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SPECIAL ISSUE PAPER

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Thick film biosensors for metabolites in undiluted whole blood and plasma samples

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Abstract The new electrochemical thick film biosensors from Roche Diagnostics are presented. Following considerations about the principal requirements that biosensors have to fulfil to be useful for diagnostic purposes, the basic design of these thick film biosensors is shown. In this paper, the new generation of biosensors for glucose, lactate and urea are presented, as well as data from a new biosensor for creatinine. All biosensors are designed for multiple use, at minimum 500 samples or 1 week in-use (depending on type of enzyme used), for determinations in undiluted whole blood or plasma, with extra electrodes to compensate for interferences. The sensors are integrated in a disposable cassette requiring 38 µl sample volume. The analytical ranges of the sensors scope well with the normal and pathological concentrations of metabolites in human blood, e.g. for glucose 0.5–40.0 mmol/L. Both biosensors and interference-compensating electrodes are developed to have a cycle time of 90 s maximum. Method comparison diagrams show excellent correlation of results obtained by biosensors compared to results achieved by reference methods. In addition, the possibility of urea and creatinine determinations in diluted urine is presented.

Keywords Electrochemical thick film biosensors · Diagnosis · Metabolites · Blood · Urine

Introduction

Since the pioneering work of Updike and Hicks [1], a variety of electrochemical biosensors for diagnostic purposes have been developed and described [2, 3, 4, 5, 6, 7, 8]. From a practical point of view two main groups of multiple-use biosensors have been commercialised, membrane-based sensors from YSI [9, 10], Eppendorf [11], Nova Biomedical [12, 13, 14] and Radiometer [15], as well as thick film biosensors from Bayer [16] or Roche [17]. Membrane-based biosensors have an advantage in fabrication by using prefabricated membrane materials with well known properties, but the necessary membrane change is sometimes rather cumbersome. Multiple-use thick film biosensors, on the contrary, are easy to exchange by the customer, but rather complex in production, because all layers have to be applied in a chemical way. Although some technological aspects such as screen printing have been developed for electrochemical thick film sensors for single use before, a variety of production problems still exist, often simply because of much lower production volumes of multiple-use biosensors compared to single-use biosensors. Additional reasons for developing thick-film biosensors are the possibility of miniaturisation, with the opportunity of introducing different parameters as well as several interference-compensating electrodes into one sensor housing with minimised additional costs. There is also the advantage of a maintenance-free base electrode which is renewed together with the biological part automatically.

When determining metabolites in undiluted samples with biosensors, several aspects have to be taken into consideration. First, and most prominent, the huge analytical range which should be covered by the biosensor, e.g. for lactate at minimum a range of 0.2–20 mmol/L is required. This is especially a problem for potentiometric devices because of their logarithmic response. Secondly, necessary co-substrates, such as oxygen for oxidase reactions, may be not present or present at concentrations too low to achieve linear response of the biosensor over the required range. Furthermore, matrix effects such as buffer capacity, pH or ionic strengh of the sample play a prominent role in creating failures in measuring undiluted samples. On the contrary, electrochemical interferences from uric acid or paracetamol can not be diminished by diluting the sample, because the ratio of analyte to interfering species remains unchanged by dilution. Although there are several reasons

Dedicated to Professor Dr. Rolf D. Schmid, University of Stuttgart, on the occasion of his 60th birthday

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why it is much better to detect metabolites in diluted samples, there are two reasons to do the determinations from undiluted samples: speed in whole blood and combination with blood gas analysis. With undiluted whole blood as sample material, haematocrit appears as an additional matrix component with capability to interfere [10, 13]. The main advantage of detecting metabolites in whole blood is the possible short time to result because no sample manipulations like producing serum or plasma are required. On the other hand, photometric reference methods such as the hexokinase method for glucose are based on plasma, and it is somehow sophisticated to compare whole blood and plasma results [18, 19, 20, 21, 22]. In addition, preanalytical errors become more expressed using heparinised whole blood as sample, due to glycolysis in the erythrocytes [23, 24].

Design of the thick film biosensors and working principles

Basically the Roche thick film biosensor cassette is made by polymer film screen printing on a polycarbonate substrate. Printing on plastics rather than ceramics give both, advantages in the possible design of the sensor and also restrictions to the exclusive use of low temperature curing inks. After printing the conductive tracks by a polymer-based Ag ink, respectively Ag/AgCl ink, which form also the amperometric counter and reference electrodes, respectively, carbon inks are printed to act as solid state contact for potentiometric sensors as well as conductive base [25] of the amperometric working electrode. In a next step, a carbon ink enriched with manganese dioxide [26] is printed to form the amperometric working electrode. Finally, the whole sensor is coated by an insulating ink, except the areas of the sensor spots (diameter 1.0–1.2 mm) as well as some contact area. A schematic of this biosensor array is given in Fig. 1. In this example, a sensor for glucose, lactate and urea is shown, with all corresponding interference-compensating electrodes as a so-called BSA-electrode to compensate for amperometric electrochemical interferences, and a potentiometric ammonium and potassium sensor to correct for accurate urea results. The same screen-printed base sensor can be used to measure creatinine, creatine and urea simultaneously.

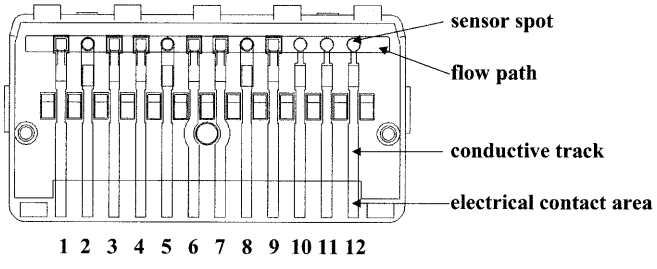

Fig. 1 Schematic of the Roche thick film biosensor platelet, showing two possible sensor combinations, one for glucose, lactate and urea and the other for creatinine and urea. Tracks: 1, 6, and 7, reference electrodes of the amperometric sensors; 3, 4, and 9, counter electrodes of the amperometric sensors; 2, working electrode for interference compensation; 5, working electrode for lactate respectively creatine; 9, working electrode for glucose or creatinine; 10, 11, and 12 are the potentiometric sensors for ammoium, potassium, and urea, respectively

Glucose and lactate sensor

The basic design and advantages of screen-printed thick film biosensors has been described elsewhere [27, 28]. A three-electrode system has been chosen by the fact that the sensor may respond with several 100 nA, making a two-electrode system not appropriate. The base sensor made from manganese dioxide in a carbon ink responds with approximately 1 $\mu A/mm^2$ to 1 mmol/L hydrogen peroxide when used without diffusional barrier. A further advantage of the manganese dioxide system is the ability of regenerating the oxygen used by the enzymatic reaction, resulting in fairly linear sensors over an analytical range much larger than required for diagnostic purposes (Figs. 2, 3). Polarisation of the manganese dioxide working electrodes can be performed at 250–450 mV, versus Ag/AgCl reference electrode, and typically 350 mV are used. This relatively low polarisation voltage allows a very good

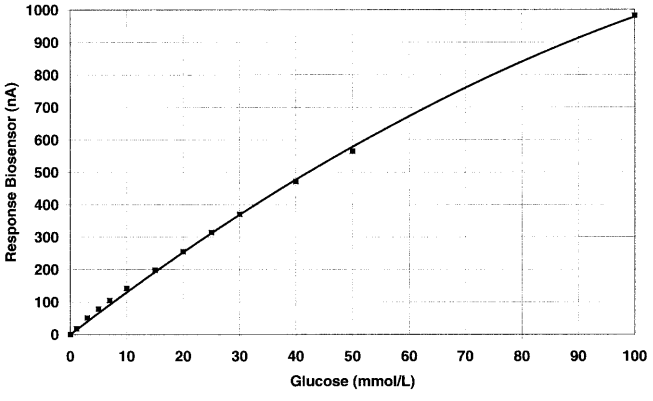

Fig. 2 Aqueous linearity of the screen-printed glucose biosensor based on a manganese dioxide electrode

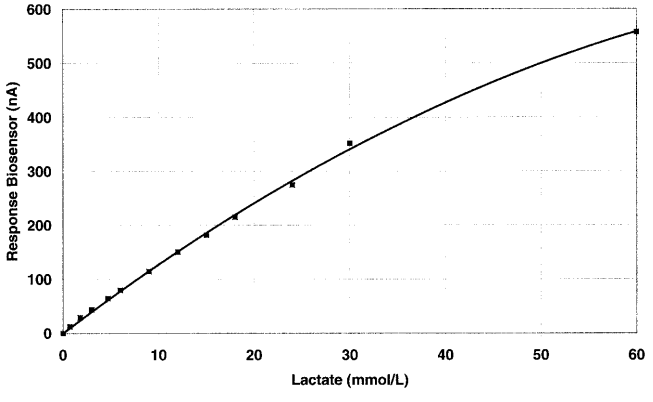

Fig. 3 Aqueous linearity of the screen-printed lactate biosensor based on a manganese dioxide electrode

Table 1 Results of an extended interference test of the polymer thick film biosensors. Interfering substances are tested at the given concentrations in a spiked certified bovine serum. Concentrations are given in mmol/L, unless stated otherwise

Sample	Concentration	pH	Glucose	Lactate	Creatinine	Urea
2 Torr[a] CO^2		8.27	–8.4%	–7.5%	18.0%	–6.8%
86 Torr CO^2		7.05	–4.9%	–9.5%	–23.1%	–7.2%
40 Torr O^2		7.36	–5.5%	–8.7%	–18.6%	–2.1%
512 Torr O^2		7.41	–3.3%	4.3%	–8.6%	–3.8%
pH 7.28		7.28	–1.1%	–4.7%	–5.8%	7.9%
pH 7.88		7.88	–2.6%	–2.9%	4.1%	–10.4%
Acetone	10.33	7.78	0.7%	2.1%	10.8%	0.2%
Acetylcysteine	0.92	7.61	1.7%	–0.4%	3.1%	–2.7%
Ampicillin	2.69	7.87	–2.9%	–2.8%	10.9%	0.7%
Ascorbate, sodium salt	0.03	7.72	–3.2%	–5.7%	0.3%	–1.3%
Acetylsalicylic acid	3.00	7.73	–7.3%	–6.9%	27.3%	2.5%
Bilirubin	0.34	7.81	–4.0%	–1.9%	–5.6%	–21.3%
Bovine albumine	7.00%	7.54	–0.6%	0.2%	5.6%	1.2%
Calcium chloride	3.00	8.18	0.6%	1.2%	14.2%	3.1%
Cefoxitin	5.56	7.94	–4.2%	–4.4%	11.5%	3.7%
Chlorpromazine	0.20	7.65	0.9%	5.1%	7.1%	–0.8%
Citrate, sodium salt	34.00	8.27	–1.7%	5.9%	2.6%	8.9%
Cyclosporin	0.004	7.91	–5.1%	–5.9%	14.9%	–2.3%
Dobesilate	0.09	7.75	2.3%	–5.6%	1.2%	14.2%
Dopamine	0.14	7.91	–3.2%	–1.7%	–1.1%	5.0%
Doxycyclin	0.10	7.55	0.0%	2.0%	–8.9%	–1.5%
Ethanol	91.17	7.80	–0.6%	0.9%	5.0%	1.9%
Gentisic acid	0.32	7.94	–5.9%	–8.7%	–16.7%	3.0%
Glutamate, sodium salt	0.86	8.21	–5.8%	–7.8%	7.2%	0.9%
Glutathion	0.33	8.03	–4.3%	–8.2%	7.6%	–4.4%
Heparin	8000 iu	7.91	–1.3%	–1.0%	6.1%	–0.4%
HEPES	20.00	7.54	–1.0%	2.2%	8.2%	4.2%
Ibuprofen	2.42	7.84	–2.6%	–1.9%	13.0%	–3.5%
Intralipid	1.00%	7.97	–3.4%	–6.6%	2.1%	–4.6%
Levodopa	0.12	7.82	–1.7%	–0.9%	–0.5%	1.3%
Lithium nitrate	3.00	8.19	0.2%	1.2%	12.8%	1.0%
Magnesium nitrate	3.00	8.20	0.6%	3.6%	14.8%	2.9%
Methyldopa	0.08	8.00	–10.0%	–8.1%	–28.4%	–9.3%
Metroinidazole	1.17	7.75	–1.6%	–1.1%	3.5%	–1.7%
Oxo-(2)-butyric acid	1.61	7.67	1.6%	2.5%	5.9%	7.1%
Paracetamol (Tylenol)	1.32	7.87	–1.9%	–3.2%	–30.0%	–0.2%
Phenylbutazone	1.30	7.87	–1.3%	0.0%	6.0%	–1.3%
Potassium thiocyanate	2.39	7.74	–1.2%	–9.8%	–3.1%	14.4%
Rifampicin	0.07	7.90	–3.5%	–2.9%	12.4%	2.3%
Salicylic acid	3.62	7.53	1.1%	–3.3%	–1.5%	0.3%
Sodium hydrogencarbonate	20.00	8.40	0.6%	2.0%	–5.6%	2.8%
Sodium hydrogenphosphate	2.00	8.16	1.1%	0.8%	12.6%	–1.5%
Teophylline acetic acid	0.42	7.86	–4.3%	–1.9%	10.4%	–2.2%
Theophyllin	1.39	7.38	6.2%	7.5%	–21.2%	4.5%
Uric acid	1.19	7.77	1.2%	–1.1%	–9.3%	–3.3%
Bovine serum mean		7.66	5.05 mM	14.9 mM	98 μM	2.63 mM
Bovine serum target		7.56	5.16 mM		106 μM	2.50 mM

[a] 1 Torr=133.322 Pa

suppression of signals from electrochemically interfering substances.

The enzyme layer, glucose oxidase for the glucose sensor and lactate oxidase for the lactate sensor, respectively, are immobilised via a self cross linking acrylate polymer [29] at room temperature. Using this immobilisation method, a good adhesion of the enzyme layer to the screen-printed manganese dioxide layer can be achieved, as well as a good immobilisation of the enzyme in the layer itself. It is assumed that both processes, matrix encapsulation and covalent enzyme immobilisation via amine groups, take place during the curing period. The enzyme in the water/acrylate prepolymer mixture is applied via a pipetting process to the working electrode, a process which can be performed automatically. In a similar way, the outermost membrane is applied as cover membrane to protect

the sensor from membrane fouling as well as acting as suppressior for certain electrochemical interfering substances. This membrane is made of a vinylacetate-vinylmaleinate-polyvinylchloride-co-polymer [30]. In addition to the sensors for glucose and lactate a third amperometric electrode is printed on the sensor platelet, identical in construction, but with bovine serum albumine (BSA) immobilised rather than the enzymes. This sensor acts as a further tool to minimise interferences by compensating all kinds of unspecific electrochemical interferences. To avoid differences in signal response due to the fabrication process, this interference compensation electrode can be calibrated by e.g. uric acid. Using all three types for eliminating interferences (polarisation at low voltage, cover membrane, and compensation electrode) nearly interference-free results can be achieved, as is shown in Table 1. The in-use stability of the lactate biosensor is limited mainly by the temperature applied in the instrument with 1–2 weeks at 37 °C, and 2–3 weeks at 25 °C. The glucose biosensor, on the contrary, has a nearly unlimited in-use stability enzymatically but membrane fouling by whole blood samples limits in-use time to approximately two months.

Urea sensor

This sensor is based on the hydrolysis of urea by urease [31] with potentiometric measurement of the ammonium ions produced by the enzymatic reaction. The base sensor is a solid state potentiometric ammonium ion sensor made of the ionophore nonactin in plasticised polyvinylchloride (PVC). The PVC is doped with amino groups (approx. 1%) to allow covalent immobilisation of the urease and acrylate prepolymer [29] onto the plasticised PVC surface. Solid state contact is made simply by dispensing the amino-PVC membrane cocktail on a printed carbon ink layer. To avoid interferences by potassium and endogenous ammonium ions, both a separate ammonium ion sensor and a potassium ion sensor, are located on the same platelet. The responses of all three potentiometric electrodes, detected via an external reference electrode, are used to calculate the urea concentration of the sample. Due to the logarithmic nature of the response, potassium ions can be compensated very well in the physiological range by using a modified Nernst equation extended by a diffusional parameter, but not at unphysiological potassium concentrations (Table 1). Although the in-use life time of the urea sensor alone is rather high (>4 weeks for a decrease of 20% in signal) the combination with screen-printed amperometric devices in the same flow channel shows detrimental effects on the urease. The sulfhydroxy-groups of the urease are easily blocked by silver ions from the amperometric counter and reference electrodes. Without further action, the resulting in-use life time would thus decrease to a few hours only. Carbon layers on the counter and reference electrodes as well as a permanent exchange of the flow-through sensor volume with uncontaminated liquid increases the in-use life time of the urea biosensor to 1–2 weeks. Further developments will include exchange of the silver-containing counter and reference electrodes by materials free of heavy metal ions.

Creatinine sensor

A variety of creatinine detecting principles were investigated, before finally the three-enzyme cascade [32] with creatininase, creatinase and sarcosine oxidase was found to be the best suited for the development of a screen-printed creatinine biosensor. Interference by creatine has to be compensated by an additional creatine sensor with creatinase and sarcosine oxidase only. To compensate for electrochemical interferences, which are much more expressed at creatinine sensors compared to glucose sensors (due to the approximately 100 times smaller normal range in biological samples), a third electrode for compensation of electrochemical interferences is required. Basically the same manganese dioxide base sensor as for glucose and lactate can be used, but the immobilisation of the enzymes has to be different. Obviously, it is quite tricky to immobilise three enzymes with different properties together. Thus a new procedure has been developed [33]. First, all three manganese dioxide working electrodes are impregnated with a droplet of sarcosine oxidase in water. This procedure leads to an intimate contact of the oxidase with the hydrogen peroxide electrode, resulting in much higher signals compared to separate membrane layers. In a second step, the creatinine sensor spot is covered by a mixture of creatininase, creatinase, glutardialdehyde and glycerol, the creatine electrode spot by a similar mixture but without creatininase. The third electrode spot is covered with BSA rather than the enzymes. The signals of all three electrodes together give a nearly interference-free result for creatinine in biological samples (Table 1). For creatinine sensors the use of cover membranes to achieve diffusional control, making the sensor response independent from the actual immobilised enzyme activity, is rather critical. One has to compromise between absolute signal height, which is rather low at physiological creatinine concentrations, and good diffusional control of the sensor. Because of that compromise an additional interference could be found, the competitive inhibition of creatinase by bicarbonate [34]. To overcome this interference, calibration reagents are used containing bicarbonate at an average physiological concentration. The creatinine sensors, described so far, without the use of any outer membrane have in-use life times of approximately 1–2 weeks, before losing half of the initial signal.

Another interesting feature of calibrating creatinine sensors is the internal conversion of creatinine to creatine in solution. Although described very early in the last century [36, 37], it has been ignored by many of the creatinine biosensor scientists in the last decades. To achieve stable liquid reagents at least three reagent parts are necessary. To calibrate the creatine sensor a solution containing the thermodynamic equilibrium of creatinine and creatine (approximately 40:60) can be used. To have a stable

Fig. 4 Picture of the fully assembled Roche thick film biosensor cassette, ready to use

reagent to calibrate the creatinine sensor, creatinine can be stored nearly creatine-free in solutions at pH around 3.0, and has to be mixed before use with an alkaline buffer to give a creatinine solution with physiological pH for calibration [35].

Biosensor cassette

Besides the polymer platelet with the screen-printed and dispensed sensor spots, the disposable sensor cassette has to be assembled as shown in Fig. 4. The upper part of the cassette, forming the flow-through channel with 38 µl internal filling volume, is fastened to the sensor platelet with a polymer gasket between both parts [38, 39]. Finally, the system has another gasket to seal the opening for liquid transport. This sensor design allows the use of many different sensors of this type in-line, e.g. the contact with the external reference electrode. Storage of these biosensor cassettes sealed in aluminium pouches dry with inert atmosphere at 4 °C is possible without loss of any signal for a minimum of six months.

Results of the thick film biosensors with undiluted biological samples

Although designed for whole blood measurements, method comparison studies have to be performed with plasma samples because of the availability of reference methods for plasma only. For glucose and lactate, it has been found that sample handling and pre-analytical errors due to glycolysis are usually more expressed than errors due to the methods themselves. Figures 5, 6 and 7 show the correlation plots of the human plasma results of the thick film biosensors for glucose, lactate and urea versus the results of a Hitachi 902. Figure 8 presents the correlation graph of the human plasma results from the thick film creatinine sensor system compared to the enzymatic method of the Roche Reflotron IV. For glucose determinations the photometric reference method with hexokinase has been used in the Hitachi.

All sensors show an excellent correlation with the reference method. There is a nearly linear relationship over the whole analytical range such as 0.5–40.0 mmol/L for glucose and urea, 0.2–20.0 mmol/L for lactate, and 20–1000 µmol/L for creatinine. One should take into consideration that these results are obtained with different sensors from different production lots and, in addition, blood results are expected to be different due to several

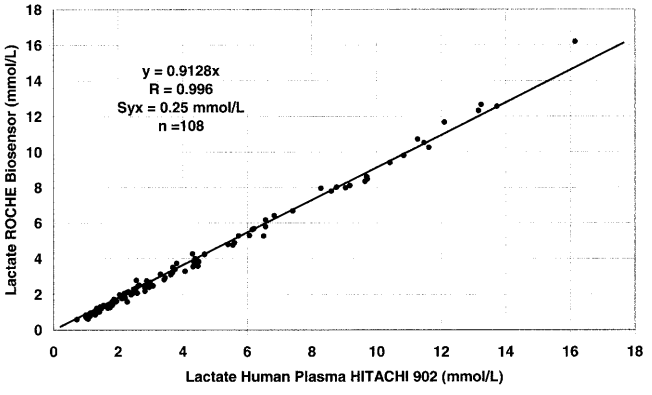

Fig. 6 Method comparison for lactate in human plasma samples

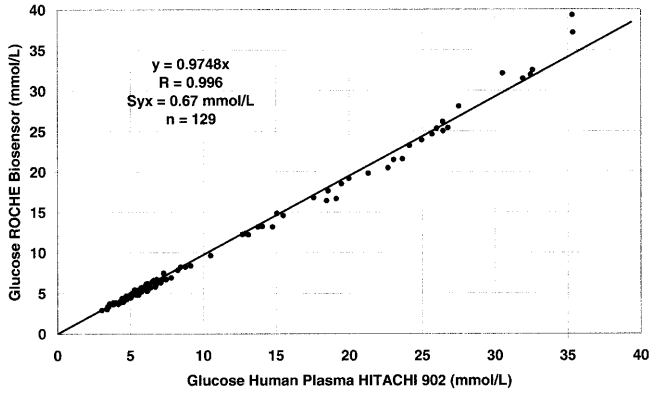

Fig. 5 Method comparison for glucose in human plasma samples

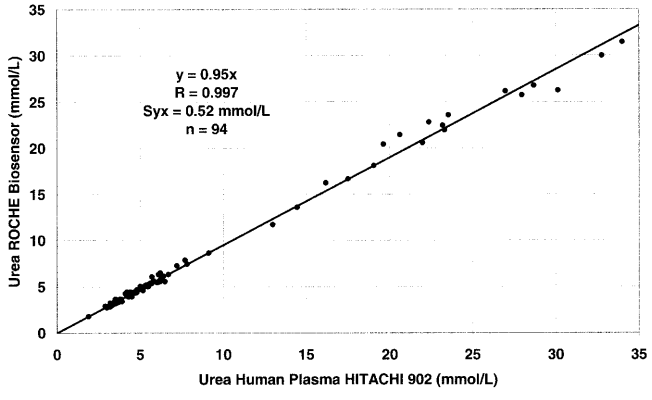

Fig. 7 Method comparison for urea in human plasma samples

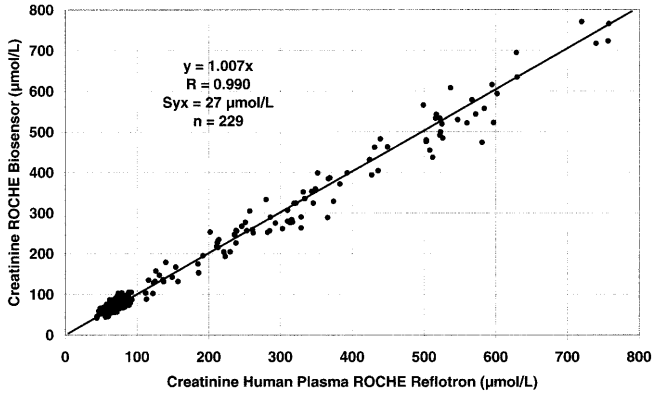

Fig. 8 Method comparison for creatinine in human plasma samples

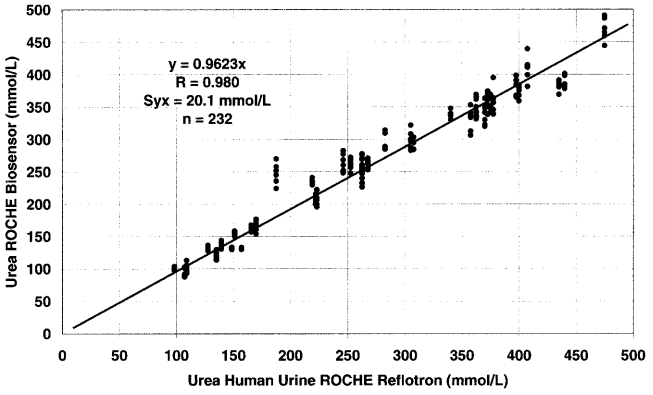

Fig. 10 Method comparison for urea in human urine samples

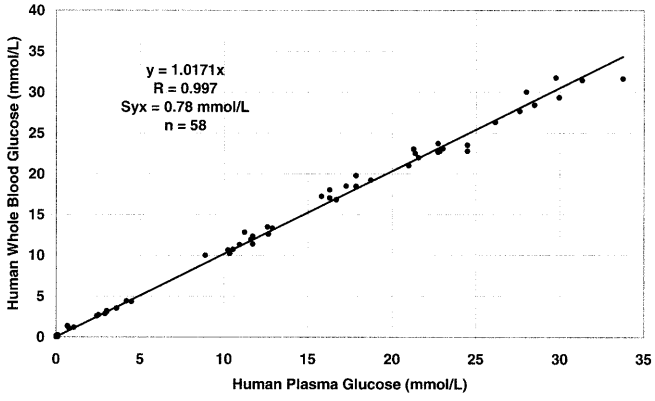

Fig. 9 Method comparison for the thick film glucose biosensor: heparinised whole blood versus human plasma results

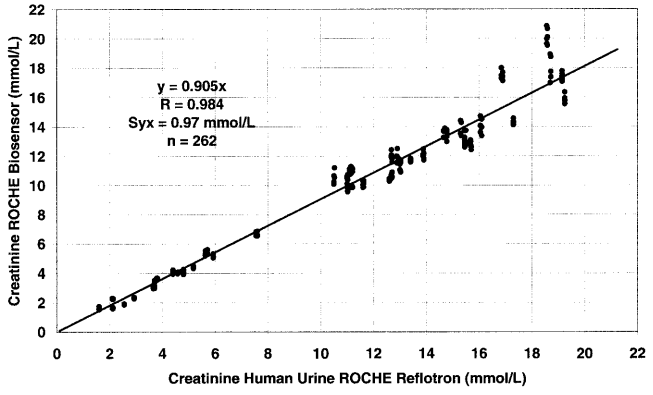

Fig. 11 Method comparison for creatinine in human urine samples

reasons [18,19, 20, 21, 22]. As an example of the possibility for using the glucose sensor both in plasma and whole blood, Fig. 9 presents a correlation study between split human whole blood and plasma samples. To achieve a signal correction of the whole blood result, the raw signal has to be recalculated with the haematocrit of the sample. Haematocrit can be measured roughly by a simple conductivity determination of the whole blood sample. Similar results have been achieved with lactate, urea, and creatinine biosensors.

Results of the thick film biosensors with diluted urine

Biosensor determinations in urine with sensors developed for plasma or whole blood measurements are somewhat difficult because of the big difference in concentration between these sample types. Creatinine in plasma is in the 100 µmol/L range, but in urine in the 10 mmol/L range. The same relation is valid for urea, leading to the necessity of diluting the urine sample before measurement. For urine this can be done easily by using a buffered physiological saline solution. The buffer substance is necessary because of the large variance of sample pH in urine. A plasma equivalent electrolyte composition is required because of the ionic interferences affecting the urea sensor. Figures 10 and 11 show the comparison of results obtained in 1:25 diluted human urine with the Roche Reflotron IV with the thick film biosensors for urea and creatinine, respectively. The correlation in both comparison plots is good. As the calibration of the biosensors to obtain these results has been done at concentrations of approx. 25 mmol/L for urea and 250 µmol/L for creatinine, these two curves show clearly the wide linear range of both sensor systems.

Conclusion

These newly developed biosensors should improve metabolite determination in biological samples with regard to minimised interference effects and maximised robustness of the system, compared to those planar biosensors using prefabricated membranes [40]. Further developments will be done to miniaturise the system: screen-printed electrode spots even in the range of 200 µm appear to be feasible, resulting in sensor arrays requiring only a few µl of blood. Because the in-use stability of the biosensor cassette is limited by the electrode spot with the shortest life-time, improvements have to be achieved to maximise in-use stability of all biosensors except glucose. Although the production yield of the existing biosensors

is rather high with 99.8% per electrode spot or >98% per fully assembled cassette, further optimisation is required if there are sensor arrays in future with many more parameters on one sensor platelet. An expansion of the menu in both directions, new metabolites such as uric acid or cholesterol, and combination with other solid state blood gas and electroyte sensors will be the goal for the future.

Acknowledgements This paper is dedicated to Prof. Rolf. D. Schmid, celebrating his 60th birthday, who headed the Institue of Enzyme Technlogy at the GBF in Braunschweig (FRG), during the author's postdoctoral studies at GBF. Prof. Schmid has established one of the biggest multinational & multicultural biosensor groups worldwide. Numerous publications as well as the famous GBF Monograph series on biosensors (edited or co-edited by Prof. Schmid) document well the major impact of Prof. Schmid's work. Several co-workers of his biosensor group have successfully developed biosensors for industry, as a result of the high level of expertise in the biosensor team at the GBF. The sensors presented within this paper are to a certain extent a result of Prof. Schmids efforts to strengthen biosensor science in Europe.

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