Continuous *in vivo* Monitoring of Blood Diffusible Calcium Using On-line Microdialysis Sampling Coupled with Flame Atomic Absorption Spectrometry

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A direct, rapid and continuous *in vivo* monitoring of diffusible calcium in the blood of living rabbits has been developed using microdialysis sampling coupled on-line with flame atomic absorption spectrometry. Microdialysates perfused through implanted microdialysis probes were collected with a sample loop on an injection valve and directly introduced into the flame atomizer by a carrier solution. An ultrapure saline solution (0.9% NaCl, pH 7.2) was used as the perfusion solution at a flow rate of 20 µl min^{-1} *via* the microdialysis probe. A 0.1% La solution in 0.5% HNO_3 solution was employed as the carrier solution at a nebulizer uptake flow rate of 2.5 ml min^{-1}. The interval for each determination was 2.5 min (2 min of sampling time, 20 s of read time and 10 s of washing time). The performance characteristics of the on-line microdialysis-FAAS system were validated as follows: linearity range, 0 – 100 mg l^{-1}; detection limit (3σ, n = 7), 3.66 mg l^{-1}; precision (RSD, n = 50), 6.2%. For the evaluation of analytical accuracy, the proposed on-line method was compared with the *in vivo* no net flux method. The use of an on-line microdialysis-FAAS system permitted the *in situ*, dynamic and continuous *in vivo* monitoring of diffusible calcium in the blood of the living rabbits after $CaCl_2$ administration with a temporal resolution of 2.5 min.

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Introduction

In the blood, calcium (Ca) exists in protein-bound, complex-bound, and ionized fractions. Ionized Ca is the biologically-active portion of Ca and is not bound to proteins in the blood. Ionized Ca concentration of extracellular fluid is tightly regulated to maintain cell membrane integrity, blood coagulation, hormone metabolism, and neuromuscular transmission.[1] Small decreases in ionized calcium can cause paresthesia and seizures; in contrast, increases in ionized calcium lead to constipation, obtundation, and coma.[1] Diffusible Ca, consisting of a complex-bound and a free ionized fraction, is also considered as a physiologically active species and may play roles in calcium homeostasis.[2] Although the predominant biological activity of calcium is attributed to its ionized fraction, any change of diffusible calcium would also result in diseases.[3] Thus, to measure diffusible calcium for clinical diagnosis is of crucial importance.

Usually ionized Ca analysis is carried out by potentiometry or fluorometry; however, expensive and specialized electrodes and costly reagents are needed.[4,5] In a clinical laboratory, among the techniques available for the determination of ionized calcium in complex biological fluids, ion-selective electrode (ISE) sensors are commonly used because of its sensitivity and selectivity.[6] However, on-line *in vivo* continuous Ca measurement using ISE is still not available. Small changes in ionized blood Ca concentration can have lethal consequences. Thus, on-line continuous monitoring of ionized blood Ca concentration is of utmost importance for the effective management of patients with unstable electrolyte status in the body fluids. For these reasons, there is a growing interest in designing and developing various types of on-line techniques for rapid, easy, and reliable analysis of Ca concentration in blood.[2,7]

Microdialysis,[8–11] a powerful sampling technique used to obtain protein-free samples, has become an important technique for continuous *in vivo* sampling of the extracellular fluid in discrete compartments of living systems. A microdialysis system is easy to automate and can be on-line coupled with many analytical techniques, such as liquid chromatography,[12] capillary electrophoresis,[13] mass spectrometry,[14] flow-injection analysis,[15–18] and electrochemical detection.[19] In addition, on-line microdialysis sampling can prevent analyte degradation during sample preparation. This is an advantage since ionized blood calcium concentration is easily affected by exposure to oxygen and pH.[20] Recently, *in vivo* monitoring of Ca in blood was documented using a flow-injection on-line microdialysis system coupled with fluorescent detection.[2]

As is well known, the three major atomization methods for atomic absorption spectrometry (AAS) are flame, electrothermal, and hydride generation techniques. In our previous studies, two hyphenated techniques of on-line microdialysis sampling coupled to hydride generation atomic absorption spectrometry[21] and electrothermal atomic absorption spectrometry[22] have been developed for *in vivo* monitoring of

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arsenic and manganese in living animals. Flame AAS (FAAS) is one of the most popular techniques for metal determination; however, the high sample volume needed would limit its on-line analysis. To our knowledge, no studies have been made to investigate the technique of on-line microdialysis coupled with FAAS for the determination of diffusible metals in biological fluids with complex matrices. The aim of this study was to develop a method of microdialysis sampling coupled on-line with FAAS for the continuous *in vivo* monitoring of diffusible Ca in the blood of living rabbits. In the present work, operating conditions and analytical performance of the proposed on-line microdialysis-FAAS system were optimized and validated. The proposed method was applied successfully to determine the nearly real-time concentration of diffusible Ca in the blood of rabbits calcium chloride ($CaCl_2$) had been administrated.

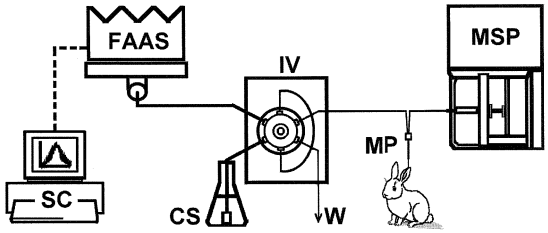

Fig. 1 Schematic diagram of the on-line microdialysis-FAAS system for continuous determination of diffusible Ca in the blood of living rabbits. MSP, microinjection syringe pump; MP, microdialysis probe; IV, injection valve; W, waste; CS, carrier solution; FAAS, flame atomic absorption spectrometer; SC, spectrometer computer.

Experimental

Reagents and vessels

High-purity water (18.3 MΩ cm) was prepared with a deionized water system (Milli-Q, Millipore) and used throughout this work. All of the reagents used were of the highest available purity and were at least of analytical grade. The perfusion solution was prepared by dissolving 0.9 g of NaCl (Merck, ultrapure grade) in 100 ml of high-purity water and the pH was adjusted to 7.2. The carrier solution was prepared by dissolving lanthanum oxide (LaO_3, Merck, ultrapure grade) in 1000 ml of 0.5% HNO_3. Stock solutions (1000 mg l^{-1}) of Ca were purchased from Merck. The working aqueous Ca standard solutions were diluted fresh daily with 0.9% NaCl.

Before each use, all containers and pipette tips were scrubbed in 20% nitric acid (Riedel-de Haën, Germany) overnight, then cleaned with high-purity water five times. The tubings used for the connections of all apparatuses were perfused with high-purity water to flush out contamination. In order to avoid contamination of Ca, metal-free syringes (10 ml Luer Tip syringe, mode 81601, Hamilton, USA) were used as the perfusion syringes throughout this work. Twenty millimeters of polyetheretherketon (PEEK) tubing (0.25 mm i.d.) was fitted to the syringe, making a homemade metal-free needle.

Instrumentation

A schematic diagram of the on-line microdialyis-FAAS system for the continuous determination of diffusible Ca in the blood of living rabbits is shown in Fig. 1. Microdialysates perfused through a microdialysis probe were collected in a sample loop on a six-port on-line injection valve for direct injection into a flame atomizer by a nebulizer uptake flow. A personal computer was used to operate the spectrometer and to acquire the atomic absorption signal.

The microdialysis system was purchased from Carnegie Medicine Associates (CMA, Stockholm, Sweden). The microdialysis sampling system consists of a microinjection syringe pump (CMA/100) and a 24 mm-long microdialysis probe (CMA/20) with a 10 mm-long and a 0.5 mm-diameter polycarbonate membrane, which is metal-free and has a molecular mass cut-off of 20 kDa. Fluorinated ethylene polypropylene (FEP) tubing (50 cm long × 0.12 mm i.d.; internal volume of 1.2 µl per 100 mm length; CMA, Stockholm, Sweden) was used to connect the microinjection syringe pump to the inlet of the probe and the outlet of the probe to the sample loop. The total dead volume of FEP tubing from probe to sample loop was approximately 6 µl. All connections of FEP tubing to the microinjection syringes, the probe and the other FEP tubing were accomplished by tubing adaptors (CMA, Stockholm, Sweden), which ensure tight, zero internal volume connections.

The on-line interface was performed with a six-port on-line injection valve (Omnifit smart actuator, Cambridge, UK). The on-line injection valve has an inert metal-free Teflon body designed for avoiding the contamination of metals. The connections and conduits were made of polytetrafluoroethylene (PTFE) connecting tubes (11 cm long × 1.0 mm i.d.; Perkin-Elmer B019-1058). A 20 cm-long delivery tube was used to connect the injection valve with a flame atomizer. An atomic absorption spectrometer (Perkin-Elmer Model 5100 PC) was used. The system was operated through a personal computer and the associated AA *WinLab*$_{Tm}$ software, version 2.0. Peak area (integrated absorbance); statistical data were printed out using a laser printer.

Procedures

To characterize the on-line microdialysis-FAAS system, we introduced the microdialysate sample directly into the injection valve in a continuous manner. Aqueous Ca standards were used to estimate the operational parameters (perfusion flow rate, sampling time, nebulizer uptake rate and detection time).

The linearity of the calibration curve was evaluated from 0 to 200 mg l^{-1} by the on-line microdialysis-FAAS system. Calculation of the detection limit (3 σ) was carried out by 7 measurements of baseline noise. To test long-term stability and precision of the method proposed, we inserted the microdialysis probe into the left-ear vein of rabbits, after which on-line sampling and detection was conducted every 2.5 min for 125 min (50 continuous measurements). The accuracy was checked by implanting the probe in the left-ear vein of three rabbits, the results of which were compared with the *in vivo* no net flux (NNF) method as described previously.[2,23,24] In the *in vivo* NNF method, the microdialysis sampling was performed by perfusing the probe with different concentrations of Ca standard solution (10, 20, 40, 60 and 80 mg l^{-1}) at a flow rate of 2 µl min^{-1}. For every change of Ca concentration, the first dialysate was discarded to avoid the residual effect of the previous measurement.

Adult male New Zealand White rabbits (2500 – 3000 g) were obtained from the Livestock Research Institute of the Republic of China (Tainan, Taiwan). These animals were specifically pathogen-free and were allowed to acclimate to their environmentally controlled quarters (25°C and 12:12 h light-dark cycle) for at least 5 days and then fasted overnight prior to the day of experimentation. The rabbits were initially anesthetized with an anaesthetic solution (ketamine 45 mg kg^{-1},

Table 1 Optimized operating conditions of the on-line microdialysis-FAAS system for continuous monitoring of blood diffusible Ca

Microdialysis sampling	
Probe (CMA/20)	10 × 0.5 mm membrane, metal-free and cut off at 20 kDa
Perfusion solution	Saline (0.9% NaCl) solution (pH 7.2)
Perfusion flow rate	20 µl min^{-1}
Sampling time	2 min
On-line interface	
Carrier solution	0.1% La in 0.5% HNO_3
Nebulizer uptake rate	2.5 ml min^{-1}
FAAS system	
Lamp type	Calcium hollow cathode lamp
Wavelength	422.7 nm
Slit width	Low 0.7 nm
Lamp current	10 mA
Oxidant	Air (10 L min^{-1})
Fuel	Acetylene (3.8 L min^{-1})
Signal type	Peak area (integrated absorbance)
Read time	20 s
Washing time	10 s

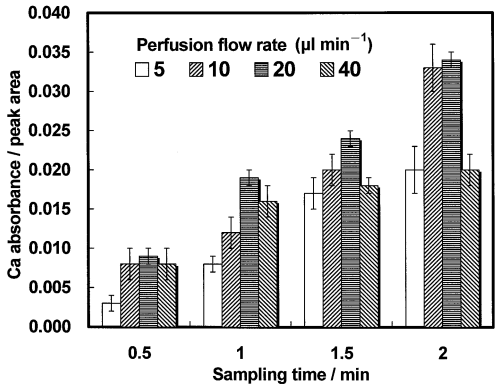

Fig. 2 Effect of perfusion flow rate and sampling time on the absorbance of 30 mg l^{-1} Ca standard solution using on-line microdialysis-FAAS system. The nebulizer uptake rate and the read time of FAAS were set at 5 ml min^{-1} and 10 s, respectively. The other conditions are as in Table 1. The error bars represent standard deviations (n = 3).

xylazine 5 mg kg^{-1} and atropine 0.1 mg kg^{-1} body weight), and continued to be anesthetized with pentobarbital (10 mg kg^{-1} h^{-1}) throughout the experimental period. The microdialysis probe (CMA/20, 10 mm dialysis membrane) was implanted into the left-ear vein of rabbit. The probe was perfused with a saline solution containing anticoagulant (20 IU ml^{-1} of heparin). The microdialysates collected over the first 2 h were discarded to prevent acute adverse effects on the animals from the surgical procedures. The probe was connected to the on-line analytical system approximately 2 h after surgery. Ca level was continuously monitored by the proposed on-line system every 2.5 min. Basal Ca levels were monitored for at least 17.5 min prior to Ca administration. When the seventh measurement was completed, $CaCl_2$ (20 mg kg^{-1} body weight) was injected into the right-ear vein of the rabbit and the Ca was continuously monitored every 2.5 min for approximately 100 min.

Results and Discussion

Optimization of the on-line microdialysis-FAAS system

The optimized operating conditions of the on-line microdialysis-FAAS system for determining of diffusible Ca are summarized in Table 1. Instead of off-line transporting the microdialysate to the FAAS, an injection valve was employed as an on-line interface. The manifold of the on-line microdialysis coupled with FAAS is shown schematically in Fig. 1. In this system design, an on-line injection valve converted the continuous sampling stream of the microdialysis system into discrete samples. In the loading step, the perfusion flow rate of 20 µl min^{-1} was converted into a 40 µl sample by the injection valve. After a 2 min loading period, the injection valve was switched to the injection position. The microdialysate sample trapped in the loop was injected into delivery tubing at timed intervals. In the injection step, the 0.1% La carrier solution was introduced into the sample loop to propel the microdialysate sample into the flame atomizer at a nebulizer uptake flow rate of 2.5 ml min^{-1}. Meanwhile, the spectrometer computer was actuated to read the atomic absorption signal for 20 s. To avoid the residual effect of the previous sample, the sample loop was washed by carrier solution for 10 s prior to the next loading step. The detailed observations are described as follows.

In the present work, a commercial metal-free probe (CMA/20) incorporating a relatively long microdialysis membrane of 10 mm was used to permit rapid and continuous sampling. In order to reduce the complexity of the matrix and to avoid Ca contamination, we perfused the implanted probe with an ultrapure saline solution (0.9% NaCl, pH 7.2). The ultrapure saline solution is similar to blood in ionic strength and pH value.

The absorbance of 30 mg l^{-1} Ca standard solution was examined in terms of perfusion flow rate (5, 10, 20 and 40 µl min^{-1}) and sampling time (0.5, 1, 1.5 and 2 min) using the on-line microdialysis-FAAS system. The results are shown in Fig. 2. The absorbance of diffusible Ca increases with the increase of perfusion flow rate and sampling time. However, as the perfusion flow rate increases to 40 µl min^{-1}, the excess high perfusion flow rate may cause the mass transport resistance of Ca diffusion and result in a low absorbance of Ca. As far as the rapid measurement and sufficient microdialysate volume are concerned, the 20 µl min^{-1} perfusion flow rate and 2 min of sampling time were favorable for the determination of diffusible Ca.

One of the diffusible Ca fractions is complex-bound Ca which mainly contains calcium phosphate. The phosphate would interfere with the determination of free ionized Ca. To eliminate the chemical interference that resulted from the phosphate, La is widely used as a chemical matrix modifier. In this study, different La concentrations (0, 0.05, 0.1, 0.2, 0.4 in 0.5% HNO_3) were investigated for the determination of diffusible Ca in whole blood. The results showed that the highest Ca absorbance was observed when 0.1% La solution used.

The effect of the nebulizer uptake rate on the absorbance of diffusible Ca was studied by adjusting the nebulizer regulator. Figure 3 shows that the nebulizer uptake rate increases with a decrease of Ca absorbance (correlation coefficient = –0.9488). These results indicate that the lower the nebulizer uptake rate, the greater the absorbance that can be detected. However, unstable signals were observed at the low nebulizer uptake rate (1.25 ml min^{-1}). In this experiment, an optimal nebulizer uptake rate of 2.5 ml min^{-1} was selected according to the analytical

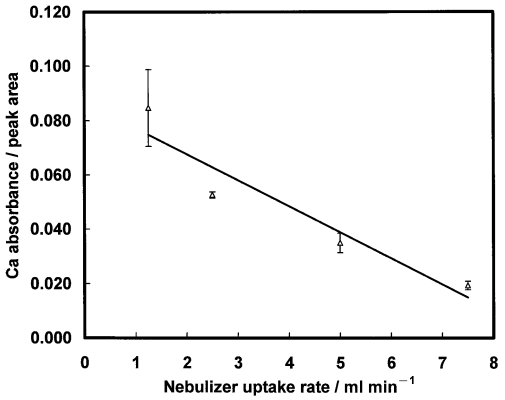

Fig. 3 Effect of the nebulizer uptake rate on the absorbance of 30 mg l^{-1} Ca standard solution using an on-line microdialysis-FAAS system. The nebulizer uptake rates of 1.25, 2.5, 5 and 7.5 ml min^{-1} were coupled with different FAAS read times of 40, 20, 10 and 5 s, respectively. The other conditions are as in Table 1. The error bars represent standard deviations (n = 3).

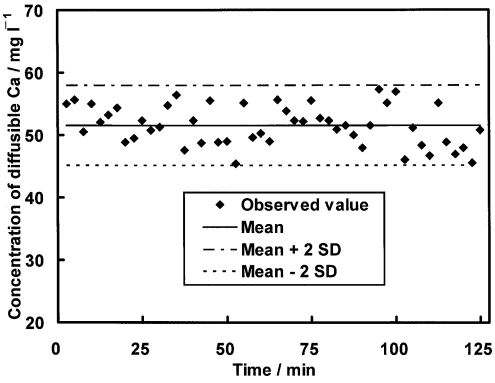

Fig. 4 Long-term stability of the proposed on-line microdialysis-FAAS system using continuous on-line sampling and detection every 2.5 min for 125 min (50 continuous measurements) in the blood of living rabbits.

stability and sensitivity.

Except for signal type and read time, all parameters for the determination of Ca using FAAS were provided from the suggested values of the instrument (Table 1). In this study, a small fixed volume of discrete microdialysate was introduced into the flame atomizer by the nebulizer uptake flow. A symmetrical or Gaussian-like signal type was formed during the process of Ca atomization. Therefore, the integrated peak area was evaluated as the absorbance of Ca. Furthermore, it is important to acquire the whole atomic absorption peak within a detectable time interval. The optimized signal read time for an atomic absorption peak was 20 s.

Method validation of the on-line microdialysis-FAAS system

The linearity of the on-line microdialysis-FAAS system was evaluated from 0 to 200 mg l^{-1} Ca. The calibration graph was linear in the range of 0 to 100 mg l^{-1}. Ca standard solutions using the on-line system were expressed by the regression equation (zero intercept): $A = 0.0015C$, $r > 0.9990$, where A is the absorbance, C the Ca concentration and r the correlation coefficient. Above 100 mg l^{-1}, the system showed a slight negative deviation from linearity. The detection limit based on three times the standard deviation of the baseline noise (n = 7) was 3.66 mg l^{-1}.

To examine the long-term stability of the on-line microdialysis-FAAS system, the microdialysis probe was inserted into the left-ear vein of the live rabbit, followed by on-line sampling and detection every 2.5 min for 125 min (50 continuous measurements). Figure 4 shows that all the data were in the range of average ±2 standard deviations. The precision of the on-line microdialysis-FAAS system for 50 measurements was 6.2% RSD.

Because no certified values for the diffusible Ca content in rabbit blood were available, the accuracy of the proposed on-line method was checked using three live rabbits and compared by employing the *in vivo* no net flux (NNF) method. The *in vivo* NNF is the most commonly used quantitative microdialysis method,[23,24] it is based on measuring the mass transport of the analyte across the dialysis membrane as a function of the perfusate concentration.[23] In the NNF method, Ca is added to the perfusate at concentration higher or lower than the expected true concentration. This generates a series of points that can be interpolated to determine the concentration of no net Ca flux, which represents the true concentration surrounding the probe. The comparisons between the on-line microdialysis-FAAS system and the *in vivo* NNF method are shown in Table 2 and are in good agreement within experimental error.

Table 2 Diffusible Ca concentration (mg l^{-1}) in the blood of living rabbits as determined by the proposed on-line microdialysis-FAAS system and *in vivo* no net flux method (mean ± SD, n = 3)

Sample	On-line microdialysis-FAAS system	*In vivo* no net flux method
No. 1 rabbit	48.3 ± 2.6	50.3 ± 2.3
No. 2 rabbit	49.0 ± 2.4	51.1 ± 1.4
No. 3 rabbit	51.7 ± 3.1	51.7 ± 1.9

Overall, the favorable analytical performance of the proposed method evaluated in terms of linearity, detection limit, long-term stability and accuracy indicates that the on-line microdialysis-FAAS system is appropriate for continuous monitoring of diffusible Ca in the blood of living animals.

In vivo study

To demonstrate the acute distribution of Ca in blood, an experiment involving the intravenous injection of $CaCl_2$ into the living rabbits was performed. Figure 5 shows the concentration profile of Ca in the blood of live rabbits. Basal microdialysate levels of Ca (53.9 ± 4.1 mg l^{-1}, n = 48) were determined at 2.5 min intervals for 120 min. Twenty mg kg^{-1} body weight of $CaCl_2$ was intravenously injected into the right-ear vein of each rabbit. Following the administering of Ca, the average time for the initial rise was 3.3 ± 1.4 min (n = 3). The average concentration of maximum Ca during stimulation was 101.6 ± 27.3 mg l^{-1} (n = 3). The average blood Ca concentration reached a maximum value at 5 min post-injection that was approximately 2-fold higher than the basal level and the value was still higher than the base line at 120 min after administration with $CaCl_2$.

Conclusions

In this work, a novel method involving on-line microdialysis sampling and FAAS detection for the *in vivo* monitoring of Ca

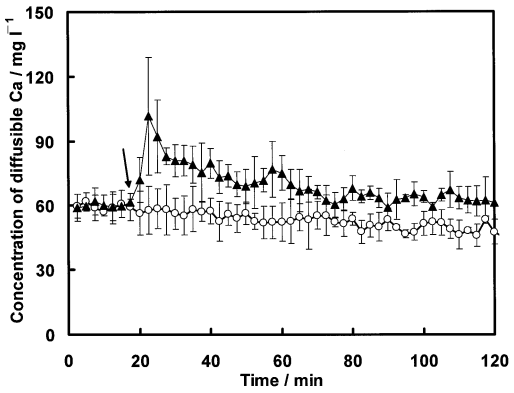

Fig. 5 The time course of Ca concentration in the blood of living rabbits following an experimental intravenous injection of Ca. ↓, right-ear intravenous injection of 20 mg kg^{-1} body weight $CaCl_2$ (closed triangle) or normal saline (open circle). Diffusible Ca in the blood of rabbits was measured using the on-line microdialysis-FAAS method. The error bars represent standard deviations (n = 3).

concentration in the blood of living rabbits was developed. On-line microdialysis that provides direct, *in situ*, dynamic and continuous sampling simplifies the pretreatment of biological samples. The design of on-line microdialysis coupled with FAAS makes it possible to determine the amounts of electrolytes in living systems. The on-line system can be readily adapted to other electrolytes by varying the detection conditions of FAAS. The proposed on-line microdialysis-FAAS analytical technique may also be employed advantageously to study the acute distribution of electrolytes in tissues, organs or biological fluids of living organisms.

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