

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
4 April 2002 (04.04.2002)

PCT

(10) International Publication Number
WO 02/27326 A2

(51) International Patent Classification⁷: G01N 33/66

(21) International Application Number: PCT/IL01/00848

(22) International Filing Date:
6 September 2001 (06.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
138788 29 September 2000 (29.09.2000) IL

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, 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, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 02/27326 A2

(54) Title: METHOD AND KIT FOR THE TRANSDERAML DETERMINATION OF ANALYTE CONCENTRATION IN BLOOD

(57) Abstract: A method is provided for determining the level of an analyte in the blood of an individual by measuring the level of the analyte in an interstitial fluid or in any other non blood fluid which does not contain red blood cells and adjusting the measurement value by the concentration of at least one reference analyte.

**METHOD AND KIT FOR THE TRANSDERMAL DETERMINATION
OF ANALYTE CONCENTRATION IN BLOOD**

FIELD OF THE INVENTION

This invention concerns a method for determination of the concentration of various analytes in the blood of an individual and kits for carrying out the method of the invention.

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PRIOR ART

The following is a list of prior art publications referred to in the present specification.

10 Aronowitz, J.L. and Mitchen, J.R., PCT Application Publication No. WO 99/13336

Ben-Aryeh, H., *et al.*, *J. Diabet. Complications*, 2:96-99 ((1988)).

15 Brown, L.R. and Edelman, E., PCT Application Publication No. WO 99/67645

Doneen, B.A., *et al.*, PCT Application Publication No. WO 99/22639.

Forbat, L.N., *et al.*, *J.R. Soc. Med.*, 74:725-728 (1981).

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Guy, R. and Rao, G., U.S. Patent 5,362,307.

Hertig, B.A. *et al.* "Time course of sweating in warm baths" in: Montagna W. *et al.* (eds.) *Advances in biology of skin, Vol III Eccrine Sweat Gland and Eccrine Sweating*, pp: 213-225, Pergamon Press, 1962

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Kuno, Y., *Human Perspiration*, Charles C. Thomas, Publisher, USA, 1956

Patrick, A.W., *et al.*, *Diabet. Med.*, 11:62-65 (1994).

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Rothman, S., *Physiology and Biochemistry of the Skin*, Chapter 19: Carbohydrates, The University of Chicago Press, 1954

Sönsken PH, *Acta Endocrinol. Suppl.* (Copenhagen) **238**:145-155 (1980).

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The acknowledgement herein of the above art should not be construed as an indication that this art is in any way relevant to the patentability of the invention as defined in the appended claims.

10 BACKGROUND OF THE INVENTION

Diabetes type I (insulin dependent) patients are required to frequently test their blood glucose levels. In 1993, a ten year long diabetes care and complications trial (DCCT) showed that the preferred mode of treatment of insulin dependent diabetes (Type 1) was by frequent small-dose administrations of insulin to such patients and determining the glucose level after each such administration. To follow such a treatment, a diabetic patient is required to puncture his skin and obtain a drop of blood for the glucose test at least three times a day. Such a frequent and repetitive puncturing is painful and often results in infection and formation of hard scar tissue and as a result, many diabetic patients neglect to sufficiently test their glucose level.

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In an attempt to minimize the harm and pain caused by skin puncturing to obtain a blood specimen, alternative methods have been developed. Those methods generally belong to one of the following approaches:

- (a) Non-invasive techniques, which are based on the analysis of transmission, scattering or reflectance spectra of various radiations, such as infra-red, photoacoustic and di-electric radiation, which permeate through the skin or other tissues.
- (b) Minimally invasive techniques, which are based on drawing out minute specimens of blood or other fluids (sometimes referred to as "*interstitial fluid*"), in a manner which causes minimal pain or damage, or none at all.

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Guy and Rao (U.S. 5,362,307) have shown a method for determining the concentration of an inorganic or organic substance in an individual by obtaining an interstitial fluid sample from the individual by a process called iontophoresis. In accordance with this method, an electric field is employed, causing the transdermal migration of ions, which carry with them non-charged molecules, such as glucose. This method was recently developed into the GlucoWatch™ product, by Cygnus, Inc., Redwood City, CA, USA.

Methods for the extraction of glucose and other analytes from the skin, employing chemicals, called "*enhancers*" or "*permeators*" (hereinafter "*permeation enhancers*") were recently described. Aronowitz and Mitchen (PCT WO99/1336) employ a gel, containing propylene glycol. The extracted fluid enters a gel and the concentration of glucose or any other analyte, is determined by a colorimetric reaction. Brown and Edelman (WO99/67645) also use a gel, containing pyrrolidone compounds and optionally other enhancers, to extract the analytes of interest from the skin. Both approaches require placement of gel on the skin for a defined length of time, which extends to more than 10 minutes, in order to ensure sufficient quantity of analyte, which will reflect the concentration of the same analyte in the blood.

Another minimally invasive method for obtaining a body fluid is that of SpecRx, Inc. Norcross, GA, USA. A minute and shallow round hole is created in the skin, extending just below the stratum corneum and a sample of interstitial fluid is collected through this hole. That fluid is then tested for its glucose content by one of the methods known in the art.

In such minimally invasive methods the concentration of the tested substance in the obtained interstitial fluid sample may not always correctly indicate the level of the same substance in the blood of the tested individual at the time in which the sample was obtained or shortly thereafter. This is mostly due to the fact that the concentration of the tested substance varies in different locations in the body and at different hours of the day. Moreover, although such minimally invasive methods attempt to reduce the pain and damage normally associated

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with blood sampling, they still often result in discomfort to the tested individual, and involve skin irritation and damage.

Attempts to detect the correct glucose level in the blood by determining the level of glucose in fluids of body samples other than blood such as saliva, urine or tears were found to be non suitable since the concentration of the glucose
5 in such fluids was shown to be variable and, more often than not, did not directly reflect the concentration of the glucose in the blood at the relevant point in time (Sönsken, Patrick *et al*, Forbat *et al*, Ben-Aryeh *et al*, Doneen *et al*).

10 SUMMARY OF THE INVENTION

In accordance with the present invention it has been unexpectedly demonstrated that glucose in the interstitial fluid is spontaneously extracted from a non-wounded skin when the skin is contacted with water, containing no skin permeation enhancers. It was shown that the concentration of glucose in the
15 blood can be inferred from the concentration of glucose in the skin water extract by the determination of a reference analyte in the extract and calculating a ratio. In accordance with the invention various electrolytes (preferably calcium ions) were found to be suitable reference analytes. The skin extraction with water was rapid: measurable levels of glucose and electrolytes were present in the specimen
20 as early as 30 seconds following the initial contact between skin and water. The phenomenon of glucose extraction from the skin into water does not seem to be related to sweating, since sweat is not secreted when the skin is submerged in water (Hertig *et al*. 1962, Kuno 1956). Moreover, the invention demonstrated the ability to follow the changing concentration of glucose in blood by following
25 glucose ingestion in skin extracts. This finding is unexpected in view of the observation that skin glucose level does not respond quickly to blood glucose variation, especially when the blood concentration falls following a hyperglycemic period (Rothman 1954).

By its first aspect, the present invention thus provides a method for
30 determining the level of an analyte in the blood of an individual by measuring the

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level of said analyte in an interstitial fluid or in any other non blood fluid which does not contain red blood cells and adjusting the measurement value by the concentration of at least one reference analyte.

By a preferred embodiment the tested analyte is glucose.

5 By an additional preferred embodiment said reference analyte is calcium ions.

The invention further provides a method for determining the level of an analyte in the blood of an individual comprising:

- 10 (i) contacting the skin of said individual with water to obtain a water specimen;
- (ii) determining the level of said analyte in said water specimen;
- (iii) determining the level of one or more reference analytes in the water specimen;
- 15 (iv) calculating the level of said analyte in the blood of the tested individual based on the measurements in (ii) and (iii).

In accordance with one embodiment of the invention, the water contacted with the skin contains no skin permeation enhancers (such as for example, N-methyl pyrrole) used for extracting the analyte from the skin.

20 The term "level" as it is to be understood in the context of the present invention relates either to a quantity or to the concentration of the tested analyte.

The tested analyte may be any substance or component found in the blood for example, sugars, proteins, organic compounds etc., which is present in detectable amounts in the non-blood fluid or sample.

25 The term "*interstitial fluid*" is the fluid that is located outside blood vessels or extracted from tissues and does not contain blood and blood cells, but may contain non-cellular blood components.

The term "*contacting*" typically refers to immersion of an individual's skin in water or touching of an individual's skin with a water volume. The contact of the skin with the water does not involve disruption or induction of discontinuity
30 of the skin and leaves the skin non-wounded.

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The skin may be contacted with the water directly and without assistance of any special means. In such a case, typically several drops of water are placed on a solid surface (e.g. plastic or glass), the skin area is washed (e.g. with detergents and water), rinsed in water and air dried and then placed in the drop of
5 water for a short period of time (typically about 30 sec.). The water is then collected from the surface and subjected to the assay. However, preferably, in accordance with the invention the skin is contacted with the water through a fine mesh (such as Polymon PES 180/31, obtainable from Sefar, Switzerland) which assists in spreading the water over the skin and thus promotes efficient extraction
10 of the analytes from the skin.

The term "*water*" encompasses any kind of water including tap water, distilled water, water to which electrolytes were added, etc. In accordance with the invention the water does not contain any permeation enhancers.

The term "*specimen*" relates to a water sample obtained by said contact of
15 the skin with water immersion of an individual's skin in water or touching an individual's skin to a water volume.

In accordance with the invention, any skin area may be contacted with the water, however it is preferable that the the skin area is a hairless skin area that does not have fat glands and, therefore, it is more accessible for testing.
20 Examples of such areas are skin on the palms of hands and fingertips. Contacting a larger area of skin will yield higher levels of analytes.

The level of the tested analyte in the obtained sample is determined using any of the methods known in the art which are suitable for determining the level of the specific analyte to be tested. By a preferred embodiment of the invention
25 the tested analyte is glucose. The level of the glucose in the body sample may be determined using any of the known highly sensitive glucose determination methods based on fluorescence, chemiluminescence, or bioluminescence. Examples of such methods are continuous monitoring of reactions that produce NADH and NADPH using immobilized luciferase and oxido reductases from
30 *Beneckea harveyi* (Haggerty, C. *et al.*, *Anal. Biochem.*, **88**:162-173, 1978 or

Jablonski, E., *et al.*, *Clin. Chem.*, **25**:1622-1627, 1979). In addition, any of the colorimetric or electrochemical methods known in the art which utilize glucose oxidase or glucose dehydrogenase or hexokinase may also be used for determining the level of the glucose in the sample (see for example Sigma Cat #: 5 315, 115-A, 510-A).

The reference analyte can be any analyte, which is normally present in the body (e.g. in the blood). In accordance with the invention one or more reference analytes may be used. The analyte can be from the group of proteins, carbohydrates, lipids, proteins or electrolytes. Electrolytes are preferable to other 10 components due to their stability and ease of measurement. Although sodium is the most abundant electrolyte in the body, calcium ions may be preferable as a reference due to (a) the tight physiological of the calcium level; (b) the abundance of sodium in nature may interfere with determination of low levels of analyte in very dilute specimens.

15 Calcium levels can be easily determined by a variety of methods known in the art. In addition, ready reagent kits for calcium determination are available from many manufacturers of *in vitro* diagnostics, such as Sigma Chemicals, St. Louis, MO, USA and Merck, Darmstadt, Germany

Calculation of the concentration of the tested analyte may be based on the 20 ratio of the concentration of the analyte, which was measured in the obtained sample to the concentration of the reference component measured in the same. For example, wherein the tested analyte is glucose and the reference component is calcium ion, the glucose concentration in the blood of the tested individual is calculated from the ratio of the glucose to calcium ion.

25 The measurement of the glucose in the water extract is corrected by the measurement of the electrolyte level in the specimen. In some cases, calculating the simple ratios of the analyte to the reference analyte may provide a good precision in calculating the level of the tested analyte. In other cases calculation of the simple ratio may result in a comparatively low precision. In such a case, 30 logarithmic transformation of the analyte measurement (using the formula: $1000x$

$\log_{10} [\text{analyte}]/[\text{reference analyte}]$) may yield a better precision and may be used in accordance with the invention.

By an additional aspect of the invention, a kit is provided for determining the level of an analyte in the blood of a tested individual comprising:

- 5 (i) a collecting member obtaining a water specimen from skin of said individual;
- (ii) a measurer measuring the level of the tested analyte in the obtained sample;
- (iii) a measurer measuring the level of a reference analyte in the sample;

10 said collecting member may be a container in which the water is collected directly from the individual's skin or a solid surface (e.g. plastic or glass) from which the water is collected into a container.

Optionally, the kit may also comprise a calculator calculating the level of said tested analyte in the blood of the tested individual on the basis of the
15 measurements obtained in (ii) and (iii) above.

The above kit may further comprise a test strip incorporating the reagents or structures necessary to carry out the measurement of the tested analyte as well as the reference analyte. In such a case, an instrument into which the test strip can be inserted or to which the test strip may be connected, is also included in the kit.
20 Such an instrument, which may be portable, is capable of detecting and analyzing the signal emitted by the test strips and optionally may translate them directly into prevalent units.

EXAMPLES

The invention will now be demonstrated by way of the following non-limiting examples.

5 Example 1: Reagents and method for luminometric determination of glucosePreparation of luminol concentrate

10 The following mixture was prepared in a disposable polystyrene test tube:
100 μ L of Sodium Luminol stock solution of 40mg/mL H₂O
14.6 μ L of p-iodophenol stock solution of 100mg/mL DMSO
1886 μ L H₂O
The mixture was stored at 4°C until use.

15

Preparation of luminometric enzyme mix

The following mixture was prepared in a disposable polypropylene test tube:

20 0.6 mL Glucose Oxidase stock of 4400U/mL H₂O
1.2 mL Horseradish Peroxidase stock of 1100U/mL H₂O
0.6 mL 0.5M Tris-HCl, pH 8.5
2.6 mL isotonic Sodium Fluoride solution (1.75%w/v in H₂O)
625 μ L 20% Bovine Serum Albumin (Cohn's Fraction V) in isotonic
25 Sodium Fluoride solution
625 μ L Luminol concentrate

For test: 5 μ L sample was placed in the bottom of a Microfuge tube. The tube was inserted into the counting chamber of a Labsystems Luminoskan
30 TL Plus. 45 μ L of the luminometric mix was added, the lid of the counting chamber was lowered into closed position and counting proceeded for 1 second.

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Example 2: Comparison between calcium and magnesium as reference analytes in the determination of blood glucose in skin extract

1 cm² pieces of polyester mesh (Polymon PES 180/31, Sefar, Rüschnikon,
5 Switzerland) were washed extensively in distilled water and dried at 70°C.
Fifty µL aliquots of distilled water were placed on the skin at different locations
of the arm. A 1cm² piece of polyester mesh was placed over each drop of water
and facilitated its spreading. Following 30 seconds, the piece of mesh was
centrifuged briefly at 10,000xg to extract the fluid. The glucose in the fluid was
10 tested by luminometry, as described in Example 1. Magnesium was tested by the
Mg-Kit and calcium ion was tested with the Ca-Kit, both kits obtained from
bioMérieux, Marcy l'Etoile, France. The skin specimen collection was
conducted under fasting conditions, so as to ensure constant blood glucose
concentration. Before calcium or magnesium determination, the pH of the
15 specimen was determined employing pH test strips of the fixed color type (Sigma
Chemical Co., St Louis, MO, USA). In all cases the pH was not more than 6.0,
thus ensuring that the cations are not complexed.

The results of the measurements are presented in Table 1 below utilizing
the following units:

20 glucose – counts per second
calcium and magnesium – OD x 1000

The glucose measurement is corrected by the electrolyte level, which
should reflect the volume of blood or interstitial fluid. By calculating the simple
25 ratios of glucose to electrolyte the precision was not acceptable (57 – 73%).
However, a logarithmic transformation of the glucose measurement (using the
formula: $1000 \times \text{Log}_{10}[\text{glucose}] / [\text{electrolyte}]$) yielded a good 13% precision,
when the Calcium level is employed for correction. Employing the magnesium
concentration for correction did not yield an acceptable precision.

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Table 1

| Specimen ID | Raw Data | | | net | | | Ratios | | Log glu ratios | |
|----------------|----------|------|-----|-------|-----|-----|--------|------|----------------|-----|
| | Glu | Mg | Ca | Glu | Mg | Ca | Mg | Ca | Mg | Ca |
| Reaction blank | 788 | 835 | 292 | -2985 | 0 | 0 | | | | |
| 1 | 23701 | 871 | 328 | 19928 | 36 | 36 | 554 | 554 | 119 | 119 |
| 2 | 55599 | 874 | 342 | 51826 | 59 | 50 | 886 | 1037 | 81 | 94 |
| 3 | 8130 | 854 | 320 | 4357 | 19 | 28 | 229 | 156 | 192 | 130 |
| 4 | 15045 | 851 | 325 | 11272 | 32 | 33 | 352 | 342 | 127 | 123 |
| Mesh Blank | 3773 | 837 | 294 | 0 | 2 | 2 | 0 | 0 | | |
| Average: | | | | | | | 505 | 522 | 130 | 117 |
| Precision (%): | | | | | | | 57 | 73 | 36 | 13 |
| Glu 0.1mg/dL | 68503 | | | 67715 | | | | | | |
| Ca 100mg/L | | | 696 | | | 404 | | | | |
| Mg 25mg/L | | 1165 | | | 330 | | | | | |

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Example 3 Effect of mesh on analyte levels in skin water extract

The significance of mesh in extracting glucose and calcium from the skin was subject to the following test.

For fingertip sampling, one hundred μL drops of distilled water were placed on a flat piece of sterile polystyrene (the lid of a 24-well tissue culture dish, Corning Glass Works, Corning, NJ, USA). In case of "No-mesh" sampling, the fingertip was washed first with detergent and water, followed by rinsing in about 50 mL of distilled water and air-dried. The washed fingertip was placed in the drop of distilled water for 30 seconds. The remaining water on the polystyrene surface was collected by a micro-pipetter and stored in a Microfuge tube. In case of mesh sampling, a 1cm^2 piece of polyester mesh was placed over the drop of water (on the polystyrene surface) and the washed fingertip was placed on the mesh for 30 seconds. Following 30 seconds, the piece of mesh was centrifuged briefly at 10,000xg to extract the fluid.

For hand sampling, the palm of the hand was washed first with detergent and water, followed by rinsing in about 50 mL of distilled water and air-dried. A one-hundred μL aliquot of distilled water was placed on the inner skin of the

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palm of the hand. In case of "No-mesh" sampling, the water was collected by a micro-pipetter and stored in a Microfuge tube. In case of mesh sampling, a 1cm² piece of polyester mesh was placed over the drop of water and facilitated its spreading. Following 30 seconds, the piece of mesh was centrifuged briefly at
 5 10,000xg to extract the fluid

The glucose in the specimens was tested by luminometry, as described in Example 1. Calcium ion was tested with the Ca-Kit, (bioMérieux, Marcy l'Etoile, France).

A comparison of the amounts of glucose and calcium obtained from
 10 fingertip and palm skin extracts with and without mesh is shown in Table 2 below.

Table 2

| Specimen | No mesh | | With mesh | |
|----------|----------------|--------------------------------|----------------|--------------------------------|
| | Glucose cps | Ca ⁺⁺ (OD x 100) | Glucose cps | Ca ⁺⁺ (OD x 100) |
| Finger | 18246 | 28 | 114654 | 83 |
| Hand | 107697 | 130 | 222287 | 269 |

15

Standard values: 0.1 mg/dL glucose = 170807 cps;

100mg/L Ca⁺⁺=379 (OD x 100)

As seen in Table 2, employing mesh increases the level of analytes in skin
 extracts.

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Example 4 Comparison between blood and skin glucose testing in a glucose loading experiment

25

Glucose loading experiment was conducted as follows:

Following an overnight fast, the capillary blood glucose level of the test subject was determined employing the OneTouch® system (LifeScan, Inc.,

Milpitas, CA, USA). Immediately thereafter the skin glucose and calcium ion levels were determined, as described in Example 1, except that a 100 μ L aliquot of water was employed.

Upon completion of the skin specimen processing, 50 gr glucose (generic, food grade) were suspended in club soda water with fresh lemon juice and ingested immediately. Forty five minutes thereafter, both capillary blood glucose and skin glucose + calcium ion measurements were taken. A third measurement of the blood and skin analytes was conducted about an hour or more later, when it could be shown that the blood glucose level was declining.

The glucose results are presented in arbitrary units derived from dividing the luminometric glucose measurement in cps (counts per second) by the value obtained by multiplying the OD (optical density) of the calcium ion test mix. Skin measurements were taken in triplicate. Standard glucose (0.1mg/dL) and Calcium (100mg/L) solutions were tested with each skin measurement. Glucose results were adjusted to a standard measurement of 200,000cps and calcium results were adjusted to ODx100 of 400.

The results of two separate experiments are shown in Tables 3 and 4 below.

Table 3 summarizes measurement conducted on the hairless skin area of the forearm:

Table 3

| Time | Blood mg/dL | Ratio | | Notes |
|-------|----------------|--------------------|--|-----------------------|
| | | Log Glucose/ OD Ca | | |
| 8:02 | 87 | 61 \pm 10 | | Fasting |
| 9:58 | 130 | 130 \pm 10 | | 45 min past challenge |
| 10:40 | 111 | 101 \pm 17 | | |

Table 4 summarizes measurement conducted on the palm of the hand:

Table 4

| Time | Blood mg/dL | Ratio | | Notes |
|-------|----------------|-------|----------------|-----------------------|
| | | Log | Glucose/ OD Ca | |
| 8:01 | 90 | 84 | +20 | Fasting |
| 8:55 | 127 | 172 | +5 | 45 min past challenge |
| 10:50 | 99 | 155 | +22 | |

- 5 In both cases it is clear that the determination of glucose in a skin extract follows the kinetics of the capillary blood glucose.

CLAIMS:

1. A method for determining the level of an analyte in the blood of an individual by measuring the level of said analyte in an interstitial fluid or in any other non blood fluid which does not contain red blood cells and adjusting the
5 measurement value by the concentration of at least one reference analyte.
2. A method according to Claim 1, wherein no skin permeation enhancers are used.
3. A method according to Claim 1, wherein said analyte is glucose.
4. A method according to Claim 1, wherein said at least one reference analyte
10 is an electrolyte.
5. A method according to Claim 4, wherein said electrolyte is a calcium ion.
6. A method for determining the level of an analyte in the blood of an individual comprising:
 - (i) contacting the skin of said individual with water to obtain a water
15 specimen;
 - (ii) determining the level of said analyte in said water specimen;
 - (iii) determining the level of one or more reference analytes in the water specimen;
 - (iv) calculating the level of said analyte in the blood of the tested
20 individual based on the measurements in (ii) and (iii).
7. A method according to Claim 6, wherein said water contacted with the skin of said individual contains no skin permeation enhancers.
8. A method according to Claim 6, wherein said analyte is glucose.
9. A method according to Claim 6, wherein said at least one reference analyte
25 is an electrolyte.
10. A method according to Claim 9, wherein said electrolyte is a calcium ion.
11. A kit for determining the level of an analyte in the blood of a tested individual comprising:

- (i) A collecting member obtaining a water specimen from skin of said individual;
- (ii) a measurer measuring the level of the tested analyte in the obtained sample;
- 5 (iii) a measurer measuring the level of a reference analyte in the sample;

said collecting member may be a container in which the water is collected directly from the individual's skin or a solid surface from which the water is collected into a container.

10 **12.** A kit according to Claim 11, further comprising a calculator calculating the level of said tested analyte in the blood of the tested individual on the basis of the measurements obtained in (ii) and (iii) above.

13. A kit according to Claim 11, further comprising a test strip incorporating the reagents or structures necessary to carry out the measurement of the tested analyte and reference analyte.

15 **14.** A kit according to claim 11, wherein said collecting member is also said measurer.