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Clinical analysis of urea in human blood by coupling a surface acoustic wave sensor with urease extracted from pumpkin seeds

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Abstract

A surface acoustic wave (SAW) urease sensor system was developed in which a SAW resonator oscillating at 61 MHz and a pair of parallel electrodes were utilized by coupling with urease extracted from pumpkin seeds. The Michaelis constant and the maximum reaction rate of urease were estimated as 2.08×10^{-3} mol/l and 8.85 kHz/min, respectively, at pH 7.5 and 25.0° C. The analytical characteristics of the urease sensor system, including influences of pH, temperature on frequency response, calibration curves, reproducibility, selectivity over enzymatic interferences were determined. The recovery of the sensor system ranged from 96 to 105% and the experimental detection limit of urea was 0.50 μg/ml (i.e., 8.3×10^{-6} M, $S/N = 3$). The SAW urease sensor system possesses the characteristics of good reproducibility and specificity. It has been successfully applied to the analysis of urea in human blood samples. The assay results are consistent with the results tested by conventional colorimetry and also support clinical diagnosis.

Keywords: Acoustic methods; Surface techniques; Biosensors; Blood; Urea; Pumpkin seeds

1. Introduction

Surface acoustic wave (SAW) devices exhibit great potential as small, reliable and very sensitive chemical sensors. The use of SAW devices as (bio)chemical sensors has grown exponentially in the last few years. SAW devices have been employed as chemical sensing devices since the original work of Wohltjen and Dessy was published in 1979 [1]. Since then, SAW devices have been applied to a variety of measurements, both chemical and physical [2–8]. A great deal of work has been directed towards their determination and several reviews have appeared [9–11].

SAW devices have found applications for a similar range of analytes as bulk acoustic wave (BAW) piezoelectric crystals. Wohltjen [12] has concluded that SAW devices may be superior to BAW devices in sensor applications mainly due to the fact that it is easy to fabricate SAW devices which operate at much higher (and more mass sensitivity) frequencies than non-overtone BAW devices. However, the large energy loss of the Rayleigh wave in liquid phases limits the use of SAW devices in the liquid phase [13].

Recently, a new type of SAW sensor system, utilizing a 61 MHz SAW resonator and a pair of parallel electrodes in series as a liquid-phase-based

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chemical sensor, was introduced in our laboratory [14,15]. This has opened up new exciting possibilities for SAW devices used as chemical sensors. This kind of SAW sensor system can respond to the changes in the physical properties of the solution medium between the two electrodes that will cause loop parameters variation in the oscillation circuit. It can be applied to the investigation of liquid phases. It has been shown previously [14,15] that the change in the oscillation frequency of a SAW sensor system in an electrolyte solution depends on the specific conductivity of the test solution. The frequency was shown to decrease with increasing electrolyte concentration added, depending on the increase of the specific conductivity. Frequency shifts (ΔF) were related to the change of specific conductivity (ΔK):

$$\Delta F = a\Delta K + b \quad (1)$$

where a and b are constants depending on the SAW device, the circuit used and other experimental conditions. We also found that in a medium conductivity region, the higher the conductivity, the higher is the sensitivity of the sensor. This advantage makes the sensor system more attractive than using normal a.c. conductimetry and a.c. impedance methods, compared with normal conductimetry. In addition, temperature drift of the sensor system is very small, only 10 Hz/° C in water.

Urease (EC 3.5.1.5) is an enzyme of high catalytic activity that catalyses the hydrolysis of urea. It is found especially in plant seeds and microorganisms, and shows a high degree of substrate specificity [16]. Urea is a chief nitrogenous end-product of protein catabolism in human body and other mammals. It is normally present at low concentrations in serum and urine, but its concentration changes under the conditions of liver and kidney diseases and nutritional deficiency. Though urea is traditionally a compound of interest, its role in the metabolism of living animals, plants or microorganisms remains to be elucidated. The determination of urea is important for diagnostic clinical processes and there is considerable interest in the determination of urea in clinical analysis. The need for a simple, quick and reliable method for the measurement of urea led to the enzymatic methods which utilize urease.

There are several enzymatic methods for the determination of urea based on the use of urease (i.e., amperometry [17], potentiometry [18–20] and methods using fiber-optic sensors [21] and conductimetry [22–26], enzyme electrodes [27–30] and ammonium ISFETs [31]). These methods are time-consuming, require extensive sample pretreatment and show poor precision and low sensitivity. So far, there were no reports on SAW devices applied to the urea/urease reaction and the analysis of urea in human blood.

In the urea/urease reaction system:

$$CO(NH_2)_2 + 3H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3^- + OH^- \quad (2)$$

The initially uncharged substrate (urea) is hydrolyzed to yield four charge-bearing species. When followed under suitable experimental conditions, frequency shift is associated with the products of the enzymatic reaction and gives useful information as well as an accurate determination in relation to the rate of the catalysed process according to Eq. 1. The estimation of kinetic parameters has usually been achieved by a Michaelis-Menten type analysis of the frequency response. The analysis gives apparent kinetic parameters such as Michaelis constant and maximum reaction rate.

In the present study, a new application of the SAW urease sensor system based on coupling the SAW device with urease extracted from pumpkin seeds is reported, with emphasis on optimum experimental conditions. The response to the urea/urease reaction of this sensor was reported for the first time. The kinetic parameters of the urease reaction were estimated. Influence of pH, temperature and interferents on the sensor system was also investigated. The results of the clinical analysis of urea in human blood samples were reported.

2. Experimental

2.1. Apparatus

During the course of this study, the 61-MHz one-port resonators used in this study were originally manufactured by Zhuzhou Radio Factory (Hunan), on y,z-cut $LiNbO_3$ crystals with aluminum metallization and mounted on 2-pin round TO-5 headers

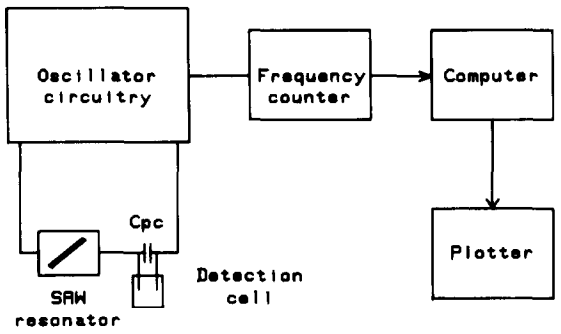

Fig. 1. Schematic diagram of the SAW urease senor system. Cpc is a parallel capacitor.

with epoxy and gold wirebonds. The aluminum interdigital transducers (IDTs) are 13000 Å in thickness. The resonators were designed with an acoustic aperture and a path length of several wavelengths. On the center of each separate $LiNbO_3$ crystal chip, there are 20 pairs of IDTs with 500 reflectors placed on each side. The nominal insertion losses of the resonators is 6.8 dB. The whole device was sealed to prevent contact with the atmosphere.

Fig. 1 shows a scheme of the SAW urease sensor system. The experimental apparatus was made of a 61 MHz SAW resonator, a home-made oscillator which had been designed to work at its resonant frequency and platinum electrodes which were placed parallel into a detection cell (its cell constant was 1.04 cm). Electrical connections between the detection cell and the oscillator board were kept as short as possible. The SAW oscillator was driven at 8 V d.c., which was supplied by a adjustable dual track d.c. power. The oscillation frequency was measured by an Iwatsu universal counter (SC7201 Model) at a resolution of 1 Hz and the data were collected at 6 points/min. The pH of the working buffer was measured by a Model EA940 Orion ion analyzer using a calibrated glass electrode and a saturated calomel electrode. A personal computer was used for data processing.

In all experiments, the detection cell was placed in an air-bath thermostating equipment (home-made) in which the temperature was monitored and controlled by a WMZK-01 temperature controller (Medical Instrument Co., Shanghai). The temperature and the stirring speed of the enzymatic reaction system were kept constant throughout in order to obtain reliable results. The volume of the detection cell was 10 ml.

2.2. Reagents

Urease (E.C.3.5.1.5, from sword, 5.0 E.U./mg), purchased from Merck, was used without further purification. Stocking standard solution of enzyme (5 mg/ml), which was used to evaluate the specific activity of urease from pumpkin seeds, was prepared in 20% glycerol solution (containing 0.02% sodium azide). Pumpkin seeds were purchased from local seed shop. Human serum albumin was obtained from Shanghai Biochemical Products Institute. Control blood plasma sample containing 10 mg/ml glucose, 2.0 mg/ml human serum albumin, 0.30 mg/ml urea, was obtained from the clinical hospital. Above reagents were stored in a refrigerator at 4° C before use. Blood samples were obtained from healthy volunteers and patients who were asked not to eat in the early morning. The samples were detected immediately without any treatment. The working buffer was 1.0 mM glycine–1.0 mM EDTA (pH 7.5). Standard urea solutions in different concentrations were prepared every week by dissolving urea in working buffer. All other chemicals used in this work were of analytical reagent grade and obtained from commercial sources. Distilled-deionized water was used throughout.

2.3. Preparation of urease extract

Urease from pumpkin seeds was extracted by a simple procedure in this work according to Ref. [32]. No further purification procedures were needed. At first, pumpkin seeds were finely ground using an electric smasher. Then 5.00 g of the powder was weighted and transferred to a 150-ml Erlenmeyer flask with a stopper. 100 ml of extracting solvent (unless otherwise noted, 20% glycerol–0.02% sodium azide was selected as the extracting solvent) was added into this vessel. The mixture was shaken for 30 min under room temperature and kept in a refrigerator at 4° C for 4 h. The solution was then centrifuged at 2000 *g* for 10 min. The urease extract was obtained after phase separation. It was stored in a brown flask and kept in a refrigerator. The crude

extract can be directly used in the measurements below. The urease concentration in this extract was estimated as 15.2 E.U./ml by the following procedure. Under the selected optimum conditions (pH 7.5, 25° C, working buffer) and at 2.0 mg/ml urea, different volumes of urease extract and commercial urease solution were used in the enzymatic reaction, respectively. Frequency response was measured vs. time and the initial reaction rate was calculated. By comparing the initial rates between commercial urease (known concentration) and urease extract, the concentration of urease extracted from pumpkin seeds can be estimated.

The experiment has shown that the activity of urease extracted from pumpkin seeds could be kept steady for about four weeks, losing only 18% of its initial activity during that time (urease extract was stored in a brown flask and kept in a refrigerator at 4° C when not in use).

2.4. Procedures

The whole experimental set-up must be turned on for at least 30 min before starting the experiment in order to guarantee stabilization. The air-bath chamber was thermostated at 25 ± 0.2° C. All experiments were conducted at 25° C. In addition, frequency shifts were collected at 1-Hz resolution. Routine noise measurements were typically determined from frequency data collected for 5 min at 6 points/min, with typical noise levels in the range of 2–25 Hz. Our sensor responses greatly exceeded the noise level. The noise was taken as the standard deviation of the residues of the linear least-squares line through the data. Usually, five such measurements were conducted consecutively. During a kinetic response experiment, baseline noise was determined using baseline frequency data collected at 6 points/min for 3 min prior to starting the enzymatic reaction.

A typical procedure was performed as follows. 9.50 ml urea solution was transferred into the detection cell. The temperature of the detection cell was thermostated at 25.0 ± 0.2° C and the solution was stirred by a magnetic stirrer. As the ionic strength varies for different samples, calculated amounts of 1.0 M NaCl solution were injected into the test solution, in such a way that, for each experimental point, a constant value of ionic strength was maintained. After a period of temperature stabilization, a steady frequency was recorded (F_1). After 0.50 ml urease extract from pumpkin was added, the enzyme catalyzed reaction was started and the oscillation frequency changed with time. Frequency (F) was recorded with time and the frequency shift (ΔF) was calculated by subtracting F from F_1.

The calibration curve was obtained by measurement of the sensor system responses to control blood plasma sample in different amounts of urea. Only 50 μl human blood sample was needed for urea measurement and the urea concentration was estimated by the calibration method.

3. Results and discussion

3.1. Optimisation of reaction medium

An important parameter to be controlled is the composition of the medium (especially the choice of buffer and its ionic strength). Therefore, it is essential to select an optimum reaction medium fitting urease activity before measuring the kinetic frequency response. Several buffers of different composition were examined. The frequency response obtained was minimum for water medium and maximum for working buffer by comparison. Therefore, working buffer (1.0 mM glycine–1.0 mM EDTA, pH 7.5) was selected for further work.

3.2. Typical kinetic frequency response

Typical responses of the SAW urease sensor to standard urea for different concentrations of urease are shown graphically in Fig. 2. The substrate urea concentration was 50 μg/ml in this experiment. It can be seen that frequency response increases with the increase of urease concentration, i.e., the reaction rate increases with the increase of the urease content. The response curve of the SAW urease sensor is analogous to the one obtained by the normal enzyme kinetic reaction [33].

3.3. Selection of extracting solvent

Fig. 3 shows the frequency response for the following four extracting solvents: 20% glycerol, 30%

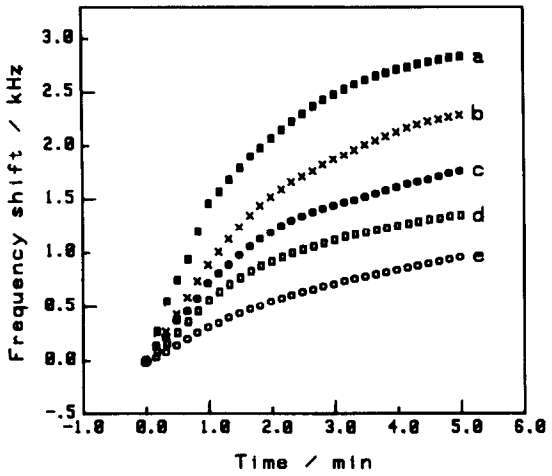

Fig. 2. Frequency response of the SAW urease sensor system for different concentrations of urease from pumpkin seeds in the reaction system: (a) 1.50, (b) 1.05, (c) 0.75, (d) 0.30, (e) 0.15 E.U/ml. 50 μg/ml urea was used in this procedure.

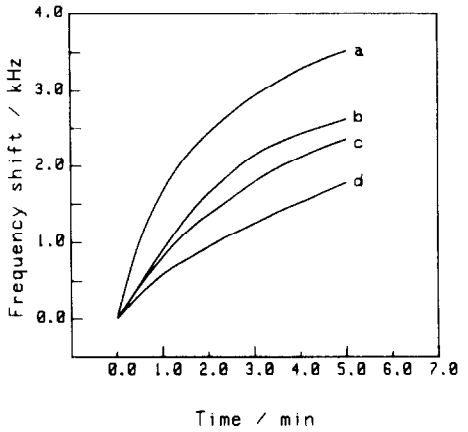

Fig. 3. Frequency responses for different kinds of extracting solvent used in the preparation of urease from pumpkin seeds: (a) 20% glycerol; (b) 30% ethanol; (c) 32% acetone; (d) water. 0.20 mg/ml urea and 0.50 ml urease extract were used in this experiment.

ethanol, 32% acetone (all v/v) and water (all containing 0.02% sodium azide). The lowest urease activity is observed for water as extracting solvent. 20% glycerol was found most favorable because of its protective effect on urease activity and was therefore selected for further investigation.

3.4. Influences of pH and temperature

The influence of pH of the sample solution on the response of the SAW urease sensor system was examined in the range of pH 3.3–9.5 at 25° C. As shown in Fig. 4, the activity of urease was maximal at pH 7.5 and at pH values > 8 the urease activity decreased rapidly. This pH optimum differs from the pH optimum of urease from jack beans (pH 6.9) [34]. This pH shift could be explained as the difference of the source of urease and the reaction medium. On the basis of these results, all subsequent measurements were carried out in a pH 7.5 test solution.

The effect of temperature on the frequency response was studied. The temperature profile for the frequency response (urease activity) is shown in Fig. 5. As can be seen, temperature evidently affects the activity of the enzyme and the sensor system yields the greatest sensitivity at 35° C. All subsequent measurements were done at 25° C by considering the measurement conditions.

3.5. Kinetic parameters

Kinetic enzymatic parameters not only depend on their sources, reaction pH and temperature but also depend on the type and concentration of buffer. For jack bean urease, the Michaelis constant K_m ranges from 1 to 5 mM, according to the Experimental conditions reported in [34,35]. In general, using an amino acid buffer (e.g., glycine) results in a greater enzymatic activity than does the simple inorganic buffer such as phosphate or carbonate. 1.0 mM glycine buffer (pH 7.5) and 25.0° C were selected for

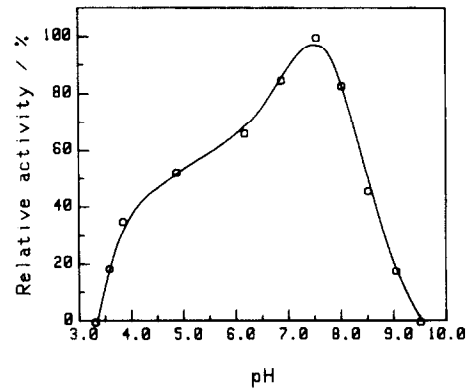

Fig. 4. Influence of pH on urease activity. 0.75 E.U./ml urease extracted from pumpkin seeds and 50 μg/ml urea were selected for this measurement.

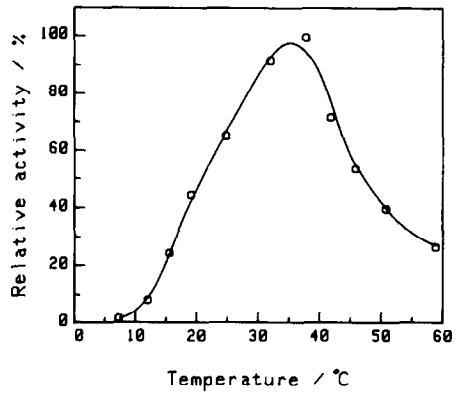

Fig. 5. Influence of temperature on urease activity. The experimental conditions were the same as in Fig. 4.

the estimation of kinetic parameters of urease extracted from pumpkin seeds. K_m and the maximum reaction rate (V_{max}) of urease were determined with the Lineweaver-Burk equation [36]. The K_m and V_{max} values for the urease extracted from pumpkin seeds were calculated corresponding to substrate (urea) concentrations ranging from 1.0 to 15 μg/ml (i.e., 1.7×10^{-5} to 2.5×10^{-4} mol/l) and their values were 2.08 mM and 8.85 kHz/min.

3.6. Calibration graphs and recovery measurements

Fig. 6 shows a relationship between the urea concentration and the frequency shift at 5 min examined using the sensor system at 25° C and pH 7.5. A

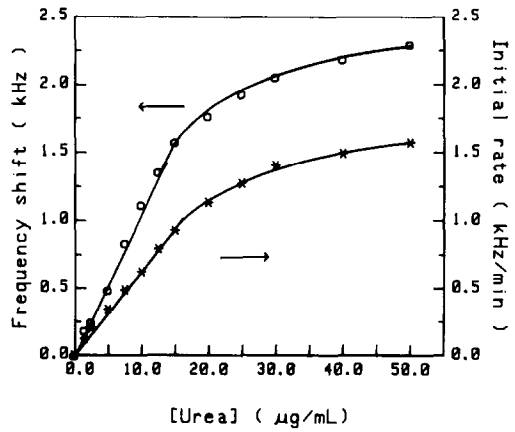

Fig. 6. Calibration graphs for the determination of urea with the SAW urease sensor system. 0.75 E.U./ml urease from pumpkin seeds was used in the urea assay.

linear relationship was obtained in the range up to 15 μg/ml urea. No detectable response was observed below 0.2 μg/ml urea compared to that of the working buffer. The calibration curve could be represented by the equation

$$\Delta F = 107.5[\text{Urea}] + 3, n = 8, r = 0.9984 \quad (3)$$

where ΔF is the frequency shift in Hz and [Urea] is urea concentration in μg/ml. The experimental detection limit of the SAW urease sensor system was 0.5 μg/ml (8.3×10^{-6} mol/l) based on three times the signal-to-noise ratio ($S/N = 3$).

As reported in Ref. [36], the initial rate (V_0) is directly proportional to the urea concentration while [Urea] $\ll K_m$ and at a constant urease concentration. So the urea concentration can be detected by using the kinetic method. A correlation of the initial rate vs. urea concentration was presented:

$$V_0 = 60.5[\text{Urea}] + 13, n = 8, r = 0.9890 \quad (4)$$

where the urea concentration is expressed in μg/ml and V_0 in Hz/min. The linearity ranges up to 15 μg/ml. The kinetic method can decrease the time of measurement (about 6 min for kinetic method and 10 min for the fixed-time method in one measurement), but it has a lower sensitivity than the fixed-time method. The difference of sensitivity can be easily found from Eqs. 3 and 4. So in the analysis of the urea concentration, the fixed-time method was employed.

The reproducibility of the urease sensor system was examined. The frequency response of this system for 40 μg/ml urea at 25° C and pH 7.5 was measured. For five measurements within one day, the variance in coefficients was 2.8%. These values were sufficient for practical use.

The average recoveries for urea with the present assay method were calculated by comparing the average frequency shift obtained from spiked control blood plasma samples of six different volumes. The average recoveries for urea over the entire concentration range of the standard solutions were 96–105% (Table 1).

3.7. Specificity of the SAW urease sensor system

This study was aimed to know the influence of in situ concentration on the specificity of the proposed

Table 1
Recovery determined with the SAW urease sensor system

Urea (μg/ml)		ΔF (Hz)	Recovery (%)	R.S.D. (%)
Added	Found			
1.50	1.44	158	96.0	3.8
2.50	2.61	284	104.4	3.5
5.00	4.84	523	96.8	3.2
7.50	7.68	829	102.4	2.8
10.0	10.2	1100	101.6	2.5
15.0	15.4	1659	102.4	2.6

All results were calculated by the calibration equation (Eq. 3) and 0.75 E.U./ml urease was used in this experiment.

method. It was also allowed to decide whether the method could be applied to real samples or not. The measurement was performed by alternately injecting a definite amount of interferents (50 μl of stock solution) into the test solution (containing 40 μg/ml urea).

As can be seen from Table 2, urea could be determined with a relative error of less than $\pm 4\%$ in the presence of foreign non-electrolytes and electrolytes, such as glucose, soluble starch, sodium chloride, human serum albumin and L-glutamic acid. Inhibitors such as Pb^{2+}, Hg^{2+} and F^- decrease reaction rate and frequency response. Hg^{2+} and F^- seriously inhibited the urease reaction among the urease inhibitors.

An inhibitor like thiourea structurally resembles urea and may be bound by urease but cannot be converted to the products. Fig. 7 shows the influence of the inhibitors thiourea and sodium fluoride on the relative initial rate of the reaction (i.e., relative activity of urease). The plot is linear ranging from 0 to 8.0 mM thiourea:

Table 2
Specificity of the SAW urease sensor system

Interferent	Concentration	Initial rate (Hz/min)	Relative response (%)
None		1495	100
Glucose	5.0 mg/ml	1450	97
Soluble starch	5.0 mg/ml	1435	96
Human serum albumin	2.0 mg/ml	1493	100
Sodium chloride	1.0 mM	1465	98
Ferrous sulfate	0.20 mM	1433	96
Lead nitrate	0.20 mM	1404	94
Mercuric nitrate	1.00 μM	1182	79
Sodium fluoride	0.50 mM	1136	76
L-Glutamic acid	0.20 mM	1437	96
Thiourea	2.00 mM	1330	89
Glycine	1.00 mM	1549	104

The concentration of urea was 40 μg/ml (0.67 mM). For experimental conditions see Experimental section.

$$\text{R.A.}(\%) = -9.2[\text{Thiourea}] + 102.8,$$
$$n = 9, r = -0.995 \quad (5)$$

The plot is linear ranging from 0 to 1.5 mM NaF:

$$\text{R.A.}(\%) = -35.8[\text{NaF}] + 97.5,$$
$$n = 7, r = -0.992 \quad (6)$$

3.8. Clinical analysis of urea in human blood

Urea concentrations of nine blood samples were determined based on the above calibration equation

Table 3
Clinical assay of urea content in blood samples

Sample	Sex	Clinical diagnostic results	Urea content (mg/ml)		
			Present method	Colorimetry	Normal value [37]
Blood plasma	Female	Healthy	0.23	0.19	
Blood plasma	Male	Healthy	0.27	0.28	
Whole blood	Female	Healthy	0.19	0.16	
Whole blood	Male	Healthy	0.21	0.25	
Whole blood	Female	Renal calculus	0.51	0.50	0.10–0.40
Whole blood	Male	Nephrotic syndrome	0.49	0.48	
Whole blood	Male	Chronic nephritis	0.46	0.43	
Blood plasma	Female	Chronic nephritis	0.35	0.37	
Blood plasma	Male	Acute cholecystitis	0.39	0.41	

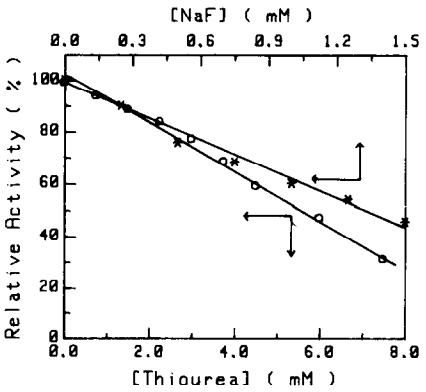

Fig. 7. Relative activity of urease as a function of the concentration of inhibitor: (○) thiourea and (*) sodium fluoride. 0.75 E.U./ml urease extracted from pumpkin seeds and 40 μg/ml urea were selected for this procedure.

and 50 μl of blood samples was used for all assays. The samples were assayed under the same conditions as the determination of calibration curves for control blood plasma with different concentrations of urea. The above samples were also determined by colorimetry [37]. The results are summarized in Table 3.

The clinical analysis of urea has shown the following. (1) For healthy adults, the urea concentrations agree with the literature range [37]. (2) For patients with liver or kidney diseases, the urea content in whole blood or blood plasma is slightly higher than the normal value. The tested results reflect those of the patients and are consistent with the clinical diagnostic results. The differences between the urea content in blood of healthy adults and patients with liver or kidney diseases have been explained pathologically [38]. Elevation of the urea concentration in blood is the most conspicuous chemical abnormality that results from kidney failure. (3) When the methods were compared, no apparent difference was found between the proposed method and the conventional colorimetric method at a confidence limit of 95%.

4. Conclusions

The proposed SAW urease sensor system, combining the inherent sensitivity of the SAW device and the inherent specificity of urease, is satisfactorily applied to clinical urea assays of small volumes of blood samples. In addition to its accuracy and precision, the assay procedure is very simple. The clinical assay results support medical diagnoses.

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