit of detection may be near (see also Fig. 3A). By plotting the antibody concentrations vs. the peak area ratios, a straight line ($y=2.0648x+0.0105$, $R^2=0.9999$) is obtained (Fig. 4). The excellent R^2 value also indicates an excellent linearity of response for the IgG_{2a} analyte.

In a separate experiment, intra- and inter-day RSD values for peak area ratio [IgG_1:IgG_{2b} (internal reference)] reproducibility were evaluated (Table 3). We obtained intra-day RSD values (0.8–1.5%, $n=6$) on 3 different days and a combined inter-day RSD value 1.3% ($n=18$). The data consistently supported the fact that this rational selection of internal reference was capable of providing good reproducibility to quantify the antibody by SDS-CGE.

We believe that the present study opens the door to quantitative analysis of IgGs from other animal sources by applying this rational approach of properly selecting an IgG internal reference. For example, we have been able to separate a murine IgG_{2b} from an ovine polyclonal antibody (data not shown), hence making the murine monoclonal antibody a potential internal reference for the quantitative analysis of the polyclonal antibody. However, it is often necessary to optimize conditions for the heat treatment of the SDS–antibody complexes in order to eliminate antibody fragmentation and to produce an efficient homogeneous peak of interest suitable for peak-area integration. IgGs from various animal sources exhibit different degrees of fragmentation under the SDS-CGE conditions [20]. One can also prepare the SDS–internal reference antibody complexes separately from the preparation of the SDS–analyte antibody complexes before carefully mixing the two components for the separation and analysis.

4. Conclusion

The present study demonstrates that SDS-CGE is an excellent method for quantitative analysis of

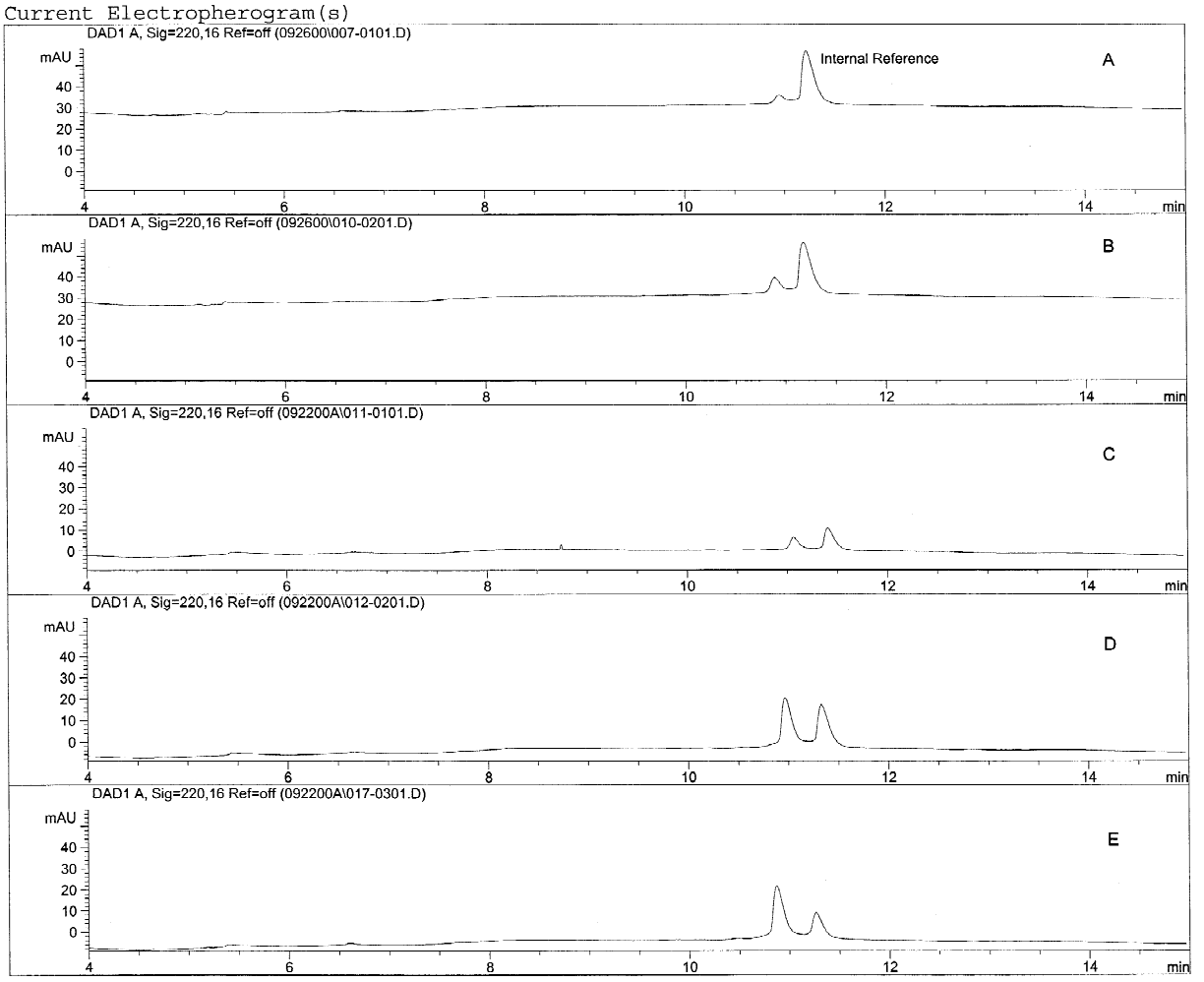

Fig. 3. Electropherograms of monoclonal antibodies (IgG_1 and IgG_{2a}). (A–E) Analyte (IgG_{2a}) concentrations (0.07, 0.13, 0.27, 0.54 and 1.07 mg/ml, respectively). Internal reference is an IgG_1.

Table 3
Intra- and inter-day reproducibility of peak area ratio

	Peak area ratio[a]		RSD (%)
	Average ($n=6$)	SD	
Day 1	1.0572	0.0086	0.8
Day 2	1.0653	0.0163	1.5
Day 3[b]	1.0565	0.0152	1.4

[a] Peak area ratio = {[peak area IgG(1)]/[peak area IgG(2b)]}.
[b] On day 3, a different lot of reagent and a new capillary were used.

monoclonal antibodies. An uniform peak can be obtained by optimizing SDS sample pretreatment temperature and its exposure time to that temperature. Separation of murine IgG_1 from IgG_{2a} or IgG_{2b} allows for a proper IgG internal reference to be chosen. Through this rational approach, we have been able to achieve consistently good peak-area ratio precision (intra- and inter-day RSD values $<2\%$) and excellent linearity ($R^2=0.9999$) of response for the analyte antibody. Limitations associated with CE sample injection repeatability are therefore circumvented for the purpose of quantifying murine monoclonal antibodies by SDS-CGE.

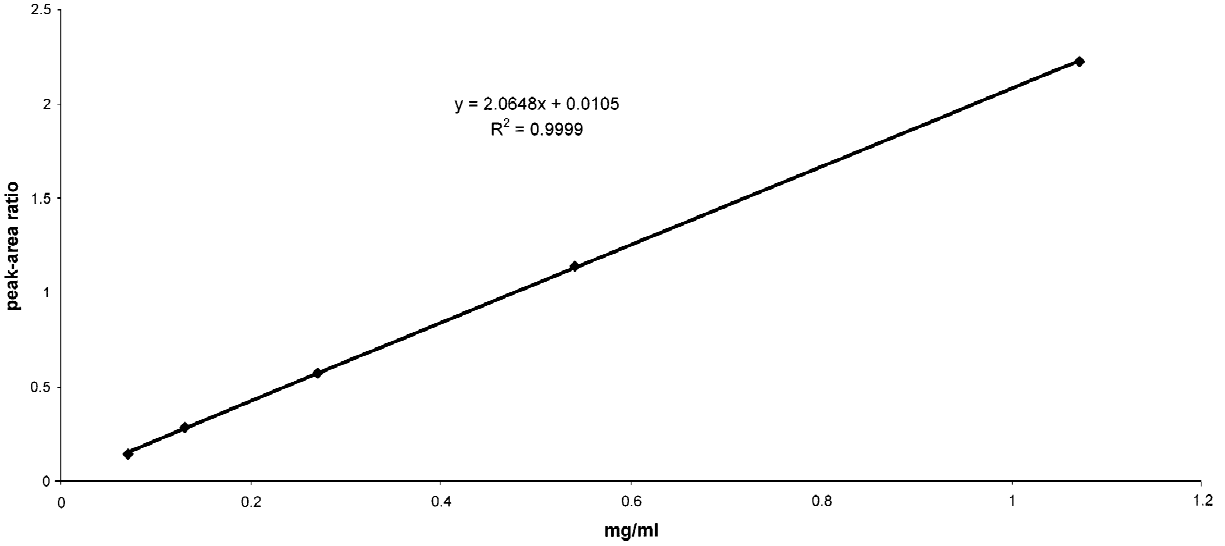

Fig. 4. Calibration curve, concentration vs. peak area ratio.

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