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Non-invasive biosensors in clinical analysis

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Abstract: Several amperometric biosensors have been developed and applied for the non invasive determination of metabolites in body fluids. Advantages of saliva or sweat analysis are the ease of sample collection and that samples can be collected more frequently with much less stress on the patient. An alcohol biosensor has been developed with a hydrogen peroxide based electrode utilizing immobilized alcohol oxidase. Immobilization parameters have been optimized to increase the stability of the enzyme. An auter hydrophobic gas membrane was used to improve the selectivity of the probe. A hydrogen peroxide based amperometric biosensor has also been developed that utilized the enzyme glucose oxidase. The biosensor was applied to the determination of sera and saliva glucose content. Two hydrogen peroxide based amperometric biosensors that utilized lactate oxidase were also developed for determination of lactate in saliva and sweat. To discriminate against electroactive substances, the biosensor for assay of lactate in saliva utilized a dual electrode with one side active and one inactive, while the biosensor for assay of sweat lactate content utilized a hydrophobic hydrogen peroxide membrane to improve selectivity. Lactate content of saliva and sweat samples were measured after an intense physical exercise. A new procedure to measure glucose via transbuccal mucosa was developed using a dual glucose probe similar to that used for lactate. Correlation between glucose in blood and in transbuccal mucosa has been evaluated.

Keywords: non invasive, biosensor, enzyme immobilization, alcohol, glucose, lactate, amperometry

1. INTRODUCTION

The measurement of metabolites in media other than blood is becoming increasingly significant because of major demands for non-invasive

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analysis. Such measurements are particularly important for patients who have to control daily parameters such as glycemia and urea, for people with problems in collecting blood (hemophiliacs, neonates, elderly people, and so on).

Although many sampling sites are available for non-invasive sensing (where there is no invasion of the body to collect body fluids such as whole blood, serum, cerebrospinal fluid or urine), until now most efforts have concentrated on sweat, saliva and the human skin itself.

In this article we shall discuss research carried out at our laboratories in New Orleans and Rome into the development of non-invasive sensors.

2. MEASUREMENT IN SALIVA

2.1. General measurements

Whole saliva is a complex mixture of parotid, submandibular, sublingual, and minor gland secretions, together with bacteria, leukocytes, sloughed epithelial cells, and crevicular fluid. The use of different stimulants of saliva secretion produces samples where the secretions from the major salivary glands occur in different proportions. The concentration of most salivary constituents depends on the flow rate of saliva. Therefore, to obtain meaningful results, the collection of saliva needs to be standardized.

Metabolite measurement in saliva is complicated by the presence of bacteria, epithelial cells and leukocytes. An additional complication is that as a result of bacterial action, the composition of saliva changes on standing. In traditional methods of analysis, saliva is therefore collected on ice to arrest the bacterial metabolism. Centrifugation stops the bacterial action and removes both the cells and the turbidity, which can interfere with many analytical techniques. Such clarification, however, may decrease the levels of certain salivary parameters.

Fifteen years ago, Horning *et al.* [1] discussed the use of saliva as a sample site in therapeutic drug monitoring. Drug monitoring is particularly useful in the management of patients for chronic therapy, especially if the therapeutic concentration range of the drug involved is narrow. Although blood is most commonly used for sampling, saliva may be preferable: the concentration of most drugs in saliva corresponds to the free or unbound plasma drug concentration, and for the consideration of pharmacological activity or toxicity, this is a more meaningful value than a value which reflects both the bound and unbound drug. Furthermore, saliva can be obtained by non-invasive techniques; this is helpful in cases where multiple serial samples are needed and in the monitoring of children.

Earlier studies demonstrated that most therapeutic agents are transferred rapidly from plasma to saliva; more recently it was shown that the concentration of drugs in saliva is proportional to that in plasma. The view that the salivary concentration of most drugs reflects the unbound drug concentration in plasma is now accepted [2]. Lithium is the most well-known example of a drug that is actively secreted in saliva and can be determined with a good serum/plasma correlation. For other drugs, for example digoxin, a substantial interindividual variation in the S/P ratio is found, which raises questions about the use of saliva for monitoring [2].

Salivary glucose does not serve as a reliable indicator of blood sugar, even though diabetic patients show elevated salivary glucose levels [3]. Microorganisms present in whole saliva utilize glucose rapidly. The whole saliva glucose levels decrease, even when saliva is collected on ice and kept chilled after collection. A convenient way to stop the bacterial metabolism after saliva collection appears to be acid precipitation, which is included in common blood glucose determinations.

Metabolism occurring rapidly in collected saliva also affects other assays. For example, the ammonia content of saliva is known to rise after standing because of the metabolism of urea and amino acids [3]. In whole saliva, the ammonia content increases by about 10%, even when stored at +4°C. Not surprisingly, the increase is greater at +20°C. The ammonia formation duration storage can be inhibited to some extent by diluting the sample before storage or by adding chloroform; acidification has no effect on ammonia formation [3]. The ammonia and urea content of saliva stored at −20°C remains unchanged for the first two weeks. Prolonged storage of saliva at −20°C results in a decrease in the ammonia content, whereas it tends to rise in frozen blood and urine. Thus, for the analysis of metabolites such as ammonia, saliva should be collected in chilled tubes and analyzed immediately after collection. If this is not possible, storage at −20°C is necessary.

Tables of metabolites, electrolytes, enzymes and therapeutic drugs monitored in saliva can be found in many references [3, 4, 5]. For metabolites such as alcohol and lactate, there are excellent correlations for their respective concentrations in saliva and blood serum and these will be discussed further. Some metabolites, like cholesterol, can be determined in saliva as well as whole blood, but exist at very low levels that make analysis difficult. Schwertner *et al.* [6] have described a new electron-capture gas-chromatographic procedure for measuring cholesterol in the nanogram and sub-nanogram range. The method is based on the derivatization of cholesterol with pentafluorobenzoyl chloride and analysis by electron-capture gas chromatography. In this study, both pentafluorobenzoyl chloride and trifluoroacetic anhydride were evaluated as derivatizing reagents. In addition, the lower limits of detection achievable with the electron-capture detector were compared with those obtained with a flame-ionization, and the use of this method was demonstrated in the analysis of cholesterol in physiological fluids such as urine and saliva.

2.2 Measurements of alcohol in human saliva

2.2.1 Introduction

The determination of alcohol concentration is probably one of the most frequently performed tests in legal cases, where it is used to establish the degree of alcohol intoxication, one of the major causes of traffic accidents [7]. Impairment of driving ability can be caused by concentrations of ethanol as low as 50 mg per 100 ml of blood [7]. Thus measurements of ethanol in blood, urine and saliva are of great practical importance for forensic purposes. Proof of one's blood alcohol concentration (BAC) is admissible evidence in simple operating under the influence (OUI) cases, in motor vehicle homicide and manslaughter cases and in serious accident and death cases.

Alcohol analysis is also carried out in industry for process control in fermentation, and in clinical laboratories. Blood alcohol concentration is currently measured using breath analyzers [8], gas chromatography [9], and enzyme catalyzed methods on automated analyzers or spectrophotometers [10]. The first of these methods, although suitable for routine police screening, is too inaccurate for medical use due to interference from other organic solvents and the widely varying ratios of blood to breath alcohol levels [11]. To translate breath alcohol levels to blood alcohol levels, a conversion factor of 2100:1 is used, on the assumption that 2100 ml of deep lung breath has the same weight of ethanol as 1 ml of blood when the two phases are in equilibrium at normal body temperature. This is true, however, for only a relatively small portion of the population. The value of 2100:1 is actually a compromise achieved by a committee that examined the results of more than 25 studies in which averages ranging from 1142:1 to 3478:1 were reported [11, 12].

Jones [13] has done extensive studies on the inter- and intra-individual components of variation in the saliva/blood alcohol ratio. In the experiments 48 male subjects were examined after they drank 0.72 g of ethanol per kilogram of body weight as neat whisky following a short fast [13]. Saliva and blood ethanol profiles were monitored at 30 to 60 min intervals for up to 7 h after intake. The analytical component of variation, inherent in an estimate of the saliva/blood alcohol ratio and expressed as the coefficient of variation, was 1.75%. The calculated saliva/blood ethanol ratios for each subject at each sampling time were reported by taking the antilogarithm of the difference (log saliva alcohol minus log blood alcohol). The mean ratio between 60 and 360 min after drinking was 1.077 ($n = 336$) with 95% confidence limits of 1.065 and 1.088. Moreover, the individual ratios showed no systematic variation throughout the absorption, distribution and elimination phases of ethanol metabolism. Thus, Jones established a sound basis for non-invasive saliva testing for blood, alcohol content.

Life Scan Corporation [14], based in Mountain View, CA, has commercialized the Alcosan™ saliva alcohol dipstick for the semi-quantitative estimation of alcohol levels. This stick incorporates a pad of paper, which contains alcohol oxidase, peroxidase, and a peroxidase-dependent colour generating system. In operation, a drop of saliva is placed on the paper, the excess is blotted off, and the stick is returned to the foil wrapper to allow the oxidation reaction to proceed to completion. The product suffers from two serious drawbacks: the method is semi-quantitative, discriminating between only three alcohol levels; one mole of dye is generated for every mole of alcohol initially present. The latter property is a drawback because it limits the range of the stick to a maximum of 0.1% alcohol, as

the human eye is unable to distinguish colours of greater intensity than those generated at this alcohol concentration. The device also depends upon the ability of a pad of paper to reproducibly hold a set amount of solution.

The alcohol concentration in the blood stream determines the concentration of alcohol in other body tissues and fluids. As a result, the concentration of alcohol in these tissues or fluids is a function of the concentration in the bloodstream. Several studies published in the literature have demonstrated a relationship between blood alcohol and the saliva alcohol concentration [13, 15].

A painless, non-invasive alcohol measurement probe has been developed herein, based on the measurement of alcohol in saliva with an enzyme-based sensor. The sensor is an amperometric hydrogen peroxide electrode that is covered with a gas permeable membrane.

2.2.2 Alcohol base sensor

The saliva alcohol device requires a specific, sensitive, reliable alcohol sensor. The two most appropriate base sensors for alcohol electrodes are the oxygen electrode and the amperometric hydrogen peroxide electrode. When these sensors are coupled with alcohol oxidase, a response to alcohol is obtained due to the following reaction:

$$\text{Alcohol} + O_2 \xrightarrow{\text{Alcohol Oxidase}} H_2O_2 + \text{Acetaldehyde}$$

The alcohol is assayed by measuring the decrease in oxygen with an oxygen electrode or by measuring the increase in hydrogen peroxide with a hydrogen peroxide electrode. Either system can be used to measure the initial rate of reaction within the first 12 s, or the steady state-current at 1 to 2 min after the injection of ethanol. Because of the potential difference in the oxygen level between the atmosphere and testing matrices (blood, saliva, urine), it was realized that the hydrogen peroxide system may well give the best results. However, both systems were investigated in development of the base sensor because the oxygen measurement system is known to be more selective, and would eliminate electroactive interferences that are a problem in the peroxide based probe.

2.2.3 Assays in saliva

The response to ethanol in saliva was measured using steady-state and rate methods. The applied potential was set to +0.650 V vs Ag/AgCl. The electrode was rinsed with deionized water and placed in 10 ml of PBS until a steady base line was achieved. The electrode was then removed from the buffer and excess liquid was removed with tissue paper. 30 μl of the sample was injected onto the tip of a 2 cm diameter Teflon rod, forming a droplet. The electrode was lowered over the rod until it was just touching the droplet, forming a 5 mm diameter contact area. The maximum rate of response was calculated from the peak height of the first derivative curve. The steady-state response was calculated by subtracting the residual current from the steady-state current, which was obtained within 1 or 2 mins.

Saliva ethanol concentration was calculated from the calibration curves; the subsequent plots are shown in Fig. 1 and Fig. 2 for two subjects as concentration versus time after ingestion. The alcohol metabolism curve obtained through analysis of saliva alcohol content resembled closely that reported by many investigators for blood alcohol content [16]: an increase in alcohol concentration in body fluids during the absorption phase and a maximum peak level, followed by a decrease, denoting the elimination of alcohol from the body fluids.

There was a good correlation between the saliva ethanol content recorded by the amperometric and the spectrophotometric methods. Cor-

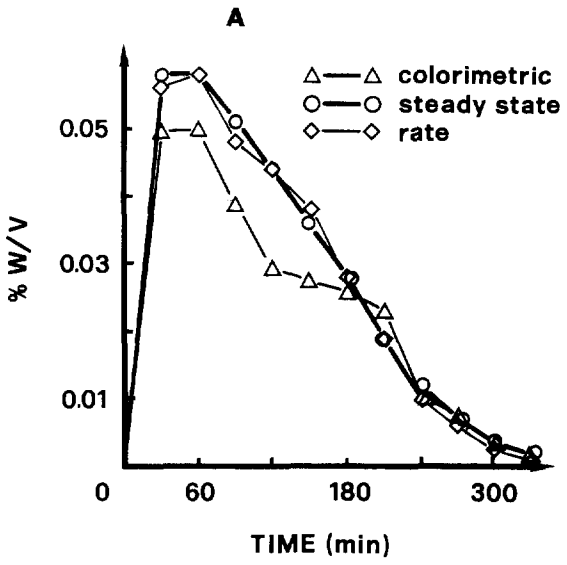

Fig. 1. Ethanol metabolism curve is shown for subject A through measurement of saliva alcohol content. (○) = steady-state, (◇) = rate, (△) = Colorimetric method.

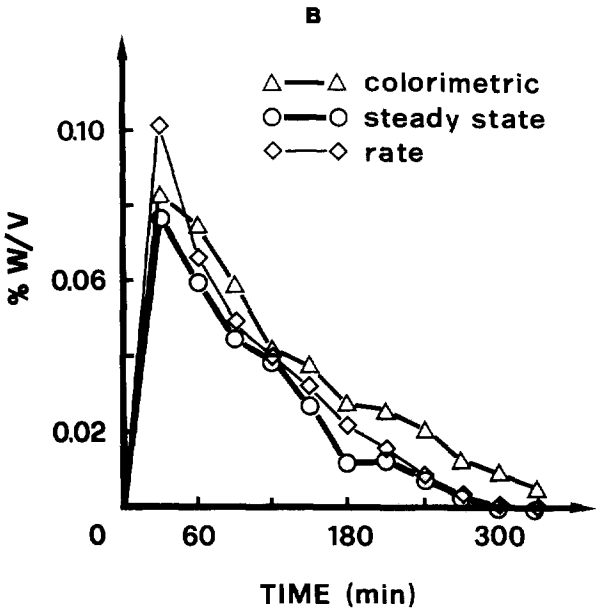

Fig. 2. Ethanol metabolism curve is shown for subject B through measurement of saliva alcohol content. (○) = steady state current, (◇) = rate and (△) = Colorimetric method.

relation plots of ethanol concentration are given for the biosensor steady-state method versus the colorimetric methods (Fig. 3), the biosensor rate method versus the colorimetric method (Fig. 4) and the biosensor rate method versus the biosensor steady-state method (Fig. 5).

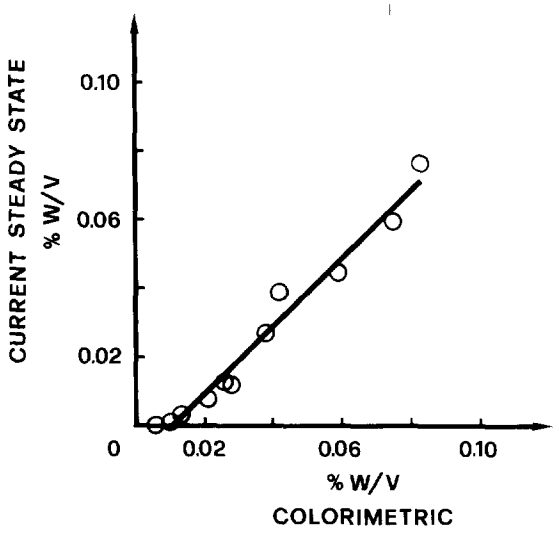

Fig. 3. Correlation between saliva ethanol content measured by steady-state current vs the colorimetric method.

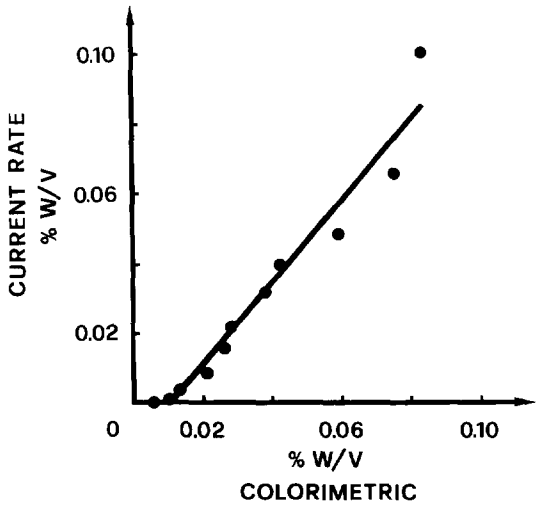

Fig. 4. Correlation curve of saliva ethanol content measured by biosensor rate method vs the colorimetric method.

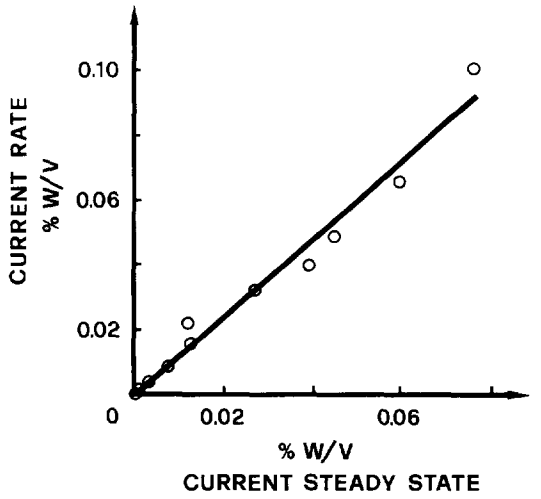

Fig. 5. Correlation curve of saliva ethanol content measured by biosensor steady-state current vs the biosensor rate method.

2.2.4 Conclusion

An amperometric alcohol sensor that shows a high degree of selectivity has been developed. The biosensor utilizes a hydrophobic membrane that enhances the linear range of the probe by slowing down the diffusion of alcohol to the enzymatic membrane. The extended linearity allows analysis of undiluted saliva samples.

The probe's response was compared with that of a reference method and a good correlation was observed between the two. The validity of

saliva as a good matrix for the detection of alcohol has been shown by many researchers, who also observed a constant saliva/blood alcohol content. Therefore blood alcohol content can be calculated from an analysis of saliva samples. The method is non-invasive; sample collection would be uncomplicated and could be carried out frequently causing little stress to the patient. A special emphasis should be placed on the sample collection technique: the variation in the sample collection during assay may cause an error in the measurements, although this can be prevented by standardization of the collection method.

2.3 Measurement of lactate in human saliva

2.3.1 Introduction

Our success with the alcohol biosensor led us to investigate the possibility of measuring lactic acid in saliva. A bibliographic search gave evidence of very scarce information on this subject. According to a biological handbook [17] lactic acid in human saliva should be around 0.2 mM. Lactate is an important metabolite that needs to be monitored rapidly in people who are sick enough to be in critical care units in hospitals, mainly to prevent heart attacks. Lactate monitors are also used in diabetes control, food analysis and sport medicine to help athletes tailor their training [18–20]. It has already been demonstrated that lactate in blood increases after meals [18, 21] and during physical exercise [22–26].

An electrochemical lactate probe operates in the following way:

$$\text{Lactate} + O_2 + H_2O \longrightarrow \text{Pyruvate} + H_2O_2$$

This reaction is catalyzed by the enzyme lactate oxidase. The hydrogen peroxide produced is measured at +650 mV with Pt and Ag/AgCl electrodes. The current change due to the oxidation of hydrogen peroxide is proportional to the lactate concentration in the sample.

This paper reports a lactate sensor that consists of dual platinum electrodes and a common Ag/AgCl reference electrode assembled so that one platinum electrode is an active lactate sensor and the other is inactive to lactate. In this way, changes in background current due to pH, temperature and ionic strength variations, together with current changes due to possible interferences affecting both sensors, could be evluated and eliminated.

2.3.2 Saliva lactate probe

The saliva lactate probe (Fig. 6) was assembled in the following way. A 100 MWCO cellulose acetate membrane was placed on the electrode surface such that the platinum and reference electrodes were completely covered. The enzyme membrane was then cut into two pieces. One piece was placed on one of the platinum electrodes and the other was immersed in boiling distilled water for 1 min, and then placed on the other platinum electrode. Finally, the probe was covered with a 0.03 μm polycarbonate membrane, which was held firmly in place with an O-ring.

For measurements in saliva, the probe was equilibrated in 4 ml of stirred phosphate buffered saline (PBS); 1 ml of freshly collected saliva was then injected and the current change for both electrodes recorded. This dual electrode did not have an internal filling solution, so the electrolytic film on its surface was due to the bulk solution. The PBS used had the same chloride concentration as blood, and because chloride in saliva is of approximately the same concentration, the reference potential was not affected by variations in chloride concentration. The reference lactate analysis was an enzymatic spectrophotometric method at 340 nm, according to Sigma procedure No. 826–UV.

2.3.3 Saliva samples

Saliva samples were collected, without salivary stimulation, from colleagues and students working in the laboratory. The fasting saliva lactate concentration should be ca. 0.2 mM. Saliva

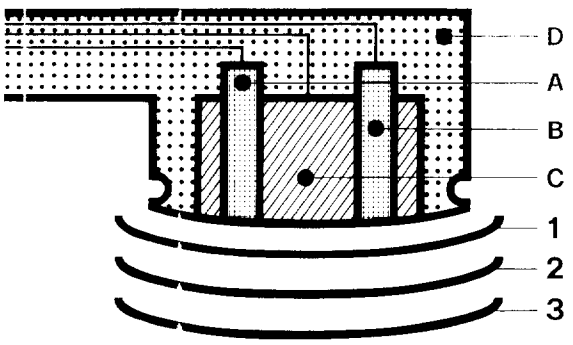

Fig. 6. Dual lactate electrode scheme and assembly. D = Plexiglas; A and B = platinum electrodes; C = silver/silver-chloride electrode; 1 = cellulose acetate membrane (100 MWCO); 2 = enzyme membrane; 3 = polycarbonate membrane.

lactate was measured by injecting 1 ml of saliva, freshly collected from a subject in the fasting state, into 4 ml of PBS. The saliva lactate response (1.8 nA) was calculated by subtracting the inactive electrode response from the active electrode response. The response corresponded to 0.21 mM lactate in the saliva sample, which is in agreement with the value reported in the literature [19]. The saliva lactate concentration was calculated from the electrode response, electrode sensitivity and dilution factors, which were 1.8 nA, 0.1 mM/4.2 nA and 5, respectively.

The main electrochemical interferents present in saliva were studied to establish their effect on the current background. Ascorbic acid and uric acid gave no current variation when injected at concentrations one and ten times higher than that present in saliva. Hydrogen peroxide (0.1 mM) gave a current variation of 6 nA over 1.5 min at the active lactate probe and 8 nA over less than 1 min at the inactive probe. The lower response at the active electrode was presumably due to catalase present as an impurity in the enzyme preparation. The catalase consumed the hydrogen peroxide at the active electrode, whereas at the inactive electrode all the hydrogen peroxide was "free" to diffuse to the platinum electrode.

As mentioned above, the lactate probe does not have an internal filling solution, so the electrolytic film reflects the bulk solution composition and, provided that the chloride concentration and the ionic strength remain constant, the background current should not change. When saliva is injected, the composition of the electrolytic film formed under the surface of each electrode changes and, hence, so does the background current. This was demonstrated by measuring saliva samples with probes that were assembled without enzyme. Both electrodes displayed the same background current variations upon saliva injections; further, the background current increased with an increase in the proportion of saliva to buffer. This increase in background current is mainly due to the relatively high concentration of ammonium ion present in saliva (1–7 mM). Ammonium chloride standard solutions were injected into buffer to obtain this concentration range. The background current variation for both electrodes was 40–70% of that obtained with whole saliva.

Attempts were made to observe possible lactate variations in the saliva of eight subjects working in the laboratory, under fasting conditions and after meals. The results are reported in Table 1. The lactate level in saliva varied widely between subjects and the level after a meal increased randomly for all the subjects.

The measurement of saliva samples was compared using two different methods. Five samples were collected from subjects in the fasting state and three samples from subjects 30 min after eating. Lactate was measured immediately after sample collection with the electrochemical method and with the spectrophotometric method. A comparison of the results is given in Table 2. Samples taken after a meal displayed the largest difference. Probe results obtained from "clear" saliva in fasting subjects were in close agreement with the results given by the spectrophotometric procedure. The lactate correlation obtained from five saliva samples from subjects under fasting conditions using the amperometric and spectrophotometric procedures gave the equation $y = 1.096x - 0.039$, with a correlation coefficient of $r = 0.962$. The x and y axes represent the lactate concentration measured, by the two methods, spectrophotometric and amperometric, respectively. If the three samples collected after meals were included, the equation would have changed drastically to $y = 0.57x + 0.157$ with $r = 0.91$. This demonstrates clearly the importance of saliva collection and how the interferents present in saliva can affect the spectrophotometric procedure: the presence of particulates or turbidity in saliva samples did not affect the electrode response but increased the response observed spectrophotometrically.

Another study performed using the dual lactate

TABLE 1 Saliva lactate measurements for eight subjects before and after eating.

Subject No.	Lactate ($mmol.l^{-1}$)	
	Fasting	After meal
1	0.20	0.56
2	0.34	0.50
3	0.70	0.76
4	0.06	0.50
5	0.50	1.20
6	0.40	0.44
7	0.32	0.56
8	0.36	0.40

TABLE 2 Comparison of saliva lactate contents measured by amperometric and spectrophotometric methods.

Sample No.*	Lactate concentration ($mmol.l^{-1}$)		
	Spectrophotometric	Amperometric	Difference
1	1.20	0.85	−0.35
2	0.12	0.09	−0.03
3	0.76	0.88	0.12
4	0.60	0.64	0.04
5	0.75	0.84	0.09
6	2.50*	1.50	−1.00
7	1.00*	0.48	−0.62
8	0.60	0.42	−0.18

*Samples 1, 6 and 7 were collected after a meal.
**Samples 6 and 7 when collected displayed a brownish and yellowish colour, respectively.

probe was the determination of lactate in saliva from a subject in the fasting state before and after physical exercise. This experiment was done because of the well-known increase of lactate in blood during exercise, when a subject is in the anaerobic mode [22]. Lactate measurements by non-invasive methods would be easier and painless for athletes involved in sports competitions and for people who exercise frequently.

Lactate was measured in a subject in a resting condition until a stable current baseline was reached; the subject was then asked to run at an initial speed of 8 km/h, increasing every 5 min by 5 km/h. After 15 min the subject went into the anaerobic mode, stopped running and rested. Saliva was collected three times before the exercise, immediately after the subject stopped running (anaerobic condition) and 15, 30 and 45 min after the exercise. The results are reported in Fig. 7, which shows an increase in saliva lactate when the subject was in the anaerobic mode and a return to the base lactate level after 30 min. Blood lactate could not be measured for comparison because of the non-availability of a micro-scale lactate blood test, but the curve obtained reflects the same trend that is obtained frequently with continuous monitoring of blood lactate [21]. The lactate probe was calibrated before and after each experiment. The probe showed no decrease in sensitivity and the results generated were reproducible.

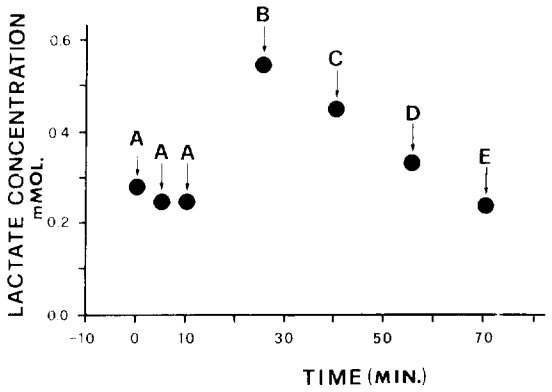

Fig. 7. Lactate acidaemic curve. A, Saliva lactate measured while the subject was in the resting state; B, C, D and E, saliva lactate measured immediately, 15, 30, 45 and 60 min, respectively, after physical exercise.

2.4 Glucose determination in human saliva

In the previous section, it was mentioned that saliva is an excellent matrix for detection of many substrates and that the number of analytes detectable in saliva are continuously increasing, as more research is carried out in this area. We investigated the applicability of saliva as a matrix for glucose detection.

Diabetes mellitus is a major metabolic disease, afflicting between 6 to 12 million people in the United States alone. Of this group, approximately 5.7 million are diagnosed as having diabetes

and those remaining are estimated to have undiagnosed diabetes. Currently, diabetes is diagnosed under abnormal conditions in either a fasting blood glucose level determination or a glucose tolerance test. After a positive diagnosis of diabetes has been established, the patient is placed on a treatment program that is designed to normalize the blood glucose level. The treatment chosen depends upon which type of diabetes the patient has. The two major primary types are known as juvenile onset diabetes (JOD), Type I, and maturity onset diabetes (MOD), Type II. These two disorders are believed to represent a gradation of the same basic disease, differing primarily in insulin insufficiency and associated metabolic abnormalities.

Type I diabetes is treated with insulin and diet control, while Type II is treated either with drugs and diet or by diet alone. Both types of treatment, however, are monitored by blood glucose level determinations, which are performed at intervals varying from several times a day for hospitalized patients to monthly intervals for non-hospitalized patients.

The aim of diabetic management is to normalize the blood glucose level. Therefore, the more frequently glucose measurements are made, the more accurately the physician is able to regulate the treatment of the patient. Accurate regulation of the blood level is essential to the health of the patient: it makes the difference between life and death.

The standard methods for measuring blood glucose levels in doctors' offices and clinics involve drawing blood from a vein in the forearm or from a finger puncture, and then measuring the glucose content of that blood using a variety of biochemical techniques. Consequently, the inconvenience, pain and cost to the patient are considerable; unfortunately this often results in infrequent testing.

Not surprisingly, it is much more desirable to measure blood glucose levels at home. Furthermore, "home" blood glucose monitoring can markedly improve control in diabetic patients, provided they receive proper guidelines, frequent teaching and adequate encouragement. A number of home methods for assessing blood glucose have become available in the past few years, and, even though they still require a blood sample to be taken, these methods are now very popular, resulting in a better control of diabetes.

There are several electronic monitoring instruments currently on the market that are designed to measure blood glucose levels at home. Most of these instruments are basically optical reflectometers that measure the colour produced on a paper strip by blood from a finger puncture. The paper strip contains an enzyme reagent system that produces the colour by reacting with glucose in the blood. The intensity of the colour is directly proportional to the amount of glucose present. The reflectometer measures this intensity and translates it into a numerical value, equivalent to milligrams of glucose per volume of blood. Typical instruments are the Ames Glucometer and the Boehringer Stattek.

The most successful and most widely used biosensor is the glucose pen – a special 13.5 cm long pencil-shaped biosensor that uses a disposable glucose oxidase sensing strip as a detector. This unit, developed by Medisense Inc., gives a reading in mg % glucose within 30 s in the form of a liquid crystal display built into the device.

A study was set up in which four subjects participated: three males and one female. The male age ranged between 27 and 39 years and they weighed between 72 and 85 Kg; the female subject was 23 years old and weighed 54 Kg. Each of the males was in good health but the female was diabetic and was periodically prescribed insulin by her physician.

All men, according to their weight, drank appropriate amounts of a sugar solution to incrrease their blood glucose level. Only men participated in the initial part of the study and, to prevent any liability, the female subject was only asked to supply saliva samples before and after insulin injections.

Saliva samples were collected in disposable plastic cups and were immediately analyzed without dilution. The analysis procedure was similar to that described above for the detection of glucose in sera samples.

Blood samples were also collected from the subject's finger tips, and were analyzed using an Ames Glucometer. There was no correlation between the saliva and blood glucose content of any of these male subjects. This was perhaps due to the effect of insulin that is released into the blood stream to normalize the blood glucose level when its concentration increases beyond a certain level. This was further investigated with the help of a subject who was diabetic. The subject was advised to collect saliva before insulin injections, when the blood glucose content was

at its highest, and afterwards, when the glucose level had dropped due to the presence of insulin. The saliva samples were collected in 5 ml vials and kept at 0°C until analysis. The subject's blood glucose levels were also registered at the times when saliva samples were collected. The saliva samples were analyzed by the glucose probe and compared to the blood glucose level. A marked increase in saliva glucose content was observed in the absence of insulin when the subject's blood glucose level was at its highest. After insulin injection, the glucose content of the saliva had dropped down on comparison with those observed in healthy individuals (Table 3). This illustrates that saliva measurements may be useful in measuring hyperglycemic patients.

The sensor, applied to glucose analysis in undiluted sera and saliva samples, already used in a previous work [27] showed very good correlation with that of the spectrophotometric method in sera measurements. Although there was no correlation between saliva and blood glucose content in healthy subjects, there was a good correlation for the hyperglycemic patient. Further studies are required to establish this correlation.

TABLE 3 Saliva glucose content of a hyperglycemic patient determined by the glucose biosensor and compared to blood glucose concentration measured colorimetrically.

	Glucose concentration (mg/dl)	
Samples	Blood	Saliva
A1	205	46.0
A2	201	44.9
A3	203	43.3
A4	211	47.6
B1	103	17.3
B2	98	16.3
B3	102	16.5
B4	105	17.9

A (1–4), Samples were collected before administration of insulin when the patient showed high blood glucose level.

B (1–4), Samples were collected after the administration of insulin when the patient's blood glucose level had decreased considerably due to the presence of insulin.

3. MEASUREMENT IN SWEAT

3.1 Introduction

The use of sweat as an analytical sample dates back almost 20 years. The analysis of sweat for electrolyte concentrations still remains the laboratory "gold standard" for the diagnosis of cystic fibrosis [28]. Here the chloride concentration in sweat provides greater discrimination between individuals with cystic fibrosis and normal individuals than does sodium [29].

An excellent correlation between the lactate concentration in sweat and that in blood was shown many years ago [4], and can form the basis of a useful analytical method. In the previous section, a dual lactate electrode was introduced that enabled analysis of saliva lactate content. However, due to the large difference between lactate concentrations in saliva and sweat at resting condition (2 mg/dl and 300 mg/dl, respectively), and due to the limited linearity of the probe developed for saliva lactate content measurement (while sufficient for use in saliva it was not ideal for measurements carried out in sweat samples), a dilution step would be necessary in the use of this biosensor that would have resulted in a decrease in sensitivity of the electrode. Hence a new probe was developed that utilized lactate oxidase by its immobilization on the novel hydrogen peroxide electrode developed by us for glucose detection. The probe performance has been thoroughly investigated and documented. [30] An electrochemical lactate probe operates according to the following reaction:

$$\text{Lactate} + O_2 + H_2O_2 \xrightarrow{\text{Lactate Oxidase}} \text{Pyruvate} + H_2O_2$$

This reaction is catalyzed by the enzyme lactate oxidase and detection is via a platinum based electrode.

3.2 Results

The effect of the following interferents on the L-lactate biosensor were investigated: ascorbic acid, acetaminophem, acetylsalicylic acid, uric acid, valine, lysine, histidine, leucine, tyrosine, threonine, arginine, sodium, chloride, calcium and potassium. The electrode was highly selective against all these compounds. For all of these

possible interferents, the electrode response was less than detectable (i.e., less than 0.01 nA) at concentrations ten times higher than that present in sweat.

The participants in the study were three males accustomed to moderate exercise, and not professional athletes. Sweat samples, 1 to 2 ml, were collected in 5 ml disposable vials, capped and either immediately assayed or kept at 0°C until assay. During each experiment, samples were collected from the upper part of the legs and arms, from the areas covering Gluteus maximas and biceps, respectively. To establish a base-line response, at least three samples were collected before exercise, while the subject was at rest. The subjects then ran at a speed of 4 to 6 miles per h for 30 min. Sweat samples were collected immediately after the run and thereafter at 5 min intervals, until the sweat lactate level returned back to normal. The biosensor was calibrated before the analysis and 20 mg/dl L-lactate controls were run throughout the study. 100 μl of each sample was pipetted into 5.00 ml of stirred PBS and the steady-state current and maximum initial rate responses were monitored. The steady-state current was measured within two minutes and the initial rate of reaction was measured in less than 10 s.

A typical plot of sweat L-lactate vs time is given in Fig. 8. All subjects showed an increase in sweat lactate after physical exercise, and the concentration decreased during the resting period. This finding is in agreement with that reported by many researchers on L-lactate metabolism after a physical exercise [20, 27, 31]. Location of sample collection is crucial and the concentration of L-lactate depends on the type of exercise. The samples collected from subjects' arms and legs showed the same trend, but the former showed much lower baseline levels and peaked at a lower maximum value.

The short term stability of sweat L-lactate was investigated by storage at 2 to 4°C for 22 h and 25°C for 5 h. The L-lactate concentration of the latter samples was periodically measured over the 5 h period. No decrease in L-lactate concentration was observed for either storage condition. This allows the samples to be collected and stored at 2 to 4°C, to be analyzed at a later time. No preservatives were added to enhance stability.

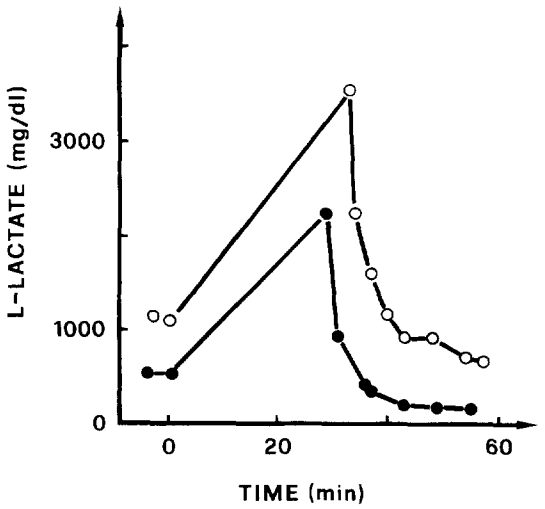

Fig. 8. L-lactate metabolism curve presented as sweat L-lactate concentration (mg/dl) vs time (min). Samples were collected from the subject's legs (○) and arms (●) before and after a physical exercise.

3.3 Conclusion

We have developed an amperometric hydrogen peroxide based L-lactate biosensor, which has shown good characteristics in terms of its selectivity, indifference to pH change and extended linearity. The biosensor was applied to the non-invasive determination of sweat L-lactate of subjects before and after physical exertion. The results were comparable with those reported by others doing the same type of experiment, but measuring blood L-lactate content.

4. TRANSCUTANEOUS AND TRANSBUCCAL MUCOSA MEASUREMENTS

4.1 Introduction

Transbuccal fluid is distinct from saliva, and is an ultrafiltrate of blood, with apparently microsolute concentrations that are similar to those of blood.

Studies performed by ourselves at Universal Sensors measuring glucose diffusion through the Transbuccal mucosa (TBM) have shown that a relationship exists between the blood glucose level and the glucose from the buccal mucosa. Xienta Inc. [32] has a patent that covers any method for performing *in vivo* measurements of a substrate, enzyme or other biochemical agent

on the skin or mucous membranes of an accessible body surface.

Transcutaneous monitoring of metabolites is not possible, due to the low solute permeability of the skin keratinized layer. Kayashima *et al.* [33] removed the top epithelial layer, and by application of negative hydrostatic pressure (400 mm Hg), were able to extract a transudate (suction effusion fluid) that served as an indirect means of assessing blood glucose during an oral glucose tolerance test.

4.2 Results

We have shown that an electrode can be fabricated for direct measurements in the TBM [34]. As shown in Fig. 9, there is a direct correlation between the response of the amperometric glucose oxidase based sensor (rate mode, curve A) with the results obtained from whole blood analysis (curve G) using the Ames glucose kit (the Glucometer). A second inactive electrode, designed to cancel out any electroactive impurities present in the TBM, showed no response in the period of the measurement (curve D). Curve C shows the changes in the steady-state. A change occurs, but is not as pronounced as in the rate mode. Excellent results were similarly obtained on a second volunteer, as shown in Fig. 10. Both the rate and steady-state modes showed a response that correlated exactly with the whole blood values. In both figures the changes in the glucose control remained constant, as expected.

The device underwent clinical trials on over 100 patients at Pharmacontrol Inc., the parent of Xienta Inc., and showed good correlations of TBM to whole blood glucose in about 50% of the patients tested. Unfortunately, this non-invasive technique showed too much variation in the remaining population to be reliable enough to enter the marketplace.

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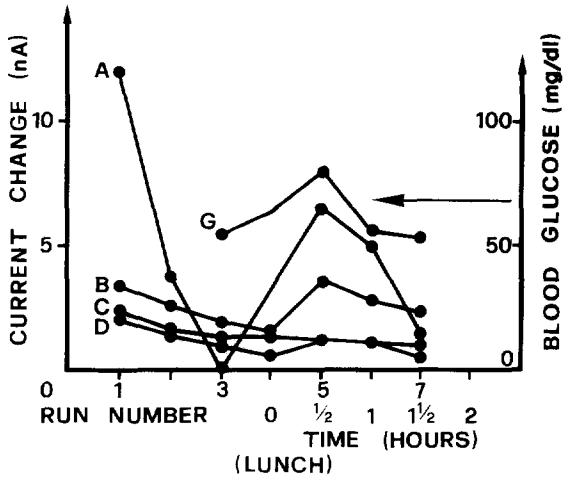

Fig. 9. Correlation study in glucose analysis by the TBM biosensor and whole blood analysis. Volunteer 1. A, Rate mode of the TBM biosensor; B, Glucose control; C, Steady-state mode of the TBM Biosensor active electrode; D, As for C, except inactive electrode; G, Glucose in whole blood (Ames Glucometer).

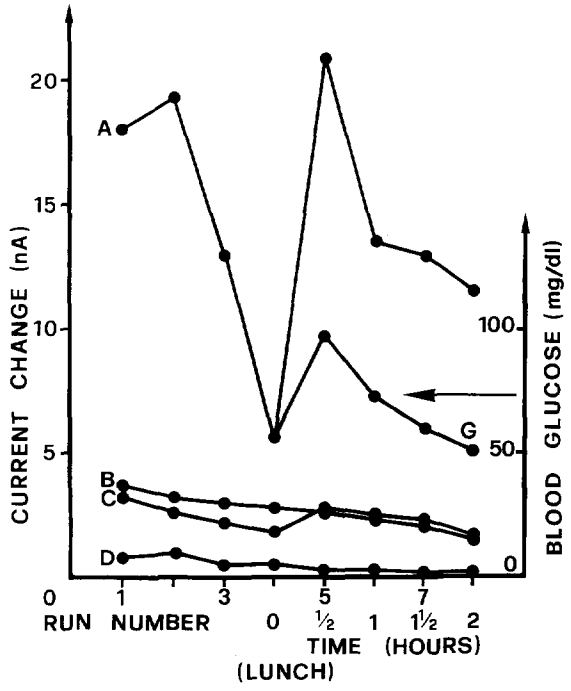

Fig. 10. Correlation study in glucose analysis by the TBM biosensor and whole blood analysis. Volunteer 2. A, Rate mode of the TBM biosensor; B, Steady-state mode of the TBM Biosensor active electrode; C, Glucose control; D, Same as B, except inactive electrode; G, Glucose in whole blood (Ames Glucometer).

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