(19) DANMARK

(10) DK/EP 4133263 T3

(12) Oversættelse af europæisk patentskrift

Patent- og Varemærkestyrelsen

(51) Int.Cl.: *G 01 N 27/447 (2006.01)* *C 07 K 16/06 (2006.01)*

(45) Oversættelsen bekendtgjort den: **2024-06-03**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2024-05-22**

(86) Europæisk ansøgning nr.: **21716415.1**

(86) Europæisk indleveringsdag: **2021-04-01**

(87) Den europæiske ansøgnings publiceringsdag: **2023-02-15**

(86) International ansøgning nr.: **EP2021058775**

(87) Internationalt publikationsnr.: **WO2021204714**

(30) Prioritet: **2020-04-07 EP 20168561**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **Ares Trading S.A., Zone Industrielle de l'Ouriettaz, 1170 Aubonne, Schweiz**

(72) Opfinder: **VASSALLO, Oscar, c/o Merck Serono S.p.A. Via Luigi Einaudi 11, 00012 Guidonia Montecelio RM, Italien**

(74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**

(54) Benævnelse: **Kapillær gelelektroforese og anvendelse deraf med komplekse biologiske molekyler**

(56) Fremdragne publikationer:
WO-A1-86/06383
US-A1- 2012 125 775
CHERKAOUI S ET AL: "Tracking of antibody reduction fragments by capillary gel electrophoresis during the coupling to microparticles surface", JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS, ELSEVIER B.V, AMSTERDAM, NL, vol. 53, no. 2, 10 October 2010 (2010-10-10), pages 172-178, XP027077563, ISSN: 0731-7085, DOI: 10.1016/J.JPBA.2010.01.039 [retrieved on 2010-02-01]
M ; Gromelski ET AL: "Agarose Gel Electrophoresis of High Molecular Weight, Synthetic Polyelectrolytes", Macromolecules J. Am. Chem. Soc. Phys. Philos. Mag. Macromolecules J. J. Chem. Phys. P. J. Photochem. Photobiol. J.; Emerald, R. L. J. Appl. Phys. P. J. J. Chem. Phys. Phys. Rev. Phys. Rev. B, 1 May 1989 (1989-05-01), pages 2270-2277, XP055730664, Retrieved from the Internet: URL:https://pubs.acs.org/doi/pdf/10.1021/ma00195a048 [retrieved on 2020-09-15]
Klaus Weber ET AL: "The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis", Journal of Biological Chemistry, 25 August 1969 (1969-08-25), pages 4406-4412, XP055124062, UNITED STATES Retrieved from the Internet: URL:http://www.ncbi.nlm.nih.gov/pubmed/5806584 [retrieved on 2020-09-10]
Wikipedia: "Wikipedia - SDS-Page", , 6 April 2020 (2020-04-06), XP055730251, Retrieved from the Internet: URL:https://en.wikipedia.org/w/index.php?title=SDS-PAGE&oldid=949434264 [retrieved on 2020-09-14]
Wikipedia: "Wikipedia - Gel electrophoresis", Wikipedia.org, 5 March 2020 (2020-03-05), XP055729543,

Fortsættes ...

Retrieved from the Internet: URL:https://en.wikipedia.org/w/index.php?title=Gel_electrophoresis&oldid=944054016 [retrieved on 2020-09-10]

DESCRIPTION

Description

Field of the Invention

[0001] The invention described herein relates to analytical capillary gel electrophoresis (CGE).

Background of the Invention

[0002] Capillary gel electrophoresis (CGE) is a sensitive and versatile technique which has emerged into the forefront of analytical methodology. Its applicability is enhanced by short separation times, facile and rapid methods of development and a requirement for very small amounts of analyte. Capillary gel electrophoresis relies on electro kinetic separation methods performed in submillimeter diameter capillaries and in micro- and nanofluidic channels. In CGE analytes migrate through electrolyte solutions under the influence of an electric field. Analytes can be separated according to molecular mass of the molecules present in the analyte.

[0003] The identity of molecules and their relative amounts in a test sample are frequently determined using CGE. Such determinations rely on the migration time(s) of the different constituents in such test sample. Considering the inter-experiment (i.e. experiment to experiment) variations in such migration times it is necessary to establish a proper calibration curve for each experiment. Such calibration curve is customarily obtained using a sample solution containing known amounts of a number of different molecules. These molecules are chosen to cover a range of molecular weights, preferably covering a range of molecular weights, which range encompasses the expected molecular weight of the analyte of interest.

[0004] In such sample/calibration solutions standard calibration molecules are customarily used. In standard CGE methods it is expected that the migration times of any of the molecules is directly correlated to their molecular weight. Thus, the only characteristic that is of relevance for the molecules in the sample/calibration solution is their molecular weight.

[0005] WO 86/06383 A1 discloses a method for the detection and determination of the molecular weights of one or more sample proteins.

[0006] It has been noted however that for complex biologic molecules this direct correlation between migration times and molecular weight is not consistently observed. Some complex biologic molecules, in particular fusion proteins and antibodies (which proteins may be

glycosylated), may have a migration time different from the expected migration time based on their molecular weight. Accordingly, the standard molecules provided in a calibration sample do not provide a calibration curve which can be used to identify the various complex biologic molecules. Misinterpretation of the identified peaks in a CGE chromatogram may than result in misidentification of the various molecules and their relative amounts in a test sample.

[0007] Therefore, there is a need in the art for an improved CGE method which would overcome these problems observed when carrying out analytical CGE with complex biologic molecules.

Summary of the Invention.

[0008] The present invention provides a solution to the problems described above. An improved method is provided for analytical CGE for complex biologic molecules. In particular, such improved method comprises obtaining a calibration curve with a partially reduced calibration solution of the analyte biologic molecule of interest. In one embodiment there is provided a method of determining the molecular weight of constituents of a test sample containing a biologic molecule using capillary gel electrophoresis (CGE), the method comprising the steps of: a.) obtaining a calibration curve for the biologic molecule in CGE comprising the steps of partially reducing a calibration sample comprising a known amount of the biologic molecule, submitting the partially reduced calibration sample to CGE, determining the migration times of the components of the partially reduced biologic molecule in the partially reduced calibration sample, and calculate the calibration curve for the biologic molecule in CGE; b.) submitting the test sample containing the biologic molecule to CGE; c.) determining the migration times of the constituents in the test sample; and d.) identifying the molecular weight of the constituents in the test sample by comparing the migration times with the calibration curve obtained in step a). Such method is particularly useful in the analysis of samples containing complex biologic molecules such as antibodies or fusion proteins.

[0009] In another embodiment there is described a method for identifying the composition of a test sample containing a biologic molecule comprising: a.) determining the molecular weight of the constituents of the sample according to the method described in the previous embodiment comprising the steps of obtaining a calibration curve for the biologic molecule in CGE by partially reducing a calibration sample comprising a known amount of the biologic molecule, submitting the partially reduced calibration sample to CGE, determining the migration times of the components of the partially reduced biologic molecule in the partially reduced calibration sample, and calculate the calibration curve for the biologic molecule in CGE, submitting the test sample containing the biologic molecule to CGE, determining the migration times of the constituents in the test sample, and identifying the molecular weight of the constituents in the test sample by comparing the migration times with the calibration curve; b.) identifying the constituents of the sample based on their molecular weight, and determine the relative amounts of each of the constituents based on the CGE performed in step a.).

[0010] In another embodiment there is described a method of determining the purity of a sample containing the biologic molecule comprising: a.) identify the composition of the sample according to the method using the method described in the previous embodiment and b.) determine the relative amount of the biologic molecule of interest present in the sample.

Brief Description of the Drawings

[0011]

Figure 1; shows the migrations times of a sample containing the customary molecular weight calibration molecules spiked with a complex biologic molecule (Cetuximab (Erbitux®)). Clearly shown is the discrepancy between the migration times of the standard sizing molecules (Std) and the cetuximab molecular subunits/species (considering their theoretical weights) (Erbitux LC and Eritux HC).

Figure 2; shows the expected molar ratio's for both light chain (LC) and heavy chain (HC) included in each of the different molecular subunits/species of a partially reduced antibody.

Figure 3; shows the different migration times and calculated molecular weights for molecular subunits/species of a partially reduced human IgG2 Non-Glycosylated antibody (anti-TIM3).

Figure 4; shows the different migration times for molecular subunits/species of a partially reduced human IgG2 Non-Glycosylated antibody (anti-TM3) and the resulting calibration curve based on the observed migration times.

Figure 5; shows the different migration times for molecular subunits/species of two partially reduced antibodies in comparison with the commercial size standard species migration, with the relative calibration curve and the huge discrepancies found in the comparison of the tree curves.

Figure 6; shows the different migration times for molecular subunits/species of a partially reduced human IgG2 Non-Glycosylated antibody (anti-TM3), the resulting calibration curve based on the observed migration times and the comparison between experimental and theoretical molar ratio relative percentages.

Figure 7; shows the different migration times for molecular subunits/species of a partially reduced IgG1 Glycosylated antibody, the resulting calibration curve based on the observed migration times and the comparison between experimental and theoretical molar ratio relative percentages.

Figure 8; shows the different migration times for molecular subunits/species of a partially reduced IgG1 with kappa light chain Glycosylated antibody, the resulting calibration curve based on the observed migration times and the comparison between experimental and theoretical molar ratio relative percentages.

Figure 9; shows the different migration times for molecular subunits/species of a partially reduced IgG1.4 moiety conjugated to Dolaflexin with a maleimide-based Cys linker Glycosylated antibody, the resulting calibration curve based on the observed migration times and the comparison between experimental and theoretical molar ratio relative percentages.

Figure 10; shows the different migration times for molecular subunits/species of a partially reduced Chimeric mouse/human IgG1 Non-Glycosylated antibody, the resulting calibration curve based on the observed migration times and the comparison between experimental and theoretical molar ratio relative percentages.

Figure 11; shows the different migration times for molecular subunits/species of a partially reduced Fusion IgG1 with Sirp-α domain linked to LC Glycosylated antibody, the resulting calibration curve based on the observed migration times and the comparison between experimental and theoretical molar ratio relative percentages.

Detailed Description

[0012] Capillary gel electrophoresis (CGE) is a frequently used analytical method to determine the composition of an analyte sample. The method can be used qualitatively and semi-quantitatively to determine the composition of an analyte sample, such as for example produced in the production of an active pharmaceutical ingredient. As an initial step in such analytical method a calibration of the method is carried out by means of a calibration curve with molecules of known molecular weight. The identity, as compared to their molecular weight, of molecules present in a sample are based on their migration times in comparison to the migration times of the molecules of known molecular weight as in the calibration curve made with the same molecule that is under investigation. The method relies on the direct correlation between migration time and the molecular weight of the molecules in the tested samples. However, migration times for complex biologic molecules in CGE may not always correlate directly to their molecular weight in comparison to the migration times for the conventional standard molecules in a calibration sample.

[0013] In figure 1, an example is provided wherein a sample containing the standard molecular weight standards is mixed with a certain amount of the antibody cetuximab (known as Erbitux®). It is clear that the subunits/species of cetuximab, which have a molecular weight of 23 kD and 53 kD have different migration times compared to the conventional standards having approximately the same molecular weight. As a result, the 23 kD cetuximab subunit/species migrates compared to the 25 kD standard with a migration time that appears to be around 30 kD. Likewise, the 53 kD cetuximab subunit/species has a migration time compared to the 50 kD (and 100 kD) standard that appears closer to a molecule of around 75 kD. Any such discrepancies may be due because such complex molecules upon exposure to reductants disintegrate in multiple components or the molecules are of such size or contain a

certain exposed charge and complex sugars that will cause their migration under an electric field and interaction with a Dextran-based gel matrix (as commonly used) in CGE to behave differently from conventional standards that are mostly un-glycosylated.

[0014] In the present invention the calibration curve is obtained with a sample of the analyte biologic molecule of interest instead of using the conventional calibration standards. Moreover, the calibration curve is obtained using a novel method for partially reducing the complex analyte biologic molecule of interest. The method of partially reducing the complex analyte biologic molecule of interest enables obtaining a calibration curve with various components (or combinations thereof) of the analyte biologic molecule of interest. For example, the analyte biologic molecule of interest is an antibody protein. Such antibody protein is a complex molecule consisting of multiple elements (subunits or species) to form the antibody protein. Two light chain and two heavy chain proteins make up a complete antibody. As shown in figure 2, partial reduction of an antibody sample results in multiple distinct elements (subunits/species) with distinct molecular weights and a single specific molar ratio. Figure 2 shows such possible subunits/species of such partial reduction of an antibody including individual light chains (LC), individual heavy chains (HC), a single combination of a light chain and heavy chain (1H1L), a combination of two heavy chains (HH), a combination of two heavy chains and one light chain (2H1L), and the intact antibody molecule (2H2L). For a human IgG2 non-glycosylated exemplary antibody (anti-TIM3) the molecular weight of each of the molecular subunit/species is known as shown in Figure 3.

[0015] The analyte biologic molecule of interest as used in the method of the present invention includes antibodies, single chain antibodies, fusion proteins, chimeric proteins and any other proteins having one or more subunits or one or more reductions sites (such as disulfide bonds). Preferably, for such analyte biologic molecule of interest the molar ratio of the molecular subunits/species is known. Although particularly useful for analyte biologic molecules the method of the present invention is not limited to a use with complex biologic molecules. Any analyte molecule comprising reducible subunits can be used in the method of the present invention.

[0016] The partial reduction of the analyte biologic molecule comprises at least two major steps. In a first step a reducing agent is employed to partially reduce the complex biologic molecule. In another step an alkylating agent is used which quenches the mixture of the complex biologic molecule and the reducing agent and prevents further reduction of the complex biologic molecule. Thus, partial reduction of the calibration sample of the analyte biologic molecule of interest comprises: a.) adding to a buffered solution containing the known amount of the biologic molecule a reducing agent, and b.) incubating the calibration sample of step a) for a period of time, wherein the length of the period of time is such that biologic molecule is not fully reduced. Within such time period wherein the biologic molecule is not fully reduced the method may further comprise: c.) adding an alkylating agent to the partially reduced calibration sample of step b, and d.) incubating the partially reduced calibration sample of step c) for a period of time.

[0017] In the above method of partially reducing the complex biologic molecule the first step (step a.) is preferable carried out at room temperature for a period of about 30 minutes or less. The step of incubating the partially reduced calibration sample with an alkylating agent (step d.) is preferable carried out at a temperature in the range of room temperature to about 75°C, preferably between about 50°C and about 75°C, more preferably at about 70°C. Incubation of the partially reduced calibration step with the alkylating agent is for a period of up to about 15 min, preferably from about 5 min to about 15 min, more preferably for a period of about 10 min. The term "about" herein is defined as an amount plus or minus 10 percent of the stated value. Thus about 50°C indicates 50°C +/- 5°C and about 30 min indicates 30 min +/- 3 min.

[0018] The concentrations used for the reducing agent and the alkylating agent in the partial reduction method of the present invention is from about 15 to 500 mM, preferably from about 30 to about 470 mM, more preferably from about 125 mM to about 375 mM, even more preferably from about 200 mM to about 300 mM. The reducing agent and alkylating agent are preferably used in a concentration ratio of reducing agent: alkylating agent of about 0.16 to 0.80. As such the concentration ratio of reducing agent to alkylating agent is in the range of 0.15:3, suitably from 1:6.5 to 3:1. More preferably the ratio of reducing agent to alkylating agent is 1:1. Preferably, the concentration of reducing agent and alkylating agent is 250 mM.

[0019] As reducing agent any conventional reducing agent can be used. Preferably the reducing agents is 2-mercaptoethanol, 2-mercaptoethylamine, dithiotreitol (DTT), Tris (2-carboxyethly) phophine (TCEP) or dithiobuthylamine (DTBA). More preferably the reducing agent is TCEP.

[0020] As alkylating agent any conventional alkylating agent can be used. Preferably the alkylating agent is selected from maleimide or a derivative thereof, 9-anthracenemethanol or a derivative thereof, 1-naphtalinemethanol, 2,2-biphenyldimethanol, 2-indanol, iodineacetmide, dithiotreitol (DTT), ammonium bicarbonate, streptozocin, N-nitro-Nethylurea, Procarbazine, temozolomide, busulfan, 2-chloro-2-methylpropane, carmustine, cyclophosphamide, 2-bromo-3'-methoxyacetophenone, 4-chloro-1-butanol, 3-chloropropionamide, bromoacetylchololine bromide, 1,10-diiodeodecane, 5-chlorovaleroyl chloride, R(-)-chloroethylnorapomorphine, ethyl methanesulfonate, methyl trifluoromethanesulfonate, tetrapentylammonium bromide, N,N-dimethylisopropylamine, 1-chloro-2,4-dinitrobenzene, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate, ethyl 7-bromoheptanoate, 2-fluorobenzyl bromide, 2,3-dihydro-3-oxo-4H-1,4-benzoxazine-4-propionic acid, ellagic acid, phenethylamine, dacarbazine, cis-1,5-dimethylbicyclo[3.3.0]octane-3,7-dione, 2,4'-dichloroacetophenone, 3,4-dichlorobenzyl chloride, trimethylsilyl bromoacetate, (3-bromopropoxy)-tert-butyldimethylsilane, diethylsulfate, 2-(2-bromoethyl)-1,3-dioxolane, 1,3-bezodithiolylium tetrafluoroborate, 4-(4-Nitrobenzyl)pyridine, 5-methoxyindole-2-carboxylic acid, phosphazine base, ethyl p-toluenesulfonate, N-Boc-5-methoxyindole, lomeguatrib, and chloroacetonitrile. As referred to herein, suitable derivatives of maleimide are propylmaleimide, N-ethylmaleimide, N-methylmaleimide, N-(2-hydroxyethyl) maleimide, N-hydroxymaleimide, N-(1-phenylethyl)-maleimide, N-(4-chlorphenyl) maleimide, 2-maleinimidoethyl-mesylate, 1-(4-aminophenyl)-1H-pyrrole-2,5-dione, N-phenylmaleimide, 1-(2-aminoethyl) maleimide, N-(2-aminoethyl)-

maleimide-trifluoroacetate, N-tert-butylmaleimide, N-benzylmaleimide, and N-ethylmaleimide. In addition, as referred to herein suitable derivatives of 9-anthracenemethanol are α-methyl-9-anthrylmethanol, 9-fluoronmethanol, methacrylacid-9-antracenylmethyl ester, 9-antracencarbaldehyde, 9-antracencarboylic acid, 9-(methylaminomethyl)-antracene. More suitable the alkylating agent is selected from maleimide, iodoacetamide and 9-antracenemethanol. Even more preferable the alkylating agent is maleimide.

[0021] As such the improved method of the present invention comprises obtaining a calibration curve with a partially reduced calibration solution of the analyte biologic molecule of interest. In one embodiment there is provided a method of determining the molecular weight of constituents of a test sample containing a biologic molecule using capillary gel electrophoresis (CGE), the method comprising the steps of: a.) obtaining a calibration curve for the biologic analyte molecule in CGE comprising the steps of partially reducing a calibration sample comprising a known amount of the biologic molecule, submitting the partially reduced calibration sample to CGE, determining the migration times of the components of the partially reduced biologic analyte molecule in the partially reduced calibration sample, and calculate the calibration curve for the biologic analyte molecule in CGE; b.) submitting the test sample containing the biologic analyte molecule to CGE; c.) determining the migration times of the constituents in the test sample; and d.) identifying the molecular weight of the constituents in the test sample by comparing the migration times with the calibration curve obtained in step a). Such method is particularly useful in the analysis of samples containing complex biologic molecules such as antibodies or fusion proteins.

[0022] The calibration curve can be obtained according to the method as described above for obtaining a calibration curve through the partial reduction of a known sample of the analyte biological molecule. Accordingly, in a method of the present invention for determining the molecular weight of the constituents of a test sample comprises, a.) obtaining a calibration curve for the biologic analyte molecule in CGE comprising the steps of partially reducing a calibration sample comprising a known amount of the biologic molecule, submitting the partially reduced calibration sample to CGE, determining the migration times of the subunits/species of the partially reduced biologic analyte molecule in the partially reduced calibration sample, and calculate the calibration curve for the biologic analyte molecule in CGE. The partial reduction of the calibration sample comprising the steps of adding to a buffered solution containing the known amount of the biologic molecule a reducing agent and incubating the calibration sample for a period of time such that the biologic molecule is not fully reduced. Such partial reduction may further include subsequently adding an alkylating agent to the partially reduced calibration sample and incubating the partially reduced calibration sample at a temperature of about 0°C to about 75°C for a period of up to 15 minutes. In a following step of the method (step b.) submitting the test sample containing the biologic analyte molecule to CGE and in step c.) determining the migration times of the constituents in the test sample and in step d.) identifying the molecular weight of the constituents in the test sample by comparing the migration times with the calibration curve obtained in step a).

[0023] The present invention further includes a method of determining or confirming the

identity of a biologic analyte molecule in a test sample. For example, such method comprises obtaining a calibration curve with a known biologic analyte molecule as described above using partial reduction of the biologic analyte molecule as calibration sample. Subsequently, using the partial reduction of the test sample and the CGE method of the present invention to determine or confirm the identity of the biologic molecule in the test sample. Such method therefore may comprise the steps of: a) obtaining a calibration curve for the biologic molecule in CGE comprising the steps of partially reducing a calibration sample comprising a known amount of the biologic molecule, submitting the partially reduced calibration sample to CGE, determining the migration times of the subunits/species of the partially reduced biologic molecule in the partially reduced calibration sample, and calculate the calibration curve for the biologic molecule in CGE; b) submitting the test sample containing the biologic molecule to CGE; c) determining the migration times of the constituents in the test sample; and d) identifying the molecular weight of the constituents in the test sample by comparing the migration times with the calibration curve obtained in step a) and determining or confirming the identity of the biologic analyte molecule in the test sample based on the identicality of the molecular weight subunits/species of the biologic analyte molecule in the test sample and the known biologic molecule in the calibration sample.

[0024] Likewise the present invention provides for a method for identifying the composition of a sample containing a biologic molecule of interest comprising determining the molecular weight of the species (constituents or subunits) of the sample as through any of the method steps described above, identifying the species (constituents or subunits) based on their molecular weight and determine the relative amounts of each of the species (constituents or subunits) as obtained from the Capillary Gel Electrophoresis (CGE). Such method of the present invention can further be applied to determine the purity of a sample containing the biologic analyte molecule of interest be determining the relative amount of the each species (constituents or subunits) to determine the relative amount of the entire biologic analyte molecule of interest compared to other molecules in the test sample which have different molecule weights.

Examples

[0025] The following example are for illustrative purposes and are not intended to limit the scope of the invention. The different migration times for molecular subunits/species of two partially reduced antibody in comparison with the commercial size standard species migration have been reported in Figure 5. The huge discrepancies, ranging from 40 to 80 kDa of difference found in the comparison of the tree curves, remarks and demonstrate the needs to have a new kind of standard calibration curve, that is precisely related to the protocol presented here.

[0026] All the examples 1-6 of molecules with their molecular characteristics submitted to Partial Reduction Protocol have shown a positive Protocol response in terms of experimental results. In all subsequent examples the Partial Reduction Protocol for the biologic molecules consisted of the following steps. The biologic molecule in a sample buffer contained 20µl

sample, 75 µl sample buffer (50 mM Acetate pH 5.5 + 2 % SDS) such that the sample contained approximately 5 mg/ml of the biologic molecule of interests. To this sample of the biologic molecule of interest in sample buffer 5 µl of 250 mM TCEP was added at room temperature. This mixture was incubated for 10 minutes at room temperature after which 5 µl 250 mM Maleimide was added. This combined mixture was then incubated for 10 minutes at 70°C. The partially reduced sample so prepared was then submitted to CGE under standard conditions (System: PA 800 Plus Capillary Electrophoresis System (Beckman Coulter); Detector: UV (DAD) at 220 nm; Capillary: Bare fused silica capillary (Beckman Coulter); 50 µm i.d. 30.2 cm, 20.2 cm effective length from the sample introduction inlet and the detector window, 200 µm aperture clip in the cartridge; Cartridge temperature: 25°C; Sample holder temperature: 15°C; Voltage and timing: 15 kV (reverse polarity) for 35-45 minutes depending on analyte; Pre-injection steps: NaOH 0.1N to clean the capillary (20 psi, 10 min), HCl 0.1 N to neutralize the capillary surface (20 psi, 5 min), Water to remove the acidic solution (20 psi, 2 min), SDS Gel to fill the capillary (70 psi, 10 min), MilliQ water per injection (inject-pressure 7 psi, 10 sec); Materials and consumables: IgG Purity /Heterogeneity Analysis KIT, SCIEX, Code A10663, SDS-MW Gel Buffer, SCIEX, Code A30341, Bare Fused-Silica Capillary - 50 µm ID, 375 µm OD, 67 cm, SCIEX, Code 33845).

Example 1

[0027] A human IgG2 Non-Glycosylated antibody (anti-TM3) was submitted to partial reduction as described above. The obtained partially reduced calibration sample was submitted to capillary gel electrophoresis (CGE) under standard conditions (as described above). As shown in Figure 6, the human IgG2 Non-Glycosylated antibody (anti-TM3) that was submitted to Partial Reduction Protocol produced all the expected molecular antibody subunits/species and the resulting calibration curve based on the different migration times fitting. The full match between experimental and theoretical relative molar ratio for LC and HC of such species was observed.

Example 2

[0028] An IgG1 Glycosylated antibody was submitted to the Partial Reduction Protocol as described above and the obtained partially reduced sample was submitted to CGE (as described above). As shown in Figure 7, the IgG1 Glycosylated antibody produced all the expected molecular antibody subunits/species and the resulting calibration curve based on the different migration times fitting. The full match was observed be noted.

Example 3

[0029] An IgG1 with kappa light chain Glycosylated antibody was submitted to Partial

Reduction Protocol as described above and the obtained partially reduced sample was submitted to CGE (as described above. As shown in Figure 8, the IgG1 with kappa light chain Glycosylated antibody produced all the expected molecular antibody subunits/species and the resulting calibration curve based on the different migration times fitting. The full match between experimental and theoretical relative molar ratio for LC and HC of such species was observed.

Example 4

[0030] An IgG1 !gG1.4 moiety conjugated to Dolaflexin with a maleimide-based Cys linker Glycosylated antibody was submitted to the partial reduction protocol as described above to obtain a partially reduced sample which was submitted to CGE (as described above). As shown in Figure 9, the IgG1.4 moiety conjugated to Dolaflexin with a maleimide-based Cys linker Glycosylated antibody produced all the expected molecular antibody subunits/species and the resulting calibration curve based on the different migration times fitting. The full match between experimental and theoretical relative molar ratio for LC and HC of such species was observed.

Example 5

[0031] A Chimeric mouse/human IgG1 Non-Glycosylated antibody was submitted to partial reduction protocol as described above to obtain a partially reduced sample which was submitted to CGE (as described above). As shown in Figure 10, the Chimeric mouse/human IgG1 Non-Glycosylated antibody produced all the expected molecular antibody subunits/species and the resulting calibration curve based on the different migration times fitting. The full match between experimental and theoretical relative molar ratio for LC and HC of such species was observed.

Example 6

[0032] A fusion IgG1 with Sirp-α domain linked to LC Glycosylated antibody was submitted to Partial Reduction Protocol as described above to obtain a partially reduced sample which was submitted to CGE (as described above). As shown in Figure 11, the fusion IgG1 with Sirp-α domain linked to LC Glycosylated antibody produced all the expected molecular antibody subunits/species and the resulting calibration curve based on the different migration times fitting. The full match between experimental and theoretical relative molar ratio for LC and HC of such species was observed.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO8606383A1 [0005]

Patentkrav

1. Fremgangsmåde til bestemmelse af molekylvægten af bestanddele af en testprøve, der indeholder et biologisk molekyle ved anvendelse af kapillær gelelektroforese (CGE), hvor fremgangsmåden omfatter følgende trin:

a) opnåelse af en kalibreringskurve for det biologiske molekyle i CGE;

b) udsættelse af testprøven, der indeholder det biologiske molekyle, for CGE;

c) bestemmelse af migrationstiderne for bestanddelene i testprøven; og

d) identifikation af molekylvægten af bestanddelene i testprøven ved at sammenligne migrationstiderne med den kalibreringskurve, der er opnået i trin a),

kendetegnet ved, at kalibreringskurven opnås ved delvis at reducere en kalibreringsprøve omfattende en kendt mængde af det biologiske molekyle, udsætte den delvis reducerede kalibreringsprøve for CGE, bestemme migrationstiderne for underenhederne/arterne af det delvis reducerede biologiske molekyle i den delvis reducerede kalibreringsprøve og beregne kalibreringskurven for det biologiske molekyle i CGE.

2. Fremgangsmåde ifølge krav 1, hvor trinnet med delvis reduktion af kalibreringsprøven omfatter:

a) tilsætning af et reduktionsmiddel til en bufferopløsning indeholdende den kendte mængde af det biologiske molekyle, og

b) inkubering af kalibreringsprøven fra trin a) i et tidsrum, hvor længden af tidsperioden er sådan, at det biologiske molekyle ikke er fuldstændigt reduceret.

3. Fremgangsmåde ifølge krav 2, hvor trinnet med delvis reduktion af kalibreringsprøven omfatter:

a) tilsætning af reduktionsmidlet til den bufferede opløsning,

b) inkubering af kalibreringsprøven ved stuetemperatur i en periode på 30 minutter eller mindre, og yderligere omfattende trinene

c) tilsætning af et alkyleringsmiddel til den delvis reducerede kalibreringsprøve fra trin b, og

d) inkubering af den delvis reducerede kalibreringsprøve fra trin c) ved en temperatur på 0°C til 75°C, fortrinsvis ved 70°C, i en periode på op til 15 minutter, fortrinsvis i 10 minutter.

4. Fremgangsmåde ifølge krav 3, hvor reduktionsmidlet og alkyleringsmidlet tilsættes opløsningen i et koncentrationsforhold mellem reduktionsmiddel:alkyleringsmiddel på 0,15 til 3.

5. Fremgangsmåde ifølge krav 3, hvor reduktionsmidlet og alkyleringsmidlet tilsættes opløsningen i et koncentrationsforhold mellem reduktionsmiddel:alkyleringsmiddel på 1:6,5 til 3:1.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 3 til 5, hvor reduktionsmidlet og alkyleringsmidlet tilsættes til opløsningen i et koncentrationsområde af reduktionsmiddel:alkyleringsmiddel på 1:1.

7. Fremgangsmåde ifølge et hvilket som helst af kravene 3 til 6, hvorved reduktionsmidlet tilsættes i en koncentration på 15 til 500 mM, og alkyleringsmidlet tilsættes i en koncentration på 15 til 500 mM.

8. Fremgangsmåde ifølge et hvilket som helst af kravene 3 til 7, hvorved reduktionsmidlet tilsættes i en koncentration på 250 mM, og alkyleringsmidlet tilsættes i en koncentration på 250 mM.

9. Fremgangsmåde ifølge et hvilket som helst af kravene 2 til 8, hvor reduktionsmidlet er Tris(2-carboxyethyl)phophin (TCEP).

10. Fremgangsmåde ifølge et hvilket som helst af kravene 3 til 9, hvor alkyleringsmidlet er maleimid eller en analog deraf.

11. Fremgangsmåde ifølge et hvilket som helst af kravene 3 til 10, hvor kalibreringsprøven inkuberes ved stuetemperatur med et reduktionsmiddel i en

periode på op til 20 minutter.

12. Fremgangsmåde ifølge krav 11, hvor perioden er ca. 20 minutter.

13. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor det biologiske molekyle er et komplekst molekyle omfattende to eller flere underenheder/arter.

14. Fremgangsmåde ifølge krav 13, hvor det biologiske molekyle er valgt blandt et rekombinant protein, et antistof og et fusionsprotein.

15. Fremgangsmåde til at identificere sammensætningen af en prøve, der indeholder et biologisk molekyle, omfattende:

a) bestemmelse af molekylvægten af prøvens bestanddele ifølge fremgangsmåden ifølge et hvilket som helst af kravene 1-14,

b) identificering af prøvens bestanddele baseret på deres molekylvægt,

c) og bestemmelse af de relative mængder af enhver af bestanddelene baseret på CGE udført i trin a.

16. Fremgangsmåde til bestemmelse af renheden af en prøve, der indeholder det biologiske molekyle, omfattende:

a) identificering af sammensætningen af prøven ifølge fremgangsmåden ifølge krav 15, og

b) bestemmelse af den relative mængde af hver underenhed eller fragment af det biologiske molekyle, der er til stede i prøven.

DRAWINGS

Drawing

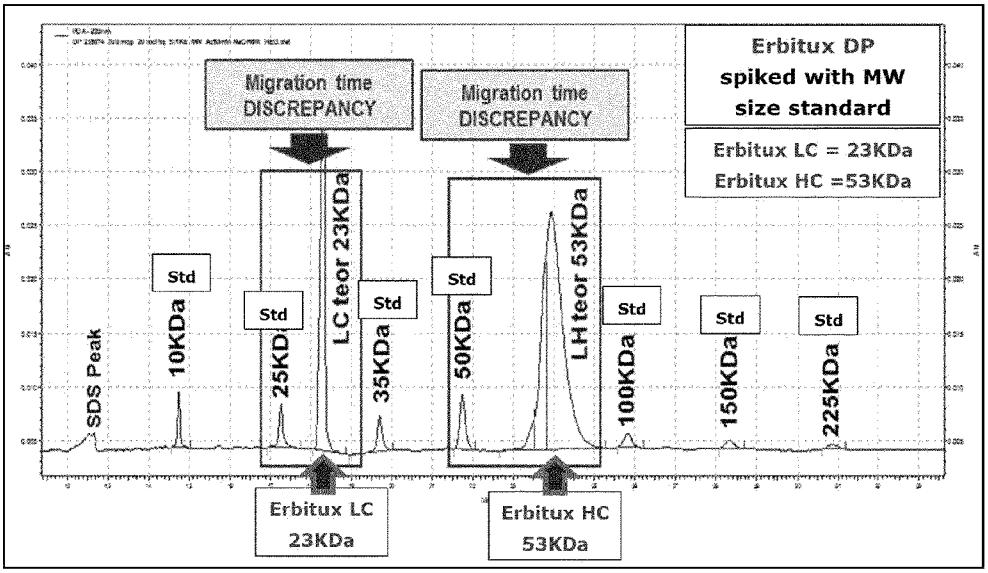


FIGURE 1

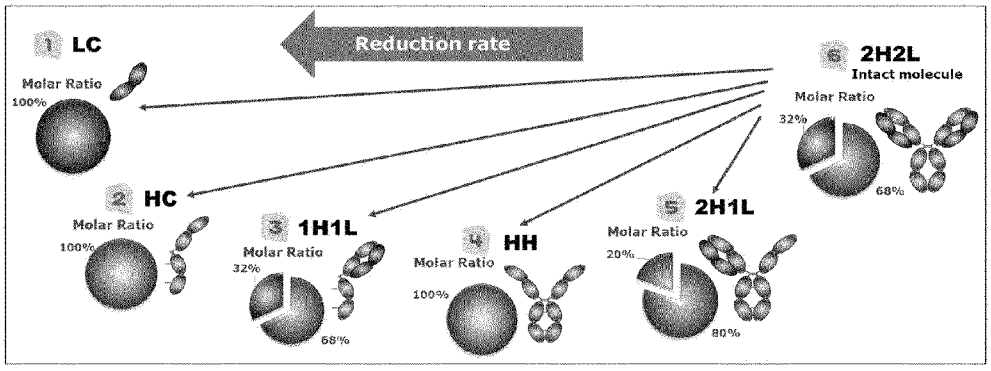


FIGURE 2

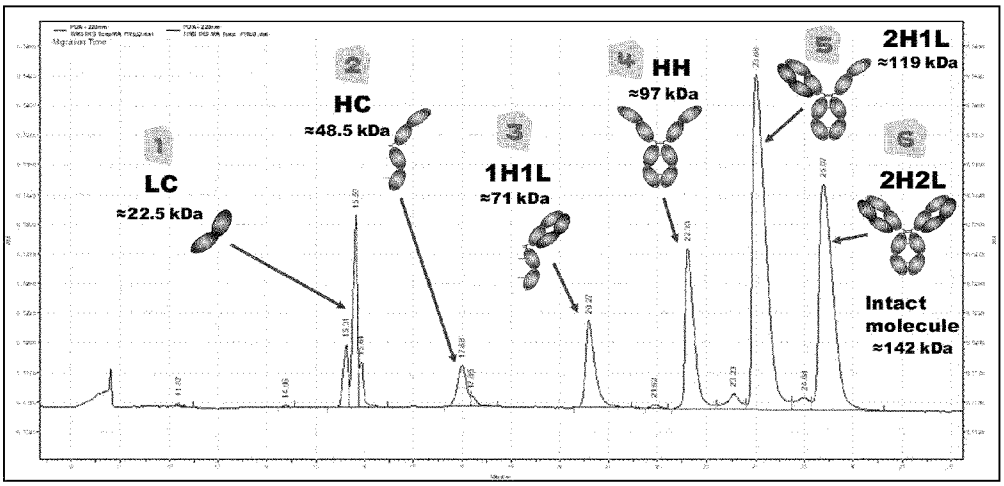
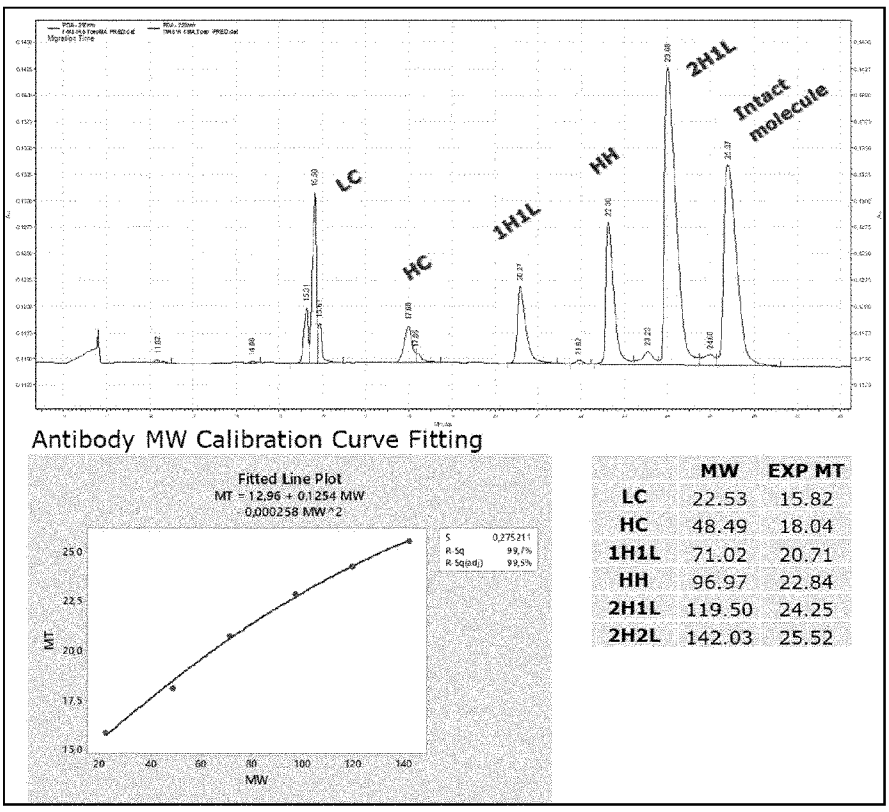


FIGURE 3



	MW	EXP MT
LC	22.53	15.82
HC	48.49	18.04
1H1L	71.02	20.71
HH	96.97	22.84
2H1L	119.50	24.25
2H2L	142.03	25.52

FIGURE 4

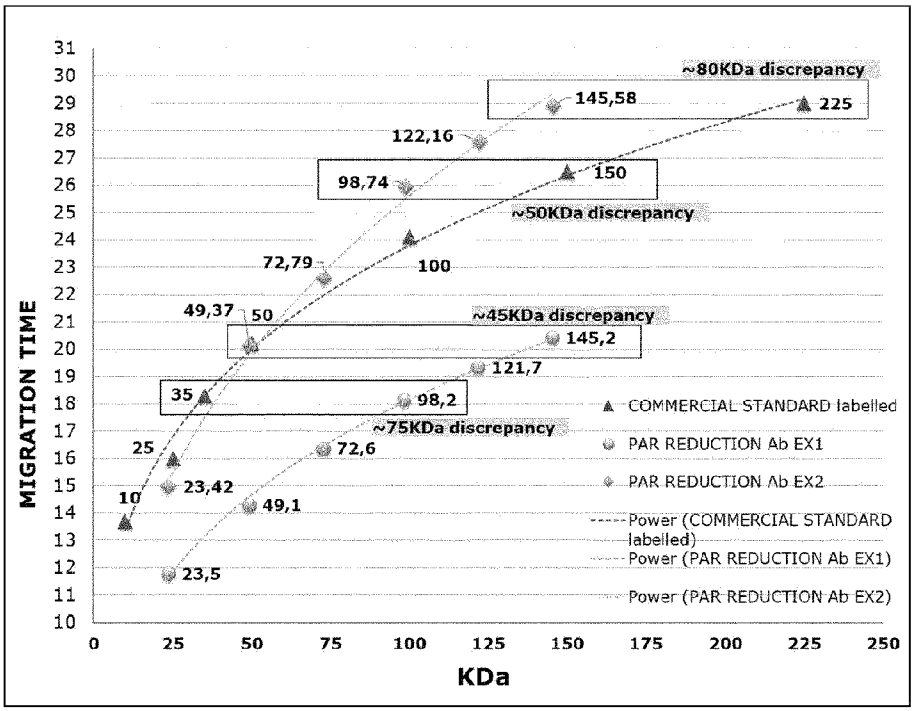


FIGURE 5

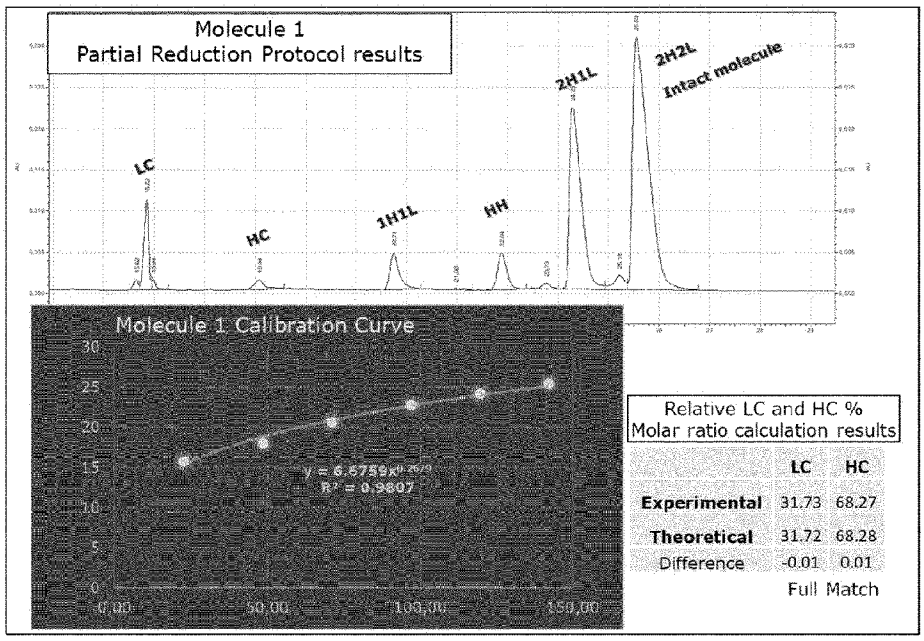


FIGURE 6

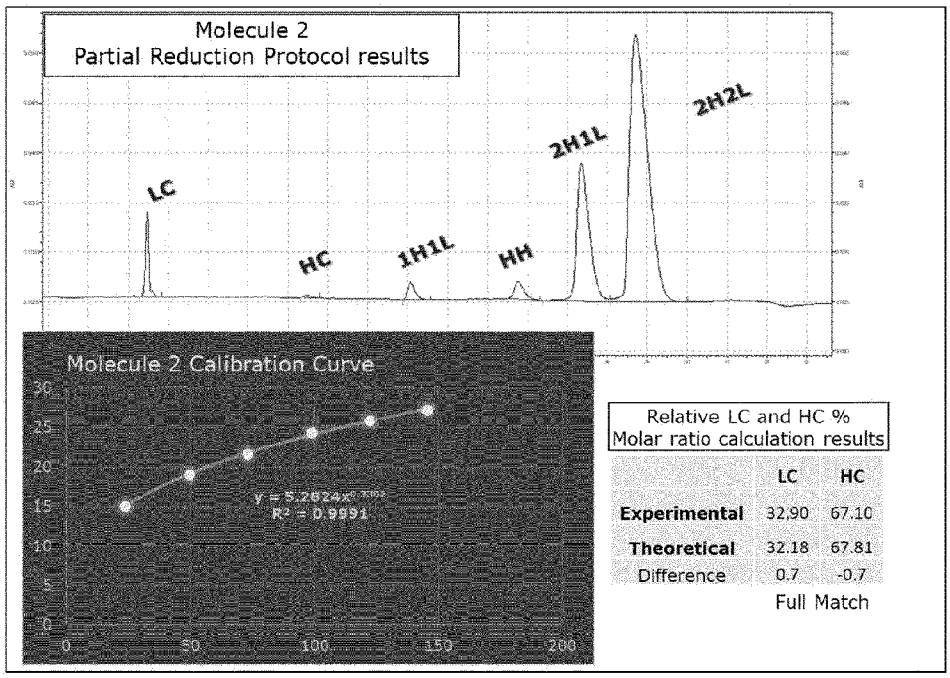


FIGURE 7

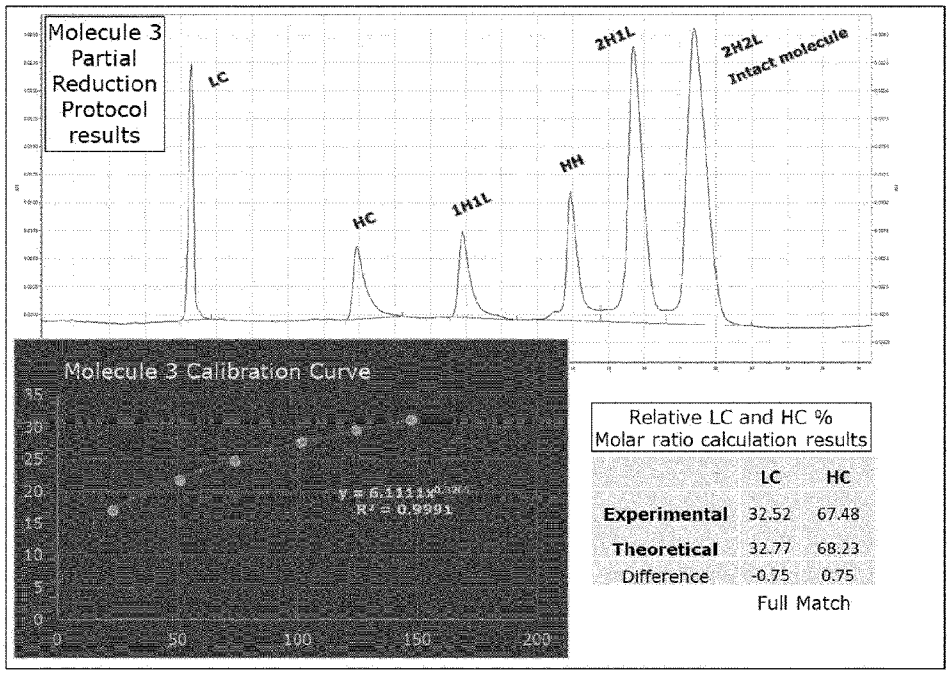


FIGURE 8

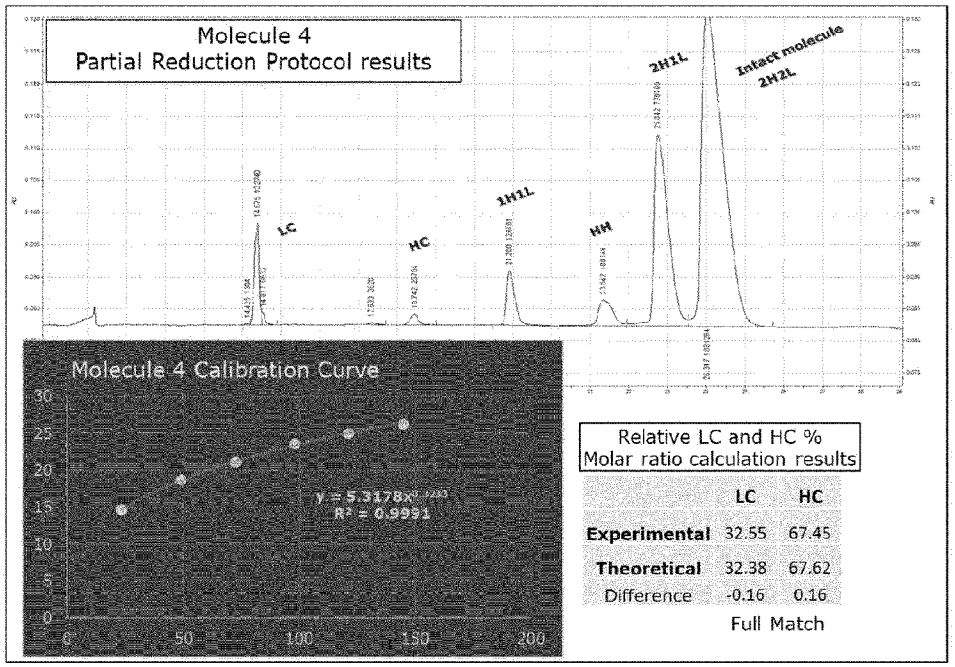


FIGURE 9

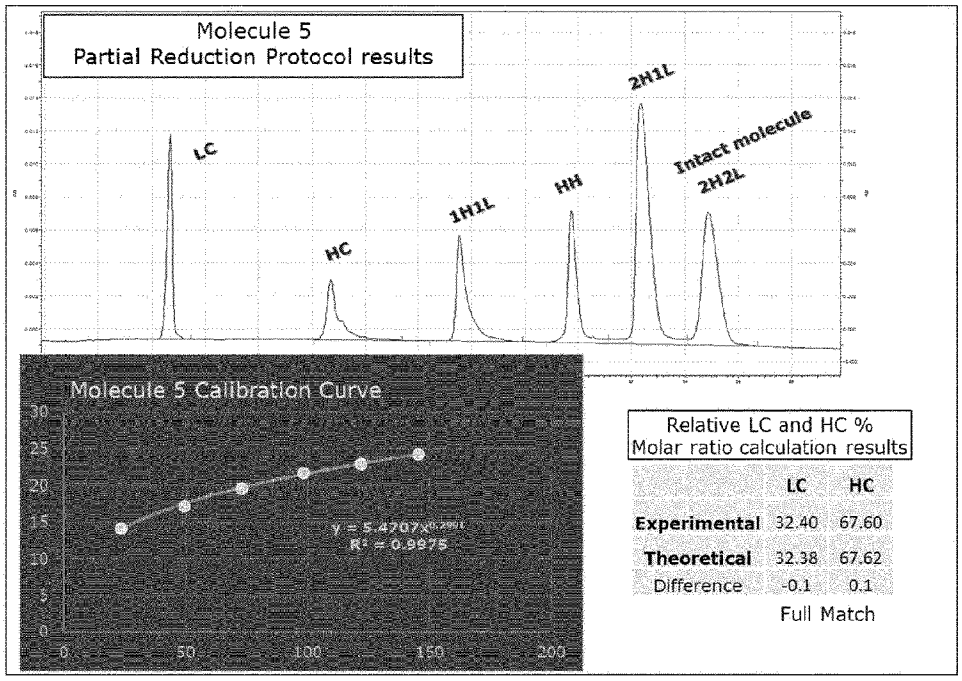


FIGURE 10

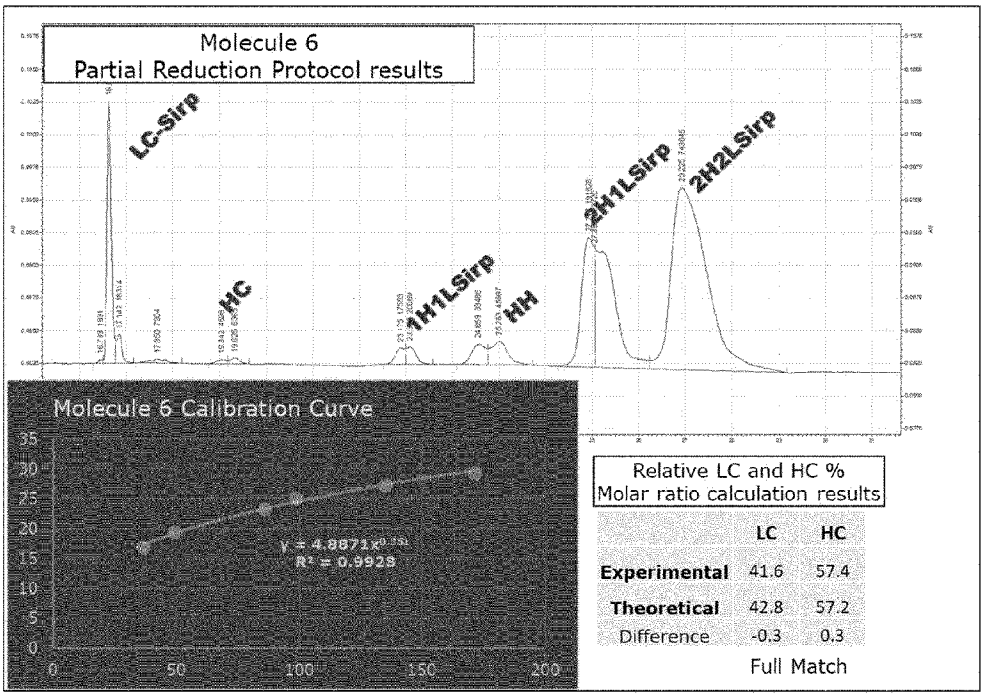

Molecule 6
Partial Reduction Protocol results
LC-Sirp
HC
1H1LSirp
HH
2H1LSirp
2H2LSirp
Molecule 6 Calibration Curve
y = 4.8871x^0.5
R² = 0.9928
0
5
10
15
20
25
30
35
0
50
100
150
200
Relative LC and HC %
Molar ratio calculation results
LC
HC
Experimental
41.6
57.4
Theoretical
42.8
57.2
Difference
-0.3
0.3
Full Match

FIGURE 11