

(21) (A1) **2,285,452**
(86) 1998/04/01
(87) 1998/10/08

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(51) Int.Cl.⁶ A01N 65/00, A01N 37/18
(30) 1997/04/01 (08/831,039) US
(30) 1997/05/02 (08/850,855) US

**(54) FRACTION DU MOMORDICA CHARANTIA ACTIVE PAR VOIE
ORALE, SES PEPTIDES ACTIFS, ET LEUR UTILISATION
DANS LE TRAITEMENT DU DIABÈTE**

**(54) ORALLY ACTIVE FRACTION OF MOMORDICA CHARANTIA,
ACTIVE PEPTIDES THEREOF, AND THEIR USE IN THE
TREATMENT OF DIABETES**

(57) L'invention porte sur un extrait hydrosoluble de M. Charantia, dit MC6, ses procédés de préparation, et son utilisation pour le traitement des troubles liés à l'hyperglycémie, le MC6 qui se déplace en formant une bande unique sur le SDS- PAGE a un poids moléculaire de moins de 10 kDa et comprend 3 peptides. L'invention porte également sur un composant du MC6, dit MC6.1 et sur ses analogues et mimétiques. Les MC6, MC6.1, MC6.2 et MC6.3 ont une action hypoglycémante, même après administration orale. L'invention porte en outre sur des méthodes d'utilisation de ces agents pour le traitement des troubles liés à l'hyperglycémie, et en particulier du diabète, les agents actifs étant de préférence administrés par voie orale.

(57) A water soluble extract of *M. charantia* named MC6, methods for its preparation and methods for its use in the treatment of hyperglycemic disorders are provided. The active MC6 is characterized by moving as a single band on SDS-PAGE having a molecular weight of less than 10 kDa, and by comprising three peptides. Also provided is a peptide component of MC6 named MC6.1, as well as analogues and mimetics thereof. The active MC6, MC6.1, MC6.2, and MC6.3 exhibit hypoglycemic activity, even following oral administration, also provided are methods of using the active agents to treat hyperglycemic disorders, particularly diabetes, where the active agents are preferably orally administered.

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A01N 65/00, 37/18		A1	(11) International Publication Number: WO 98/43484 (43) International Publication Date: 8 October 1998 (08.10.98)
(21) International Application Number: PCT/US98/06450		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 1 April 1998 (01.04.98)			
(30) Priority Data: 08/831,039 1 April 1997 (01.04.97) US 08/850,855 2 May 1997 (02.05.97) US			
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(54) Title: ORALLY ACTIVE FRACTION OF MOMORDICA CHARANTIA, ACTIVE PEPTIDES THEREOF, AND THEIR USE IN THE TREATMENT OF DIABETES

(57) Abstract

A water soluble extract of *M. charantia* named MC6, methods for its preparation and methods for its use in the treatment of hyperglycemic disorders are provided. The active MC6 is characterized by moving as a single band on SDS-PAGE having a molecular weight of less than 10 kDa, and by comprising three peptides. Also provided is a peptide component of MC6 named MC6.1, as well as analogues and mimetics thereof. The active MC6, MC6.1, MC6.2, and MC6.3 exhibit hypoglycemic activity, even following oral administration, also provided are methods of using the active agents to treat hyperglycemic disorders, particularly diabetes, where the active agents are preferably orally administered.

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ORALLY ACTIVE FRACTION OF MOMORDICA CHARANTIA,
ACTIVE PEPTIDES THEREOF, AND THEIR USE IN
THE TREATMENT OF DIABETES

5

INTRODUCTION

Technical Field

The field of this invention is diabetes.

Background of the Invention

Non insulin dependent diabetes mellitus (NIDDM or
10 Type II) is the fourth-leading cause of death in the
United States and affects from 5 to 7% of the total world
population, with an increasing prevalence in western
countries. In diabetes, the body either does not produce
enough insulin or the insulin which is produced is not
15 effective, resulting in increased blood glucose level, a
condition technically known as hyperglycemia. Although
diabetes can affect people of any age, the majority of
diabetics are over 45 years old. The disease tends to
run in families, and the risk factor of acquiring the
20 disease increases in overweight individuals.

Diabetes is a chronic disease with no cure and is
linked with several other disorders. It is the leading
cause of blindness in people ages 25-74. Ten percent of
all people with diabetes develop kidney disease.

25 Diabetes is the most frequent cause of non-traumatic
lower limb amputation. The risk of leg amputation is 30
times greater for people with diabetes. People with
diabetes are two to four times more likely to develop

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heart diseases, and are five times more likely to suffer from stroke.

The cause of diabetes is still a mystery, although both genetics and environment appear to play a role.

5 There are two types of diabetes: Insulin dependent (Type I) and Non-insulin dependent (Type II). Type I diabetes is an autoimmune disease frequently occurring in children and young adults. The autoantigen responsible for triggering Type I diabetes is still unknown and patients 10 have to take i.v. insulin daily to survive for their life.

Type II diabetes is a metabolic disorder resulting from the body's inability to make a sufficient amount of insulin or to properly use the insulin that it does 15 produce, and is considered the most common form of the disease. Although insulin secretion and insulin resistance are considered the major defects, the precise genetic factors involved remain unknown. Patients with diabetes usually have one or more of the following 20 defects. These are: less production of insulin by the pancreas; oversecretion of glucose by the liver; impairment of glucose uptake by the skeletal muscle; defects in glucose transporters (Glut-1, Glut-4); desensitization of insulin receptors; and defect in the 25 metabolic breakdown of polysaccharides.

The current therapy utilizes four classes of oral hypoglycemic agents besides i.v. insulin. These are summarized below:

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Class	Approved Drugs	Mechanisms of Action	Limitations
Sulfonylureas	4 (1st gen.) 2 (2nd gen.)	Acts on pancreas to release more insulin	Resistance development
Biguanides	Only one (metformin)	Reduces glucose secretion by liver. Also improves insulin sensitivity.	Liver problem Lactic acidosis
5 "-Glucosidas e Inhibitor	Only one (acarbose)	Interferes with digestive process. Reduces glucose absorption.	Only at postprandial level.
Thiazolidine -dione	Only one (troglitazone)	Reduces insulin resistency	AAAdd-on@ with insulin Not for people with heart and liver disease.

As is apparent from the above table, each of the 10 current agents available for use in the treatment of diabetes has certain disadvantages. Accordingly there is continued interest in the identification and development of new agents for use in the treatment of diabetes.

M.charantia is a tropical plant whose fruits are 15 used as a vegetable. Several groups have reported on the hypoglycemic activity of M.charantia, both in mammal models (Shibib et al., Biochem. J., 292, 267-270 (1993); Ali et al., Planta Med. 59, 408-412 (1993); Akhtar et al., Planta Med. 42, 205-212 (1981)) and in humans 20 (Leatherdale et al., Br. Med. J. 282, 1823-1824 (1981); Aslam et al., Lancet, I. 607 (1979)). However, the hypoglycemic component and the mechanism of action remains unknown.

The isolation of an 11 kDa peptide obtained from 25 M.charantia having insulin like activity is reported in: Khanna et al., "Hypoglycemic Activity of Polypeptide-p

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from a Plant Source," 20th Annual Meeting of the American Society of Pharmacology, Purdue University, West Lafayette, July 29-August 3, 1979; Baldwa et al., Upsala J. Med. Sci. (1977) 82:39-41 and U.S. Pat. No. 3,945,988.

5 In all of these reports, the insulin like polypeptide was non-orally administered, e.g. i.v. or subcutaneously.

A recent report indicates that the crude alcoholic extract of *M.charantia* lowers plasma glucose level partly by stimulation of glycogen synthesis in the liver and it 10 is unlikely that it acts as an insulin secreting agent (Sarkar et al., Pharmacol. Res., 33, 1-4 (1996)).

SUMMARY OF THE INVENTION

A water soluble fraction of *M.charantia* (MC6) and 15 methods for its preparation and use in the treatment of hyperglycemic disorders are provided. MC6 is characterized by migrating as a single band on SDS-PAGE electrophoresis having a molecular weight of less than 10 kDal, having a size smaller than recombinant insulin, 20 comprising three peptides, and being free of high molecular weight protein contaminants. Also provided is a specific peptide component of MC6, labeled MC6.1, and peptide derivatives of MC6.1 which are shorter in length than MC6.1, MC6.2 and MC6.3, which exhibit hypoglycemic 25 activity and may be administered orally to treat a variety of hyperglycemic disorders.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 A shows the results of gel electrophoresis on SDS-20%-PAGE of various water soluble fractions of

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M.charantia; Fig. 1 B shows the purified MC6 preparations; and Fig. 1C shows the results of HPLC of MC6;

Fig. 2A is a graphical representation showing the 5 effect of an unpurified aqueous extract of M.charantia and a control on plasma glucose levels over time in SD rats; Fig. 2B. is a graphical representation showing the effect of orally administered MC6 on blood glucose levels over time in SD rats;

10 Figs. 3 A & B provide graphical representations of the effect of MC6 on blood glucose levels in rats suffering from SZC induced diabetes;

Fig. 4 is a graphical representation demonstrating that oral administration of MC6 does not increase serum 15 insulin level; and

Fig. 5 is a graphical representation demonstrating that oral administration of MC6 is as effective as I.V. administration of r-insulin in reducing blood glucose levels.

20 Figs. 6(a) & (b) show the results of SDS-PAGE analysis of MC6.1 in a 4-20% tris-glycine gradient gel using Coomassie Blue and Silver stain, respectively.

Fig. 7 provides a graphical representation of the results obtained from purification of MC6.1 using C18 RP-25 HPLC.

Figs. 8 a & b provide the results of isoelectric point analysis for MC6.1.

Fig. 9 provides the results of NMR analysis of MC6.1

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Fig. 10 provides a graphical representation of the effect of MC6.1 on blood glucose levels.

Fig. 11 provides a graphical representation of the hypoglycemic activity of MC6.1, 6.2 and 6.3 in oral 5 glucose tolerance tests in normal rats.

Fig. 12 provides a graphical representation of the glucose lowering ability of MC6.2 in diabetic rats.

Fig. 13 is a graphical representation of the hypoglycemic effect of MC6.2 in non-obese diabetic mice.

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DESCRIPTION OF THE SPECIFIC EMBODIMENTS

A water soluble fraction of *M. charantia* named MC6, an active peptide component thereof named MC6.1, and peptide derivatives thereof, MC6.2 and MC6.3, methods for their preparation and use in the treatment of 15 hyperglycemic disorders are provided. MC6 is characterized by comprising three peptides that move together as a single band on SDS-PAGE with a molecular weight of 10 kDa and has a size smaller than recombinant insulin. MC6 and MC6.1 exhibit hypoglycemic activity and 20 are orally active. In further describing the subject invention, the characteristics of MC6 and MC6.1 will be further described in greater detail, followed by a description of methods for its preparation and use in the treatment of hyperglycemic disorders, particularly in the 25 treatment of humans suffering from diabetes.

The derivatives, MC6.2 and MC6.3 are, respectively, 11-a.a and 7-a.a peptide derivatives of the 18-a.a. peptide, MC6.1.

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Before the subject invention is further described, it is to be understood that the invention is not limited to the particular embodiments of the invention described below, as variations of the particular embodiments may be 5 made and still fall within the scope of the appended claims. It is also to be understood that the terminology employed is for the purpose of describing particular embodiments, and is not intended to be limiting. Instead, the scope of the present invention will be established by 10 the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise all 15 technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

MC6 of the subject invention is a water soluble fraction or extract of the plant species *M.charantia*. 20 Although MC6 may be derived from one or more tissues or components of the *M.charantia*, including the leaves, stems, roots, fruit, seeds and the like, including the whole *M.charantia* plant, MC6 is usually derived from *M.charantia* fruit, preferably unripe *M.charantia* fruit 25 that has been separated from seeds.

MC6 is characterized by moving as a single band on SDS-20% PAGE, where the molecular weight of the single band is less than 10 kDal, where the strength of the electric field applied during SDS-20% PAGE is 100 V. The

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MC6 has a size smaller than recombinant insulin, and is therefore less than about 6 kDal. MC6 is further characterized by eluting as three peaks following High Performance Liquid Chromatography (HPLC), where the 5 conditions of HPLC are reverse phase on C8 or C18 columns. Under these conditions, the first peak elutes at 11.33 min, the second peak elutes at 25.35 min and the third peak elutes at 34.67 min.

The MC6 of the subject invention is a preparation 10 free of high molecular weight contaminants, e.g. proteins, where high molecular weight contaminants is used in this application to denote molecules having a molecular weight in excess of about 50 kDal. Usually, the MC6 is free of any contaminants having a molecular weight 15 in excess of 10 kDal.

MC6 exhibits hypoglycemic activity in mammals. By hypoglycemic activity is meant that upon administration of MC6 to the mammal, the blood glucose level of the mammal decreases, where the amount of decrease in the 20 blood glucose level is proportional to the amount of MC6 administered to the mammal. MC6 exhibits hypoglycemic activity whether it is administered orally or intravenously, and thus is orally active or orally bioavailable, by which is meant that it is not 25 inactivated by passing from the gastrointestinal tract into the mammal.

The MC6 may be prepared using any convenient means which provide for separation of the MC6 from the source *M.charantia* tissue. One means of obtaining MC6 from

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M.charantia tissue is to first make a raw suspension of the tissue by macerating the tissue in the presence of a solvent, e.g. phosphate buffered saline (PBS), water and the like, where maceration may be accomplished using a 5 blender or other maceration means. The particulate matter of the resultant suspension is then separated and discarded from the liquid phase, where this step may be accomplished by centrifuging, usually at a speed between about 10,000 and 16,000 rpm, followed by filtration of 10 the supernatant, where filtration will generally be through filters having pore sizes ranging from 0.5 to 0.10 μ , usually from about 0.45 to 0.22 μ , where the filtration may be under vacuum. The resultant filtrate is then passed sequentially through 30 kDal and 10 kDal 15 cutoff membranes, where such membranes are known in the art and representative membranes include Amicon M.W. cut off filters, and the like, to obtain a water soluble fraction of the initial M.charantia tissue which contains MC6. The resultant water soluble fraction may be used as 20 is or further processes for subsequent use, where further processing may include dehydration, e.g. lyophilization, and the like. The resultant water soluble fraction can also be stored in liquid form at from 1 to 5, usually 2 to 4 C in the presence of preservatives, such as sodium 25 benzoate, and the like.

MC6.1 is a specific peptide component of MC6 that migrates as a single band in SDS-PAGE analysis (4-20 % tris-glycine gradient gel) and has a molecular weight as determined by SDS-PAGE of less than 2.5 kD. MC6.1 has an

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isoelectric point of 8.2. MC6.1 is 18 amino acid residues in length, where the amino acid sequence of MC6.1 is :

K-T-N-M-K-H-M-A-G-A-A-A-G-A-V-V-G (SEQ ID NO:01)

MC6.1 exhibits hypoglycemic activity in mammals. The
5 MC6.1 of the present invention is MC6.1 which has been separated from its natural environment, e.g. is present in MC6, is in isolated or pure form, and the like.

Also provided are peptide analogues and mimetics of MC6.1 which exhibit hypoglycemic activity. The MC6.1
10 analogues and mimetics will comprise, as an active motif sequence, at least 8 amino acids, usually at least about 12 amino acids, more usually at least about 18 amino acids, and fewer than about 40 amino acids, more usually fewer than 30 amino acids. Specific peptides are MC6.2,
15 an 11-a.a. derivative of MC6.1 having the sequence:

K-T-N-M-K-H-M-A-G-A-A (SEQ ID NO:02)

and a 7-a.a.. derivative having the sequence:

K-T-N-M-K-H-M (SEQ ID NO:03)

It is understood that up to about three substitutions or
20 deletions may be made in the subject sequences, where the change will not be more than about 20 number %, usually not more than about 10 number % of the number of amino acids in the active motif. Preferred are conservative substitutions, as known in the art, including
25 substitutions within the large hydrophobic group: isoleucine, leucine, valine and phenylalanine; between serine and threonine; glycine and alanine; asparagine and

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glutamine; aspartic acid and glutamic acid; or lysine, arginine and histidine.

In addition to purification from its natural source, the MC6.1, as well as peptide analogues and mimetics 5 thereof, such as MC6.2 and MC6.3, may be prepared in accordance with conventional techniques, such as synthesis (for example, use of a Beckman Model 990 peptide synthesizer or other commercial synthesizer).

Peptides may be produced directly by recombinant methods 10 (see Sambrook et al. *Molecular Cloning: A Laboratory Manual*, CSHL Press, Cold Spring Harbor, NY, 1989) or as a fusion protein, for example to a protein that is one of a specific binding pair, allowing purification of the fusion protein by means of affinity reagents, followed by 15 proteolytic cleavage, usually at a site engineered to yield the desired peptide (see for example Driscoll et al. (1993) *J. Mol. Bio.* 232:342-350).

The oligopeptides (i.e. MC6.1, MC6.1 analogues, MC6.1 mimetics) may be extended to provide convenient 20 linking sites, e.g. cysteine or lysine, to enhance stability, to bind to particular receptors, to provide for site-directed action, to provide for ease of purification, to alter the physical characteristics (e.g. solubility, charge, etc.), to stabilize the conformation, 25 etc. The oligopeptides may be joined to non-wild-type flanking regions as fused proteins, joined either by linking groups or covalently linked through cysteine (disulfide) or peptide linkages. The oligopeptide may be linked through a variety of bifunctional agents, such as

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maleimidobenzoic acid, methyldithioacetic acid, mercaptobenzoic acid, S-pyridyl dithiopropionate, etc.

The oligopeptides may be joined to a single amino acid at the N- or C-terminus of a chain of amino acids, or may be 5 internally joined. For example, the subject peptides may be covalently linked to an immunogenic protein, such as keyhole limpet hemocyanin, ovalbumin, etc. to facilitate antibody production to the subject oligopeptides.

Alternatively, the subject oligopeptides may be 10 expressed in conjunction with other peptides or proteins, so as to be a portion of the chain, either internal, or at the N- or C- terminus. Various post-expression modifications may be achieved. For example, by employing the appropriate coding sequences, one may provide 15 farnesylation or prenylation, such that the subject peptide will be bound to a lipid group at one terminus, and will be able to be inserted into a lipid membrane, such as a liposome.

The subject oligopeptides may be PEGylated, where 20 the polyethyleneoxy group provides for enhanced lifetime in the blood stream. The subject oligopeptides may also be combined with other proteins, such as the Fc of an IgG isotype to enhance complement binding, or with a toxin, such as ricin, abrin, diphtheria toxin, or the like, 25 particularly the A chain. The oligopeptides may be linked to antibodies for site directed action. For conjugation techniques, see, for example, U.S. Patent Nos. 3,817,837; 3,853,914; 3,850,752; 3,905,654;

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4,156,081; 4,069,105; and 4,043,989, which are incorporated herein by reference.

MC6, MC6.1, MC6.2 and MC6.3 (as well as analogues and mimetics thereof) of the subject invention find use 5 in the treatment of diseases characterized by the presence of elevated blood glucose levels, e.g. hyperglycemic disorders, such as diabetes mellitus, including both Type I and Type II diabetes, as well as other hyperglycemic related disorders, such as obesity, 10 increased cholesterol kidney related disorders, and the like. By "treatment" is meant that the MC6, MC6.1, MC6.2 and MC6.3 (as well as analogues and mimetics thereof) are administered to at least reduce the blood glucose level in the host suffering from the hyperglycemic 15 disorder. In treating hyperglycemic disorders with MC6 and the subject oligopeptides, the MC6 or oligopeptides are administered to the host in an amount sufficient to reduce the blood glucose level in the host to an acceptable range, wherein acceptable range means $\pm 10\%$, 20 usually $\pm 8\%$, and more usually $\pm 5\%$ of the normal average blood glucose level for the host.

A variety of hosts may be treated according to the subject invention to reduce their blood glucose levels, where such hosts are mammalian and include livestock, 25 valuable or rare animals, pets, such as dogs and cats, and humans.

Of particular interest are methods of treating human hyperglycemic disorders, such as diabetes, including both Type I and Type II diabetes, where MC6, MC6.1, MC6.2,

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MC6.3 and analogues and mimetics thereof, are administered to the human suffering from the hyperglycemic disorder to at least reduce the blood glucose level of the human, where the blood glucose level is reduced to

5 about the normal blood glucose range for the human.

Although, the active agents may be administered to the human using any of the convenient techniques described above, of particular interest is the oral administration of active agents.

10 For therapy with the fraction MC6 or the MC6.1, 6.2 and/or 6.3 peptides, the active compounds may be administered to the host suffering from the hyperglycemic disorder using any convenient administration technique, where such techniques include intravenous, intradermal, 15 intramuscular, subcutaneous, oral, and the like, where oral routes of administration are of particular interest. The dosage delivered to the host will necessarily depend on the route by which the dosage is administered, but will generally range from about 50 to 500 mg/70 kb human 20 body weight, usually from about 100 to 200 mg/70 kg human body weight. In treating human hyperglycemic disorders with MC6, the dosage of active fraction or compound administered to the human will generally range from about 50 to 500, usually from about 100 to 200 mg/70 kg of 25 human body weight.

In using the active fraction or compounds according to the subject invention, they may be combined with a physiologically acceptable vehicle to produce a pharmaceutical composition. The nature of the

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physiologically acceptable vehicle with which the active fraction or compound is combined to produce the pharmaceutical composition will necessarily depend on the method by which the pharmaceutical composition is 5 intended to be administered. Illustrative vehicles include water, e.g. sterile water for injection, saline, and the like. Of particular interest will be physiologically acceptable vehicles suitable for use in the oral administration. Such vehicles are known in the art 10 and include water, e.g. deionized water; saline, e.g. phosphate buffered saline, lyophylized powder in the form of tablets and capsules, where such forms may include various fillers, binders etc., and the like. The amount of active ingredient present in the pharmaceutical 15 composition will be selected in view of the method by which the pharmaceutical composition is to be administered, and may be determined empirically by those of skill in the art.

For therapy, MC6.2 and/or MC6.3, and oligopeptide 20 analogues or mimetics thereof may be administered orally, topically or parenterally, e.g. by injection at a particular site, for example, subcutaneously, intraperitoneally, intravascularly, intranasally, transdermally or the like. Formulations for oral 25 administration include those listed above suitable for use with MC6. Formulations for injection will comprise a physiologically-acceptable medium, such as water, saline, PBS, aqueous ethanol, aqueous ethylene glycols, or the like. Water soluble preservatives which may be employed

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include sodium bisulfite, sodium thiosulfate, ascorbate, benzalkonium chloride, chlorobutanol, thimerosal, phenylmercuric borate, parabens, benzyl alcohol and phenylethanol. These agents may be present in individual amounts of from about 0.001 to about 5% by weight and preferably about 0.01 to about 2%. Suitable water soluble buffering agents that may be employed are alkali or alkaline earth carbonates, phosphates, bicarbonates, citrates, borates, acetates, succinates and the like, such as sodium phosphate, citrate, borate, acetate, bicarbonate and carbonate. Additives such as carboxymethylcellulose may be used as a carrier in amounts of from about 0.01 to about 5% by weight. The formulation will vary depending upon the purpose of the formulation, the particular mode employed for modulating the receptor activity, the intended treatment, and the like. The formulation may involve patches, capsules, liposomes, time delayed coatings, pills, or may be formulated in pumps for continuous administration. The specific dosage can be determined empirically in accordance with known ways. See, for example Harrison's, Principles of Internal Medicine, 11th ed. Braunwald et al. ed, McGraw Hill Book Co., New York, 1987.

Generally, a therapeutically effective dose of the active fraction or peptides, analogues and mimetics thereof, will be in the range of about 0.005 - 10, more usually from about 0.01 - 1 mg/kg of host weight. Such a dose will be sufficient to achieve the desired

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hypoglycemic activity. Administration may be as often as daily; usually not more than one or more times daily, or as infrequent as weekly, depending upon the level of drug which is administered. The amount oligopeptide

5 administered will generally be adjusted depending upon the half life of the peptide, where dosages in the lower portion of the range may be employed where the peptide has an enhanced half life or is provided as a depot, such as a slow release composition comprising particles,

10 introduced in a matrix which maintains the peptide over an extended period of time, e.g., a collagen matrix, use of a pump which continuously infuses the peptide over an extended period of time over a substantially continuous rate, or the like. Heller, Biodegradable Polymers in

15 Controlled Drug Delivery, in: CRC Critical Reviews in Therapeutic Drug Carrier Systems, Vol. 1, CRC Press, Boca Raton, FL, 1987, pp 39-90, describes encapsulation for controlled drug delivery, and Di Colo (1992) Biomaterials 13:850-856 describes controlled drug release from

20 hydrophobic polymers.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

25 A. Isolation and purification of the active component of Momordica Charantia extract

Fresh unripe fruits Momordica Charantia were washed, wiped and the seeds were removed. The pulp was blended

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in either phosphate buffered saline (PBS) or in deionized water. The suspension was centrifuged at 12,000 x g and filtered through 0.22 : sterile filters (obtained from Whatman) under vacuum. The clear supernatant was passed sequentially through 30 kD, 10 kD and 3 kD cut-off membranes obtained from Amicon filtration. 0.45 μ (1st step), 0.22 μ (2nd step) and 10 kD (3rd step) cut-off fractions were analyzed by SDS-20% polyacrylamide gel, and the results are provided in Fig 1A. The 3rd step purified fraction showed a single band with a size smaller than human insulin. It is evident from the gel analysis that the purified fraction is free of various high molecular weight protein bands. Several batches of 10 K cut-off fraction labeled MC6 were purified in gram quantities, as shown in Figure 1B. The resultant purified fractions were lyophilized to obtain MC6 free of uncombined water.

100 to 500 mg of the lyophilized MC6 was subjected to high performance liquid chromatography (HPLC) (reverse phase HPLC using TFA/acetonitrile linear gradient using C8 or C18 column (analytical)) and the results are provided in Fig. 1C. The HPLC analysis showed three major peaks with retention time of 17.35, 25.35 and 34.67 minutes respectively, demonstrating that MC6 comprises 3 peptides.

B. Hypoglycemic activity of MC6

The hypoglycemic effect of purified fraction was compared with the starting crude extract preparation. SD

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rats (200 gram avg. obtained from Charles River, MA) were starved for 3 hours and an aliquot of blood was collected from the tail vein for measuring the basal plasma glucose level. 5 ml crude extract preparation or 1 ml of 5 purified fraction MC6 (500 :g) in PBS (as prepared in Part A above) was administered orally to each rat. Control animals received an equal volume of PBS. Rats were anesthetized using suritol (i.p.) (0.08 μ g/ml/kg, propanolol (1.7 μ g/min/kg)). The jugular vein of each 10 rat was cannulated and glucose was infused (18 mg/min/kg body weight) in 5 mM KCl and 140 mM NaCl solution for 3 hours. Blood was collected from the carotid artery at 30, 60, 90, 150 and 180 minutes. The plasma glucose level was estimated by the established calorimetric 15 methods described in Kadish et al., Clin. Chem. (1968) 14: 116.

The results are presented in Figure 2A. The results demonstrate that animals given crude extract have a significant decrease in blood glucose level at all 20 points, the effect being substantially high at 60 minutes (with a relative difference of almost 100%). In parallel, as shown in Figure 2B, a 1 ml aqueous purified fraction of MC6 (500 :g) in PBS given orally has an effect similar to the crude extract, suggesting that the 25 isolated polypeptide pool of MC6 is the major active ingredient in the crude extract.

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C. Effect of MC6 on preventive and treatment modes

The next set of experiments was carried out to demonstrate the efficacy of the purified fraction in both the preventive and the treatment modes of diabetes in 5 animal model. Diabetes was induced in male SD rats (200-225 g each) using streptozotocin (40 mg/kg body weight) (obtained from Sigma Chem., MO). In the preventive model, animals received orally 1 ml (500 :g) of the purified fraction MC6 daily effective day 0. In 10 the treatment model, animals received orally 1 ml of purified fraction MC6 (500 :g) at the onset of the disease on day 5. Serum glucose levels were monitored daily for a 15-day period. As shown in Figure 3A and 3B, purified fraction MC6 is highly effective in both 15 preventive and treatment modes ($p<0.001$ at day 10).

The effect of MC6 in the preventive mode of streptozotocin-induced diabetes strongly supports the suitability of using MC6 in treating Type I autoimmune diabetes as well.

20 D. Effect of MC6 on serum insulin level

Serum insulin levels of animals at different days from the experiment described in Part C were measured to examine the role of MC6 in the regulation of the systemic insulin during the hypoglycemic effect. The quantitative 25 measurement of serum insulin level was carried out by radioimmuno assay (LINKO assay) as described in Hales & Randle, Biochem. J. (1963) 88:137. Figure 4 shows that oral administration of this fraction had no effect on

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enhancing the serum insulin level, suggesting that the effect of this fraction is not mediated through insulin secretion by the pancreas. In Fig. 4, Diabetes was induced with streptozotocin. The untreated group was 5 given PBS only; the SZC group was given 40 mg/kg SZC; the preventative group was give MC6 orally from day 1; and the treatment group was given MC6 from day 5. Insulin was measured by standard RIA. The data represent average of triplicate determination.

10 E. Oral administration of MC6 is equally effective as systemic injection of insulin

In the next experiment, orally administered purified fraction MC6 (500 :g) was compared with i.v. injection of insulin (150 mU) in SD rats. Animals were divided into 15 four groups and starved for three hours. Group 1 received PBS only; group 2 was given MC6 orally 60 minutes before glucose infusion; group 3 received 150 mU r-insulin (Humalin-R® of Novo-Nordisk) i.v. at 60 minutes following glucose infusion through jugular vein and group 20 4 received 500 :g MC6 in 1 ml PBS (i.v.) at 60 minutes following glucose infusion through jugular vein. In all cases, glucose infusion was at 18 mg/min/kg. Blood samples were collected from the carotid artery at different time intervals and quantitative plasma glucose 25 levels were measured calorimetrically.

The results are presented in Figure 5. The results show that oral administration of MC6 is comparable to i.v. injection of r-insulin at 120 minute time period.

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Furthermore, systemic injection of this fraction and r-insulin showed identical hypoglycemic profile. These results demonstrate that the purified fraction is effective orally and systemically.

5 F. Characterization MC6.1

MC6.1 was further characterized by SDS-PAGE analysis on 4 to 20% tris-glycine gradient gel using either coomassie blue or silver stain for visualization of resolved bands. The results are provided in Figs. 6a and 10 6b.

G. Further Purification of MC6.1

MC6.1 was further purified and analyzed by C-18 RP HPLC (gradient 0.1% TFA buffer A; 60 % AcN in 0.1% TFA. The results are provided in Fig. 7.

15 H. Isoelectric Point of MC6.1

The isoelectric point of MC6.1 was analyzed and found to be 8.2. The results are provided in Figs. 8a and 8b

I. NMR analysis of MC6.1

Proton NMR analysis was made of MC6.1 and the 20 results are provided in Fig. 9.

J. Hypoglycemic Activity of MC6.1

The hypoglycemic activity of MC6.1 was assayed in a manner analogous to Example B. above. SD male rats weighing on average 220 g were used. The rats were orally

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given 50 μ g MC6.1 in 500 μ l. Control rats were administered PBS. The results are provided in Fig. 10. The results demonstrate the MC6.1 exhibits hypoglycemic activity following oral administration.

5

EXAMPLE 2

Figure 11 represents the hypoglycemic activity of MC6.1 and its derivators, MC6.2 and 6.3 analogs in oral glucose tolerance test in normal rats. All three peptides were effective in lowering blood glucose in this 10 experiment as compared to vehicle treated animals.

Glucose lowering ability of MC6.2 (11-amino acid peptide) was further evaluated in diabetic rats.

Streptozotocin at a dose of 40 mg/kg body weight was injected in SD rats of average 200g. As shown in Figure 15 12, oral treatment of diabetic rats (average blood glucose 330 mg/dl) with MC6.2 at a dose of 100 μ g/rat (0.5 mg/kg body weight) lowered the blood glucose level by significantly as compared to the control group.

The hypoglycemic effect of MC6.2 (11-amino acid 20 peptide) was evaluated in the treatment mode in non-obese diabetic (NOD) mice. As shown in Figure 13, oral administration of MC6.2 in NOD mice daily has significant effect on blood glucose level as compared to vehicle treated animals.

25 These results demonstrate that the N-terminal 7-11 amino acid residues of MC6.1 are important for reducing blood glucose level which is useful for the development of therapeutics for the treatment of human diabetes.

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It is evident from the above results and discussion that a novel extract of *M.charantia* and specific peptide agents are provided that are useful in the treatment of hyperglycemic disorders, particularly diabetes. Since the 5 active agents of the invention do not cause an upregulation in the amount of insulin produced by the body, they provide a useful alternative mechanism for regulating blood glucose levels. Furthermore, since the MC6 and the peptide MC6.1, 6.2 and 6.3 are orally active, 10 they provide significant advantages over conventional insulin products that have been used in the treatment of diabetes which must be introduced by more invasive and less convenient means of administration, such as i.v.

All publications and patent applications cited in 15 this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing 20 date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Although the foregoing invention has been described in some detail by way of illustration and example for 25 purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Natpro, Inc.

5 (ii) TITLE OF THE INVENTION: ORALLY ACTIVE FRACTION OF
MOMORDICA CHARANTIA, ACTIVE PEPTIDES THEREOF, AND
THEIR USE IN THE TREATMENT OF DIABETES

(iii) NUMBER OF SEQUENCES: 3

10 (iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Fish & Richardson, P.C.
- (B) STREET: 2200 Sand Hill Road, Suite 100
- (C) CITY: Menlo Park
- (D) STATE: CA
- (E) COUNTRY: U.S.A.
- (F) ZIP: 94025

15 (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ for Windows Version 2.0

20 (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: Unknown
- (B) FILING DATE: April 1, 1998
- (C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/850,855
- (B) FILING DATE: 02-MAY-1997

- (A) APPLICATION NUMBER: 08/831,039
- (B) FILING DATE: 01-APR-1997

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- (A) NAME: Suyat, Reginald J
- (B) REGISTRATION NUMBER: 28,172
- (C) REFERENCE/DOCKET NUMBER: 08948/006WO1

35 (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 650-322-5070
- (B) TELEFAX: 650-854-0875
- (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids

- 26 -

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5 Lys Thr Asn Met Lys His Met Ala Gly Ala Ala Ala Gly Ala Val
1 5 10 15
Val Gly

(2) INFORMATION FOR SEQ ID NO:2:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Thr Asn Met Lys His Met Ala Gly Ala Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

25 Lys Thr Asn Met Lys His Met
1 5

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WHAT IS CLAIMED IS:

1. The water-soluble composition MC6
2. The composition according to Claim 1 characterized by moving a single band on SDS-20% PAGE.
- 5 3. The composition according to Claim 2, wherein said single band has a molecular weight of less than 10 kDal and has a size smaller than r-insulin.
4. The composition according to Claim 1 comprising three peptides.
- 10 5. The composition according to Claim 1 which exhibits hypoglycemic activity and is active by oral administration.
6. The water-soluble composition MC6 characterized by migrating as a single band of less than 10 kDal on SDS-15 20% PAGE comprising three peptides, exhibiting hypoglycemic and is active by oral administration.
7. A pharmaceutical composition comprising MC6 in a physiologically acceptable vehicle.
8. The pharmaceutical composition according to Claim 7, 20 wherein said physiologically acceptable vehicle is an orally acceptable vehicle.

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9. The pharmaceutical composition according to Claim 8, wherein said orally acceptable vehicle comprises deionized water or phosphate buffered saline.

10. A method of reducing the blood glucose level of a 5 host, said method comprising:
administering MC6 to said host.

11. The method according to Claim 10, wherein said MC6 is present in physiologically acceptable vehicle.

12. The method according to claim 10, wherein said 10 administering is oral or I.V.

13. The method according to Claim 10, wherein said host suffers from a hyperglycemic disorder.

14. The method according to Claim 13, wherein said hyperglycemic disorder is diabetes.

15 15. The method according to Claim 14, wherein said diabetes is Type I diabetes.

16. The method according to Claim 14, wherein said diabetes is Type II diabetes.

17. A method of reducing the blood glucose level of a 20 mammalian host resulting from a hyperglycemic disorder, said method comprising:

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orally administering a pharmaceutical composition of MC6 to said host.

18. The method according to Claim 17, wherein said mammalian host is a human.

5 19. The method according to Claim 18, wherein said hyperglycemic disorder is diabetes.

20. The method according to Claim 19, wherein said diabetes is selected from the group consisting of Type I and Type II diabetes.

10 21. A method for reducing the blood glucose level in a human having hyperglycemia resulting from diabetes, said method comprising:

orally administering to said host a pharmaceutical composition containing MC6.

15 22. The method according to Claim 21, wherein said diabetes is selected from the group consisting of Type I and Type II diabetes.

23. A peptide selected from the group consisting of MC6.1, MC6.2, MC6.3 and analogues or mimetics of MC6.1,

20 MC6.2 and MC6.3.

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24. The peptide MC6.1 according to Claim 23, having the amino acid sequence:

K-T-N-M-K-H-M-A-G-A-A-A-G-A-V-V-G (SEQ ID NO:01).

25. The peptide MC6.2 according to Claim 23, having the 5 amino acid sequence:

K-T-N-M-K-H-M-A-G-A-A (SEQ ID NO:02).

26. The peptide MC6.3 according to Claim 23, having the amino acid sequence:

K-T-N-M-K-H-M (SEQ ID NO:03).

10 27. A pharmaceutical composition comprising the peptide MC6.1, MC6.2, MC6.3, analogue or mimetic thereof, or mixtures thereof, in a physiologically acceptable vehicle.

15 28. A method for reducing the blood glucose level of host, said method comprising:

administering the pharmaceutical composition of Claim 27 to said host.

29. The method according to Claim 28, wherein said administering is oral or i.v.

20 30. The method according to 28, wherein said host suffers from a hyperglycemic disorder.

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PCT/US98/06450

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31. The method according to Claim 30, wherein said hyperglycemic disorder is diabetes.

32. The method according to Claim 31, wherein said diabetes is Type I or Type II diabetes.

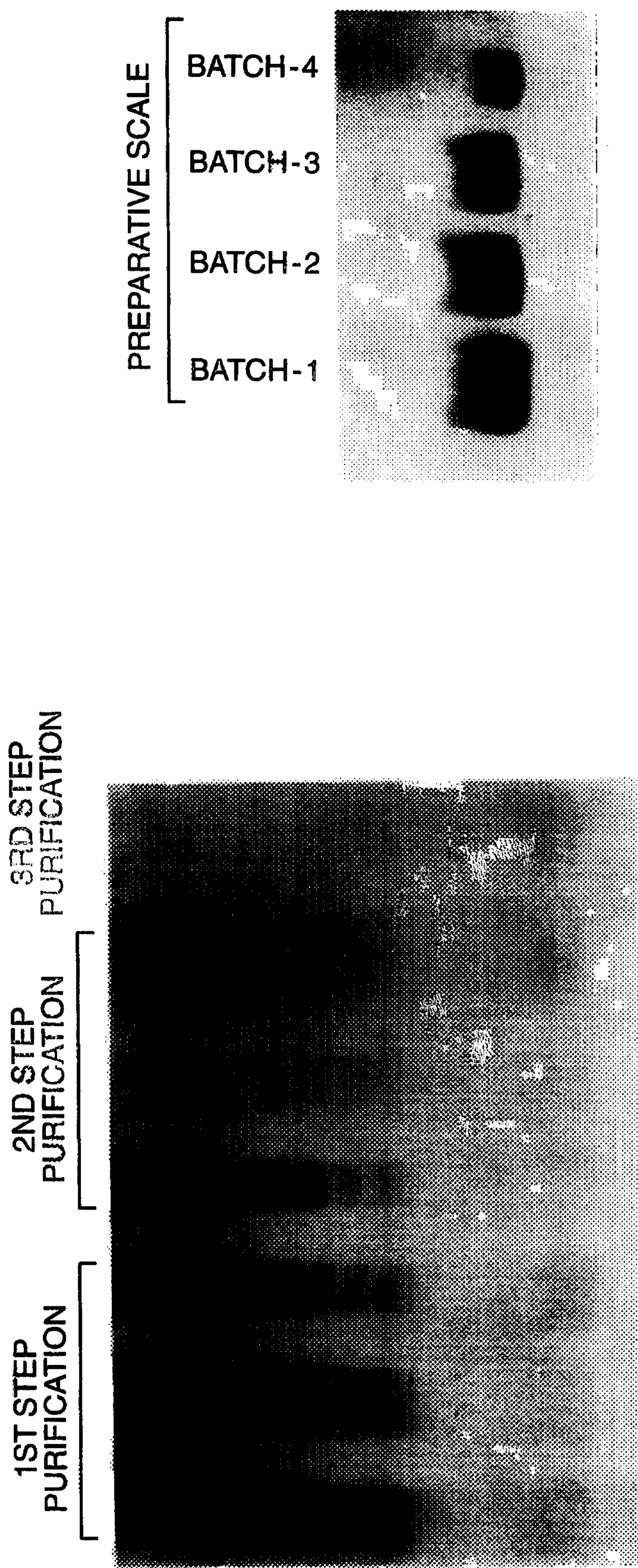


FIG. 1B

FIG. 1A

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CHROMATOGRAM 1 MEMORIZED
WARNING NO PEAK

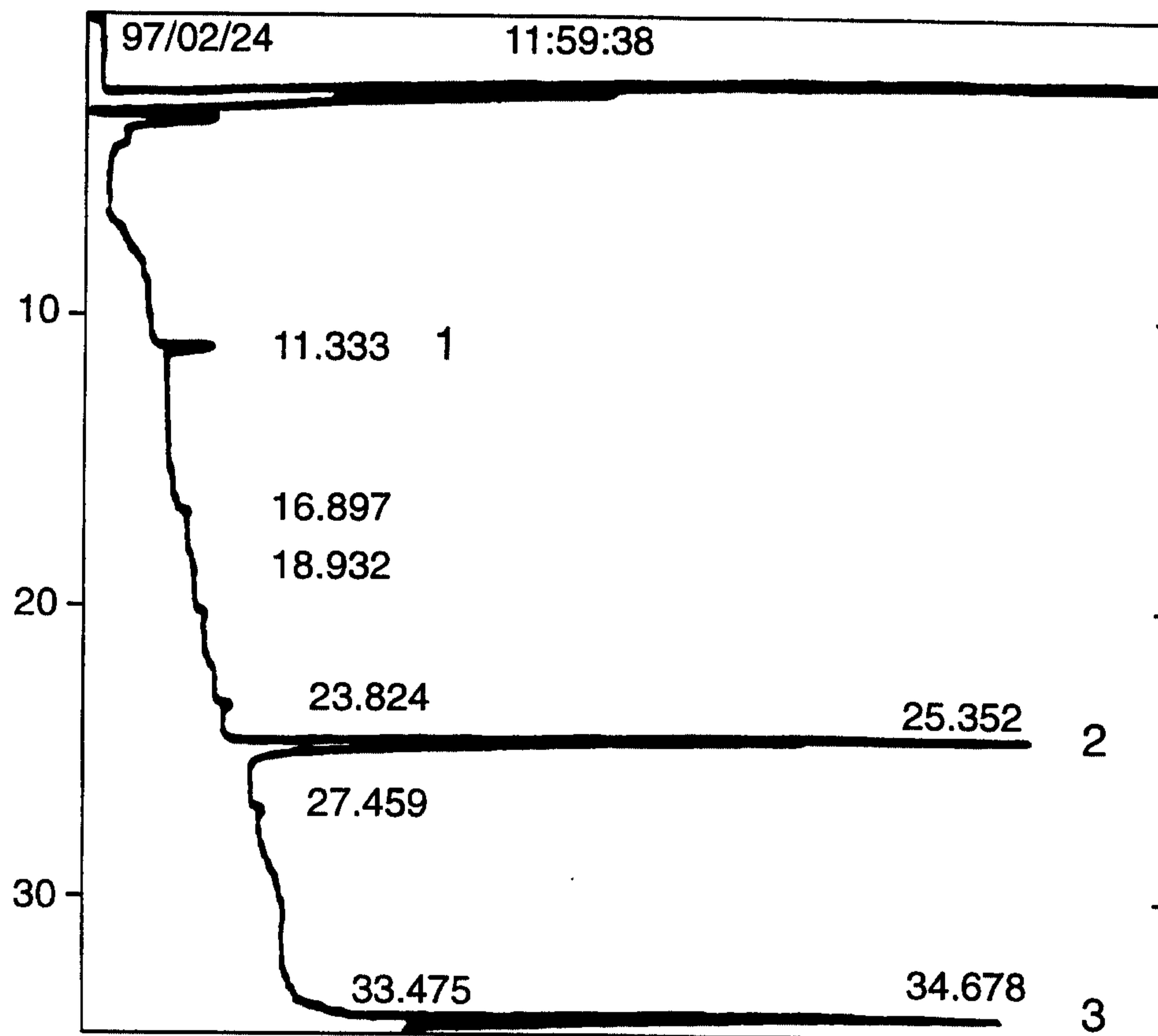
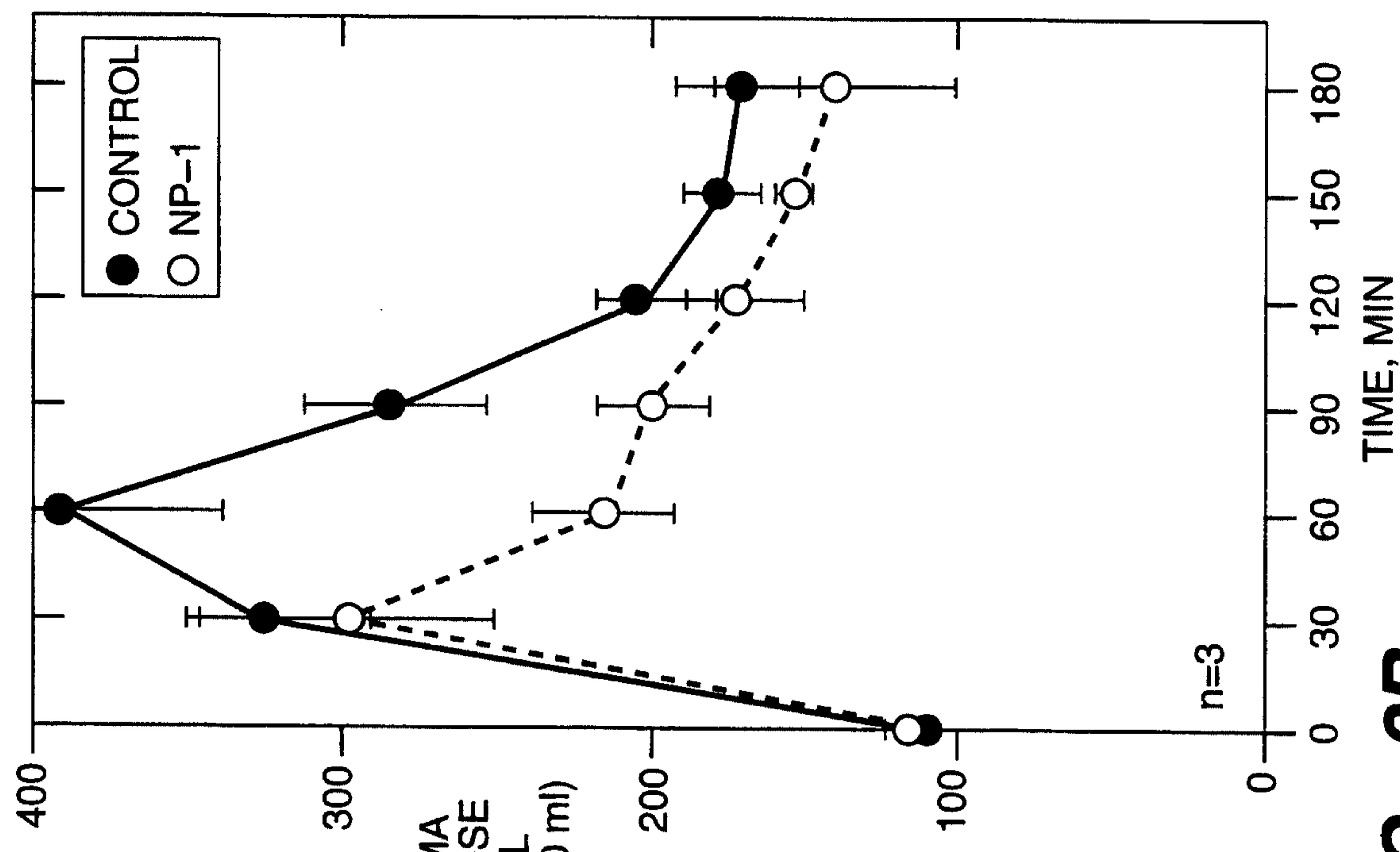


FIG._ 1C

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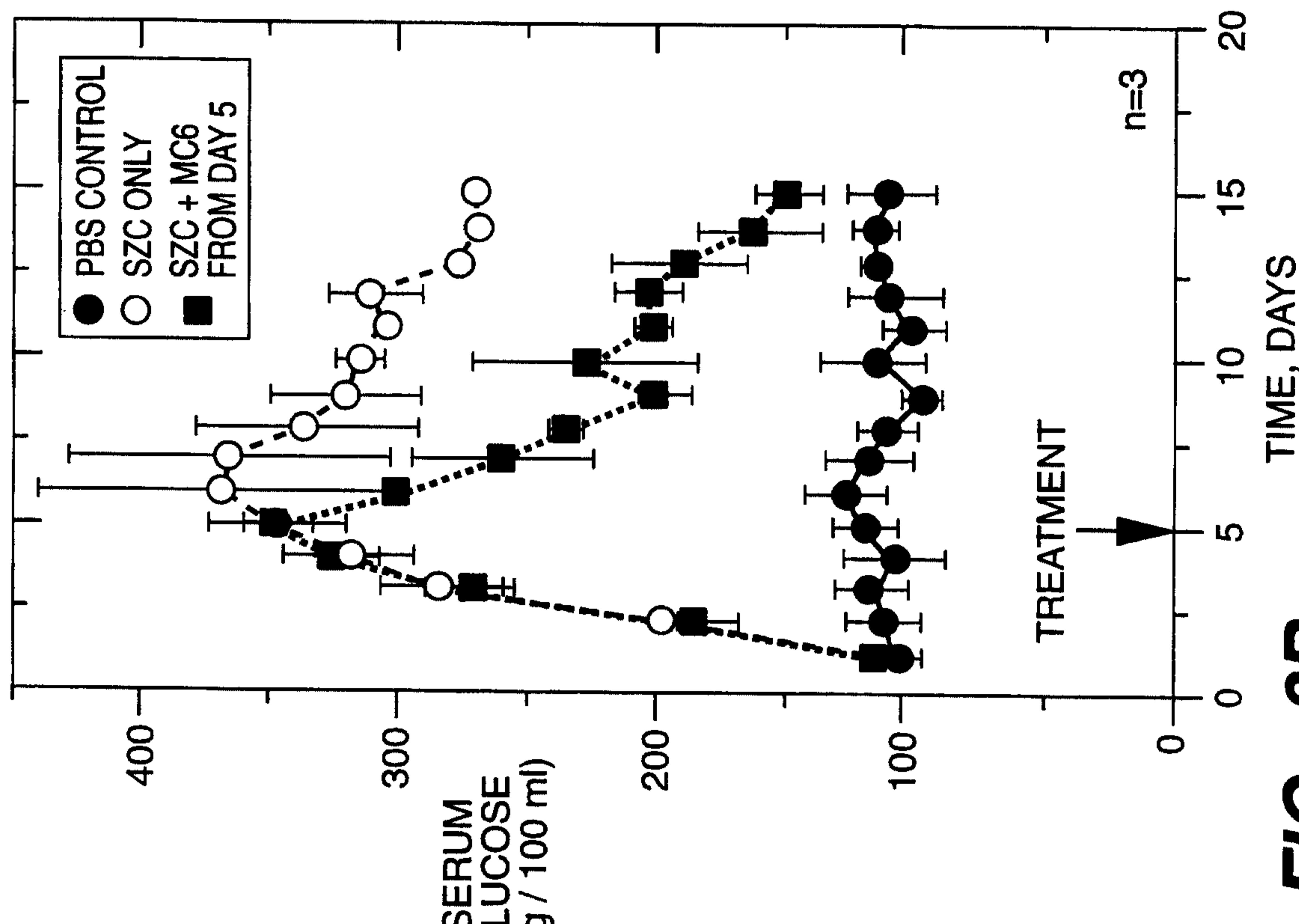


FIG._3B

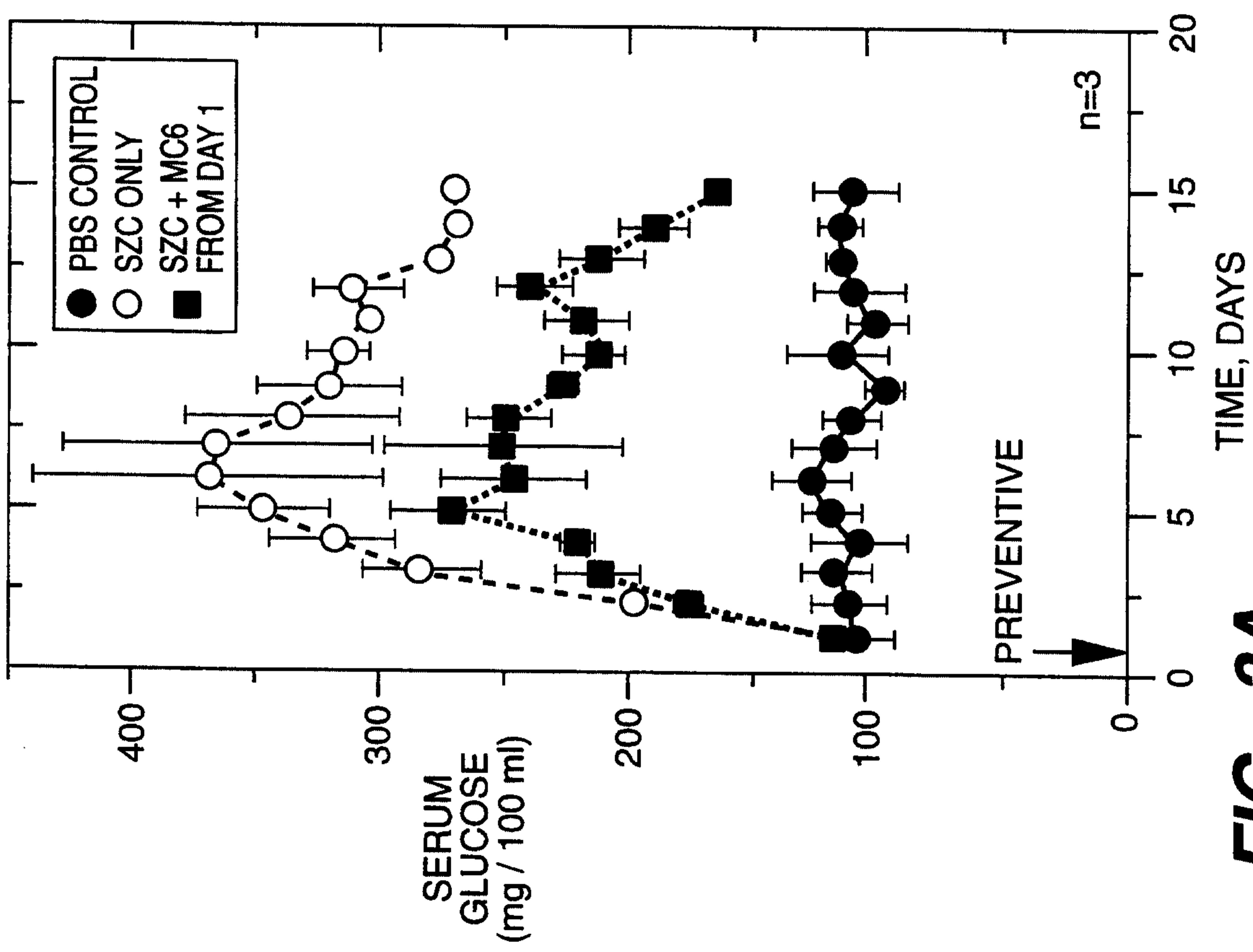


FIG._3A

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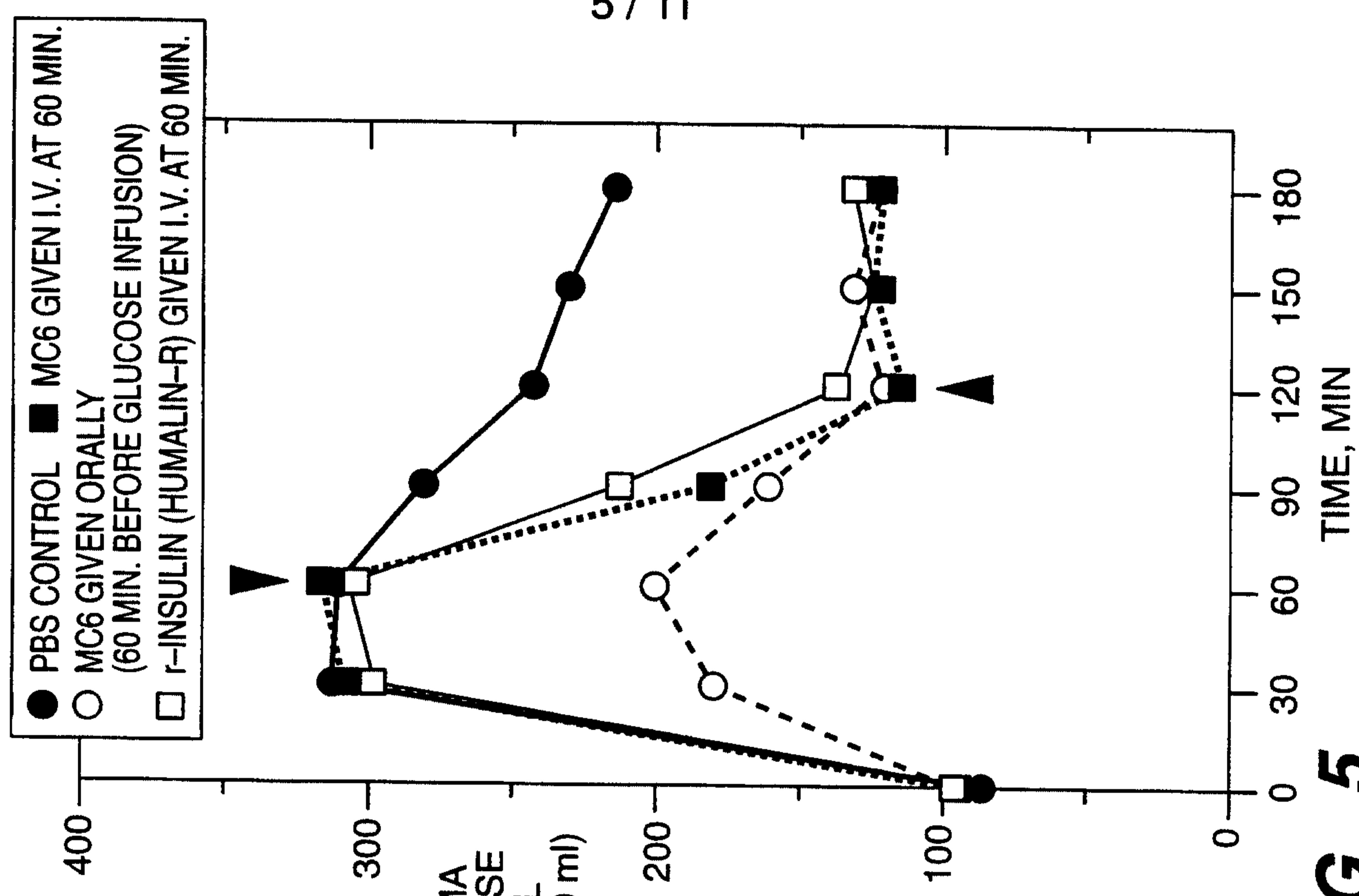


FIG. 5

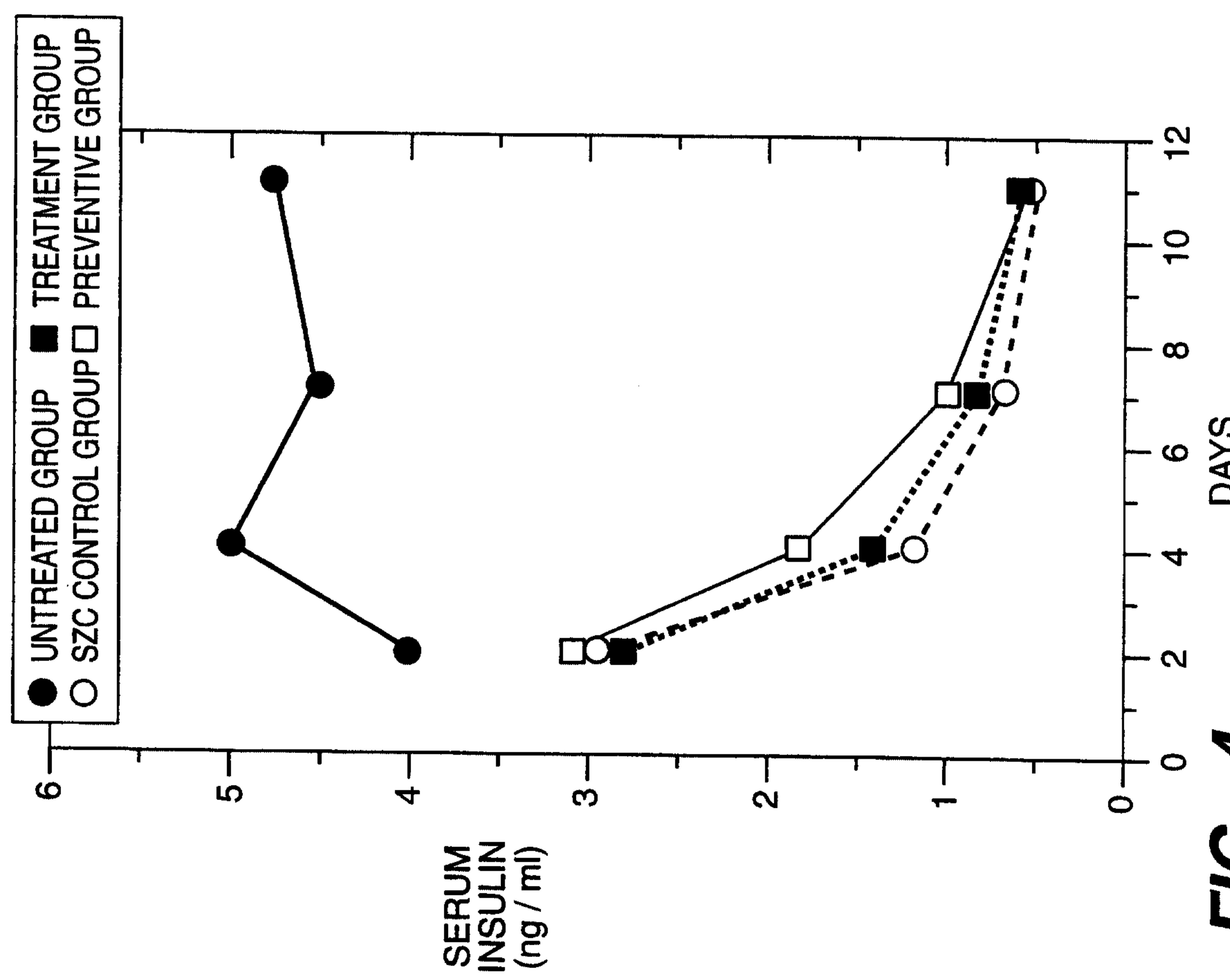


FIG. 4

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MC6.1, 100 ng

MC6.1, 50 ng

r-Insulin

LMM, kD

36 — 31 — 21 — 14 — 7 — 3.5 — 2.5 —

LMM, kD

r-Insulin

MC6.1, 5 μ gMC6.1, 10 μ g

— 36 — 31 — 21 — 14 — 7 — 3.5 — 2.5 —

SUBSTITUTE SHEET (RULE 26)

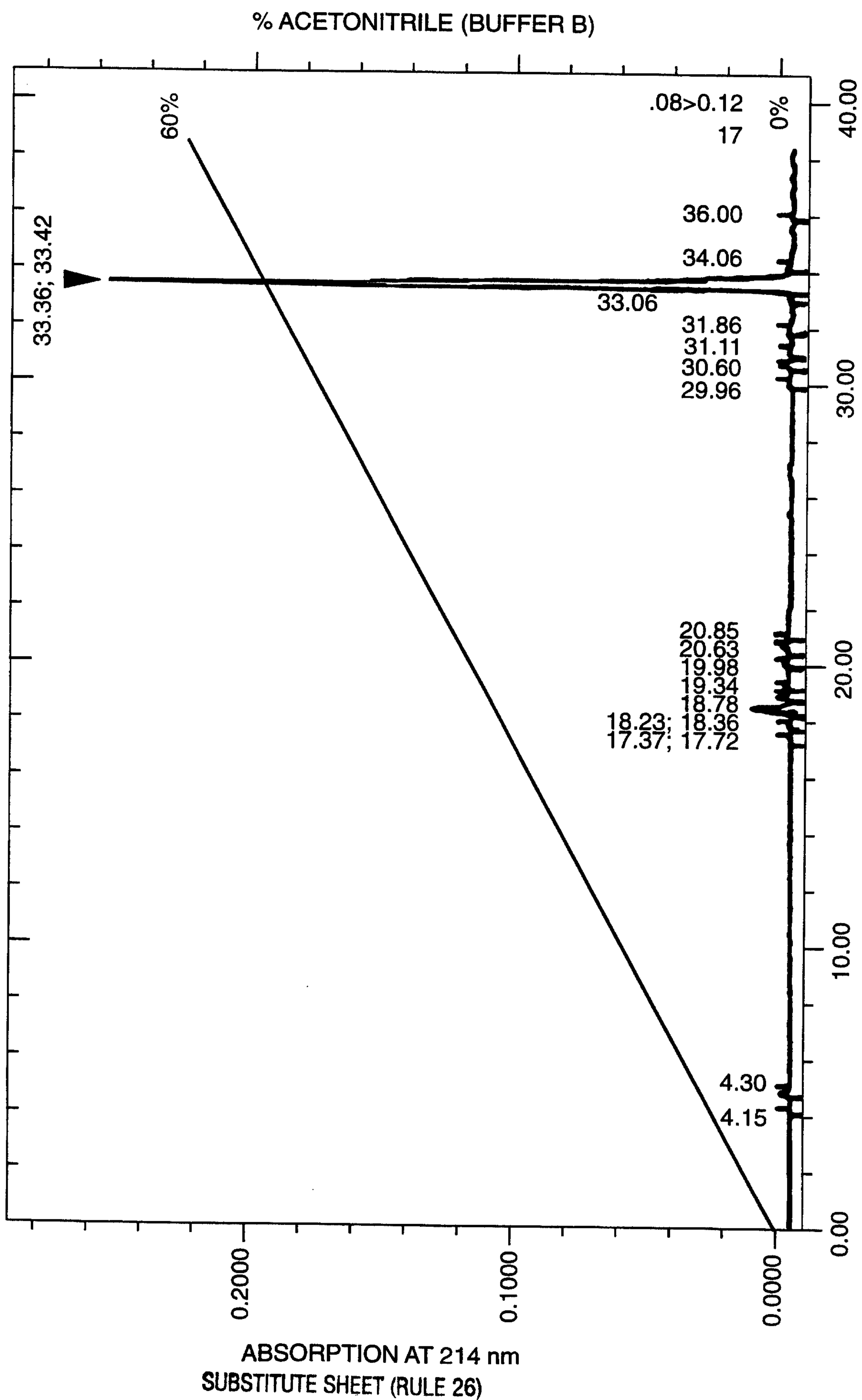
SILVER STAIN

FIG._6B

CB STAIN

FIG._6A

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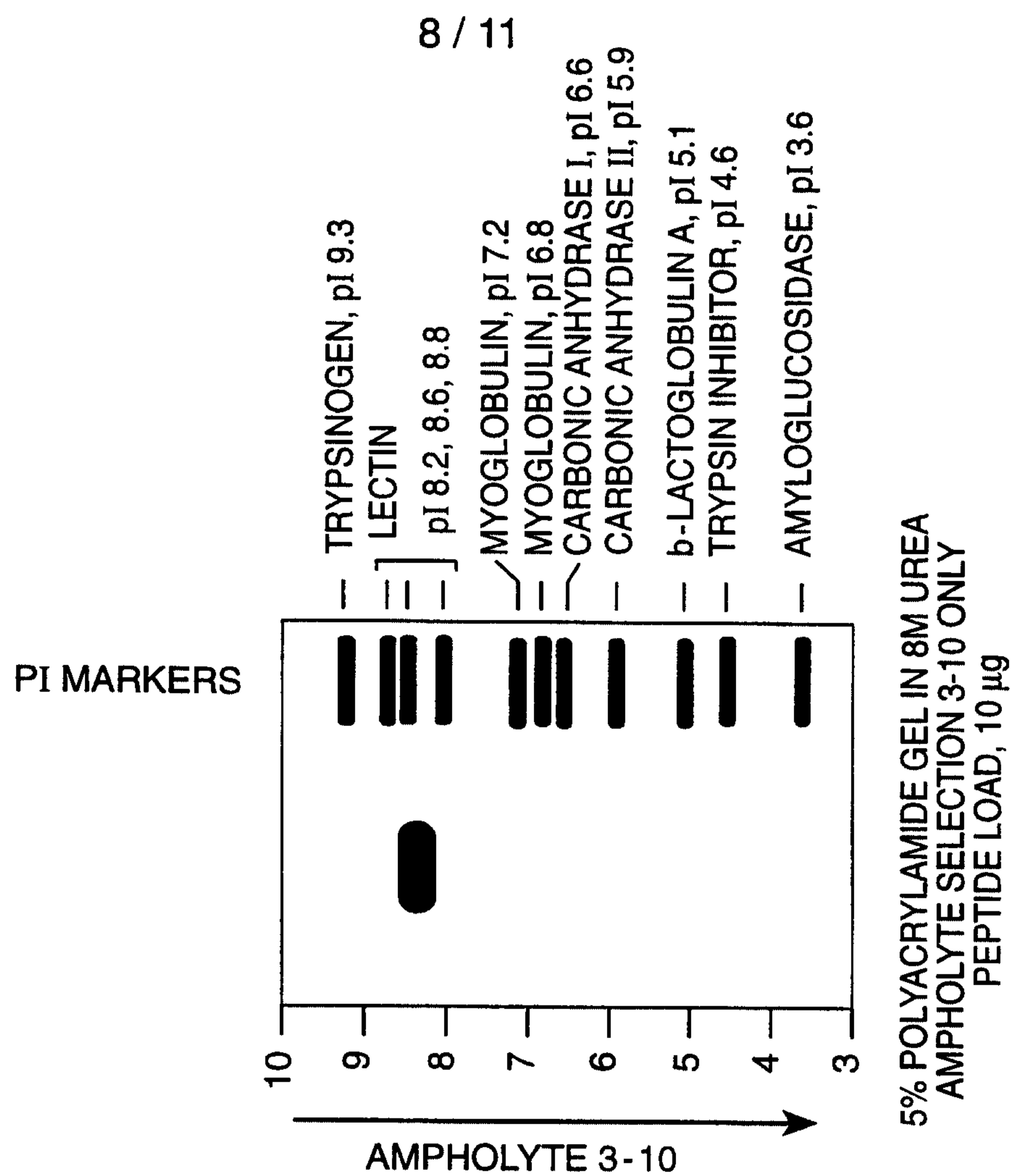


FIG. I

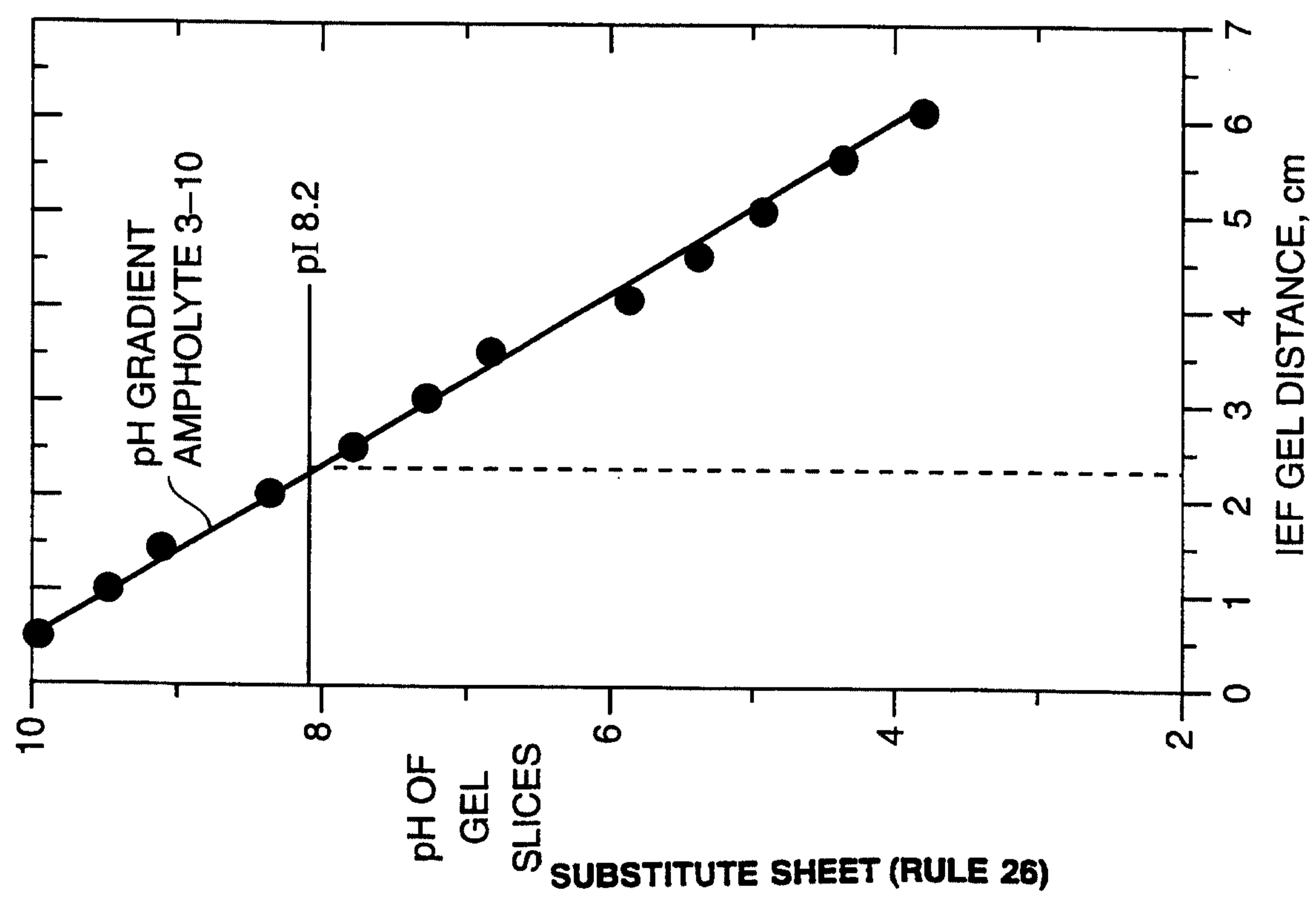
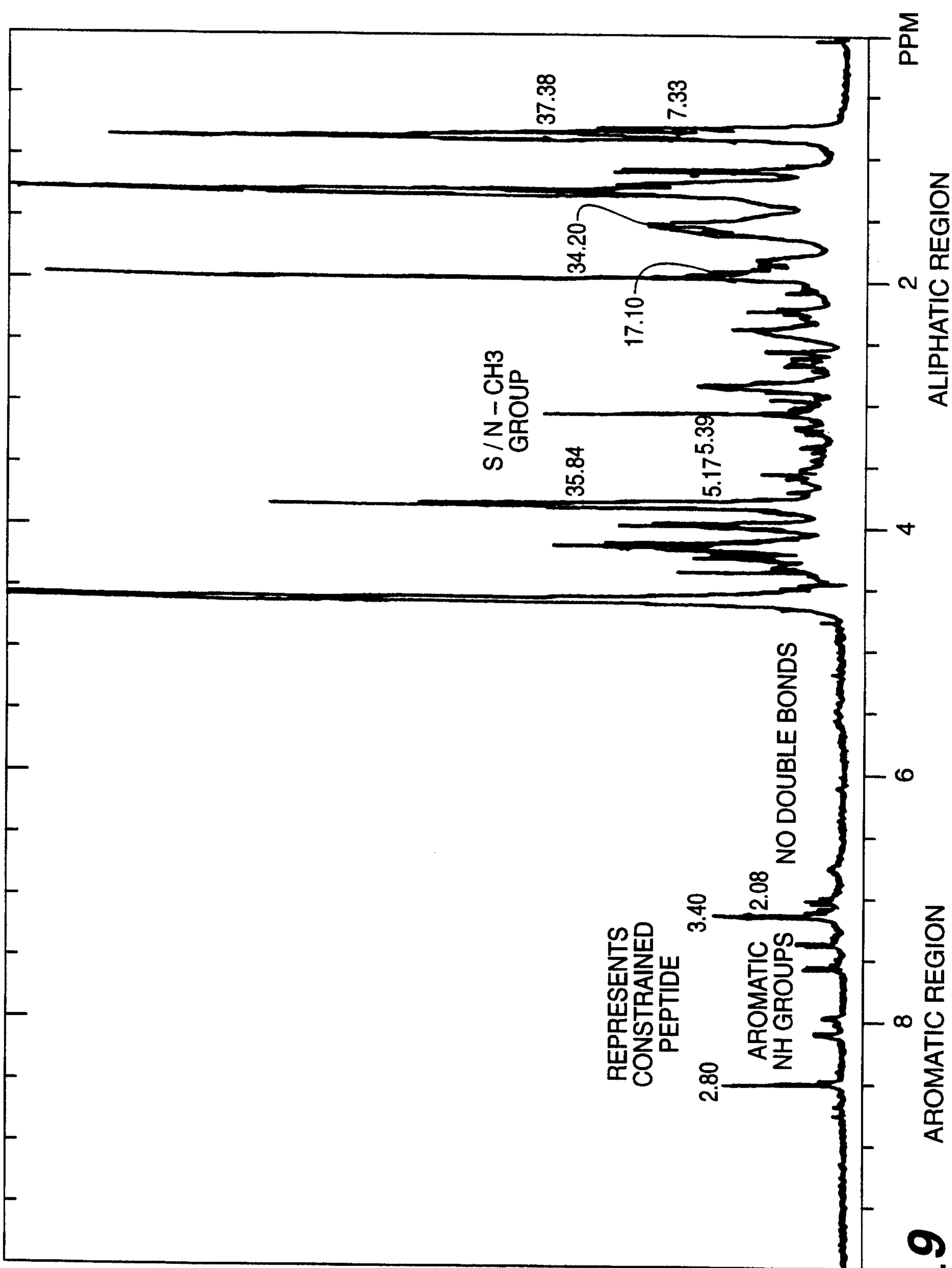


FIG. 8A

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SUBSTITUTE SHEET (RULE 26)

FIG. 9

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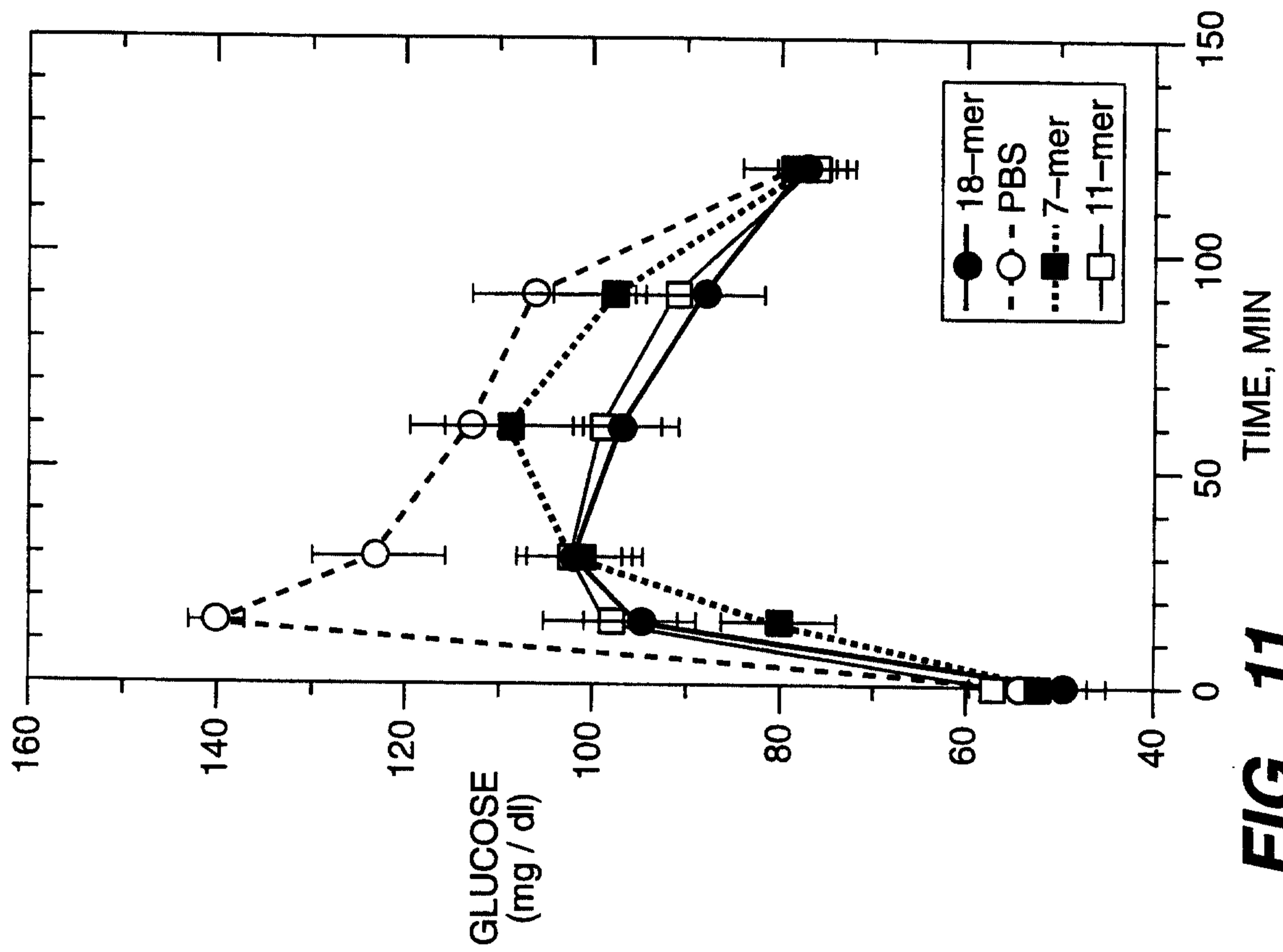


FIG. - 11

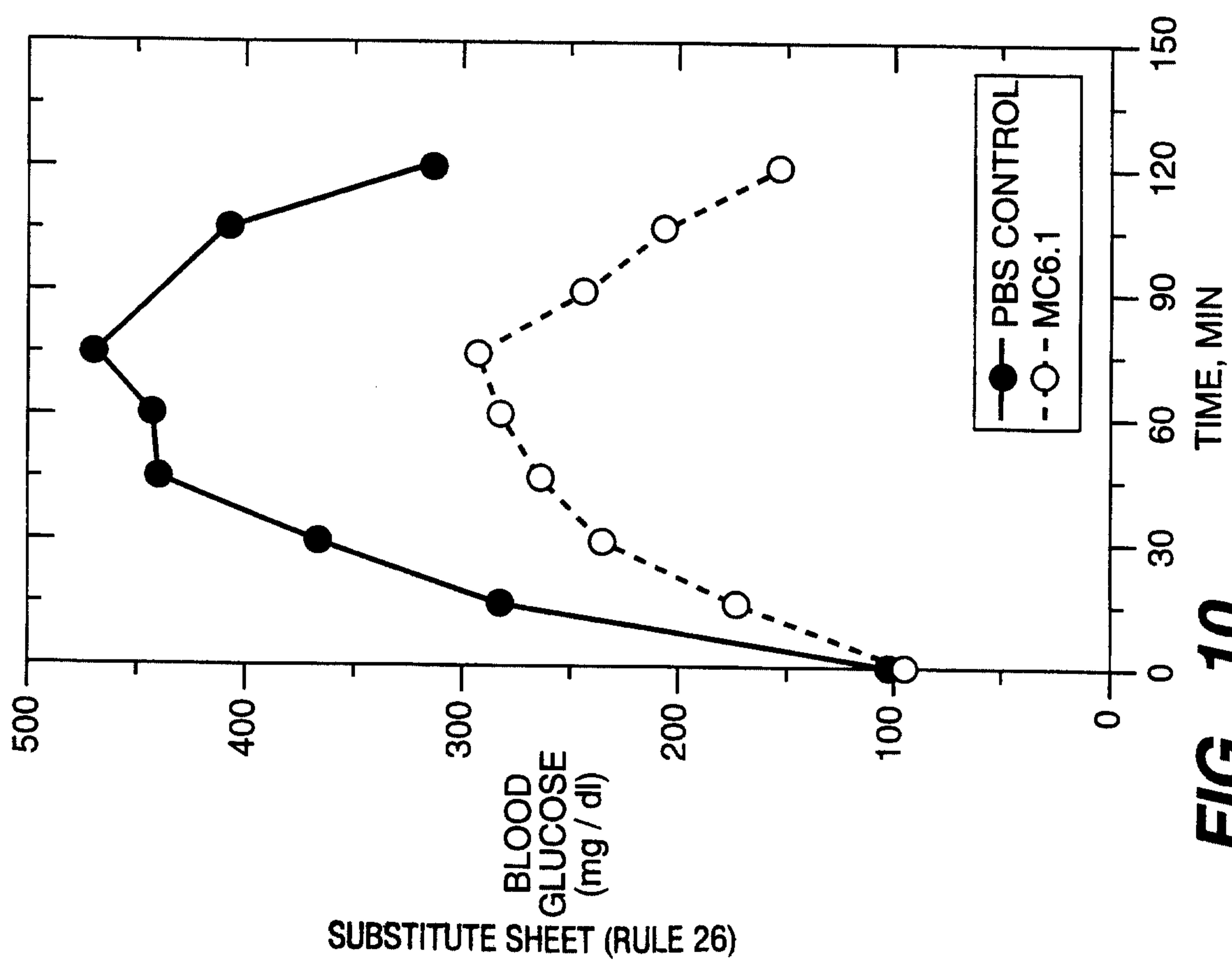


FIG. - 10

SUBSTITUTE SHEET (RULE 26)

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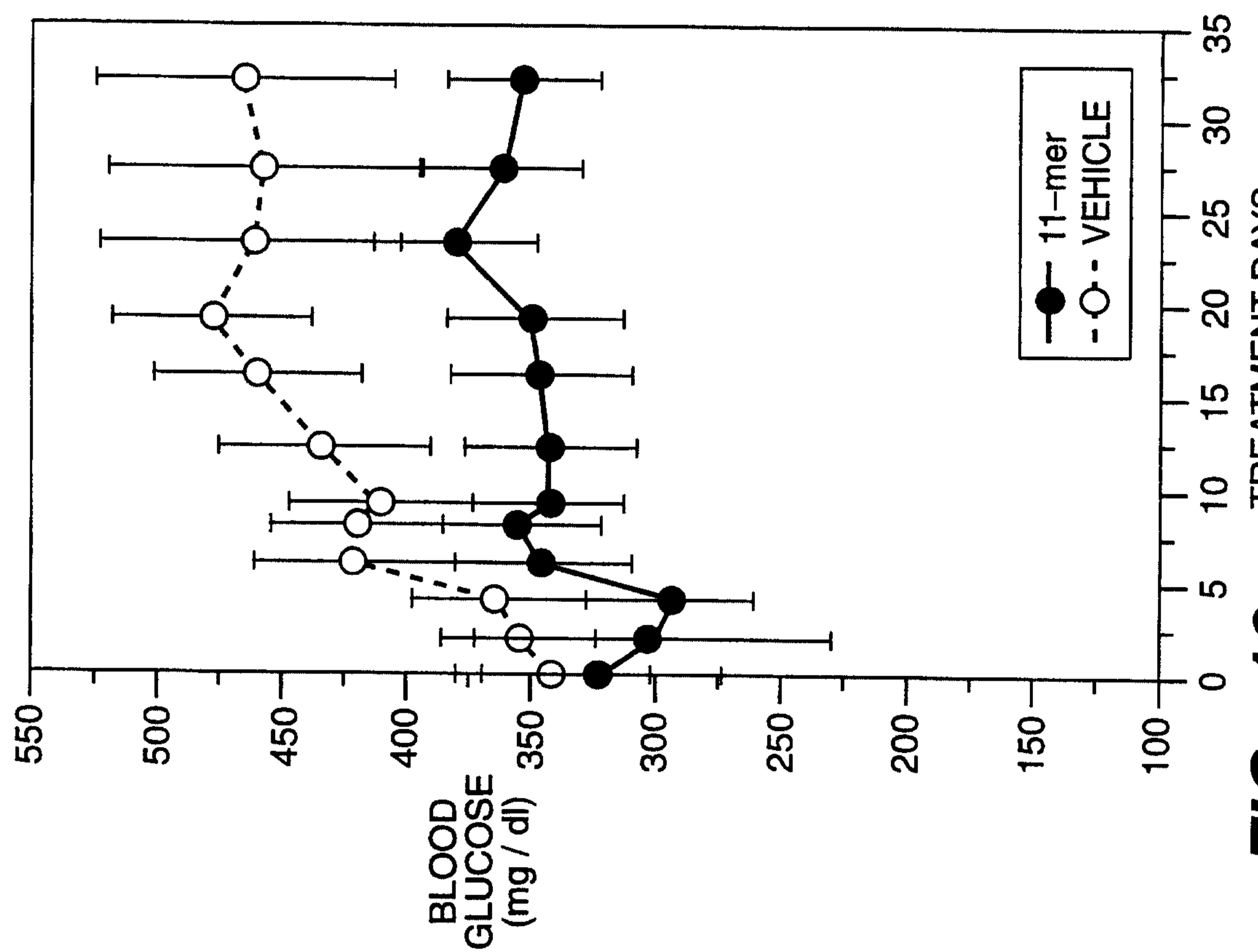


FIG. 13

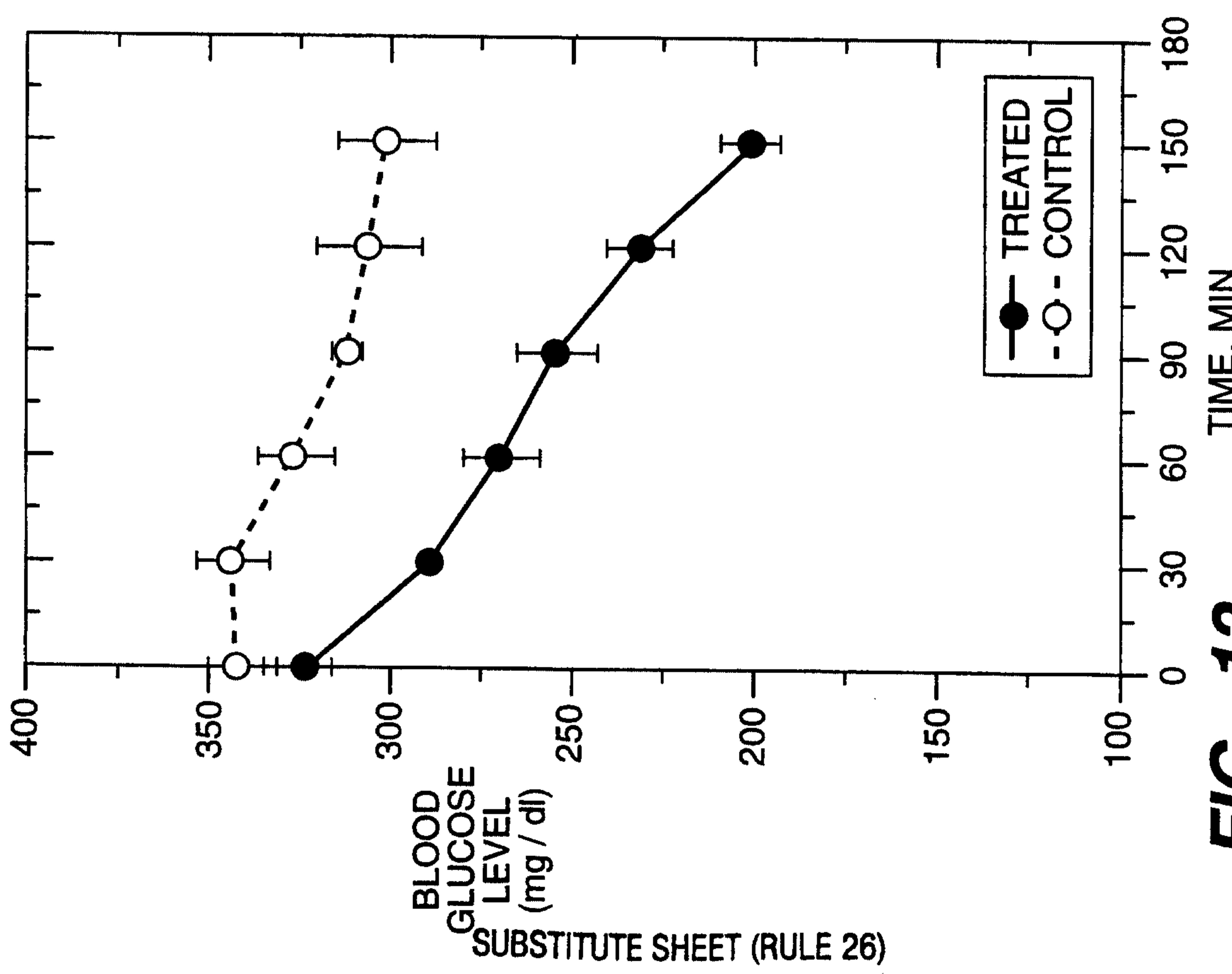


FIG. 12

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