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Biomedical point-of-care microanalyzer for potentiometric determination of ammonium ion in plasma and whole blood

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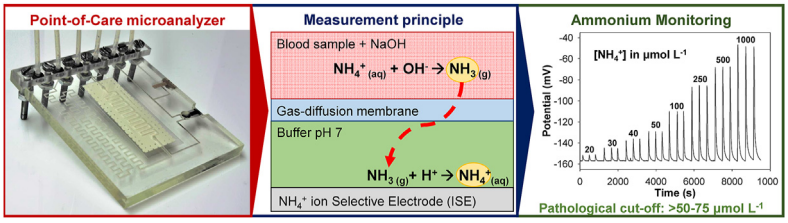
HIGHLIGHTS

- Low-cost Point-Of-Care analytical microsystem with integrated gas-diffusion, dialysis membranes and potentiometry.
- Direct whole blood and plasma ammonium monitoring.
- Physiological and pathological blood ammonium levels are covered with accuracy and precision.
- Simple and automatic analytical method for ammonium determination.

GRAPHICAL ABSTRACT



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ABSTRACT

Some inborn errors of metabolism and other diseases can result in increasing blood ammonium (hyperammonemia episodes), which can cause serious neurological complications in patients or even death. Early diagnosis, follow up and treatment are essential to minimize irreversible damages in brain. Currently, adequate analytical instrumentation for the necessary ammonium bedside determination is not available in all health centers but only in clinical laboratories of reference hospitals. We therefore have developed a low cost and portable potentiometric Point-of-Care microanalyzer (POC) to address this problem. It consists of a cyclic olefin copolymer-based microanalyzer, the size of a credit card and working in continuous flow, which integrates microfluidics, a gas-diffusion module and a potentiometric detection system. The analytical features achieved are a linear range from 30 to 1000 µmol L^{-1} NH_4^+, a detection limit of 18 µmol L^{-1} NH_4^+ and a required sample volume of 100 µL, which comply with the medical requirements. Plasma and blood samples are analyzed with no significant differences observed between ammonium concentrations obtained with both the proposed microanalyzer and the reference method. This demonstrates the value of the developed POC for bedside clinical applications.

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1. Introduction

A large group of inborn errors of metabolism (IEM), which may impair urea cycle, a liver metabolic pathway in charge of ammonium elimination, present the accumulation of high levels of ammonium in blood as main pathophysiological mechanism [1,2]. This fact is clinically known as hyperammonemia [3,4]. Within a large group of diseases, primary disorders of the urea cycle, different organic acidemias and other metabolic conditions affecting liver function, which can cause a secondary dysfunction of the urea cycle, are included. Urea cycle disorders (UCDs) are IEM of amino acids due to genetic defects in different urea cycle enzymes and transporters, which are involved in the conversion of ammonium/ammonia into urea [5–9]. These defects result in episodes of hyperammonemia, the clinical outcomes of which may range from subtle neurological manifestations during childhood/adulthood to hyperammonemic coma at few days after birth or during the evolution of the disease. Similarly, any chronic or acute liver disease that leads to liver failure may end up with hyperammonemic encephalopathy as one of the most severe consequences, since the detoxification of ammonium/ammonia through the urea cycle occurs in liver [10]. The range of usual ammonium concentrations in healthy individuals ranges from 11 to 50 μmol L^{-1} in adults, and up to 75 μmol L^{-1} in newborns (according to some authors). Values beyond 200 μmol L^{-1} are considered as severe episodes of hyperammonemia that can cause irreversible neurological damage. When patients present grossly elevated ammonium values (between 500 and 1500 μmol L^{-1}) mortality is a common complication [7,11–15]. Early and fast diagnosis and thus, rapid and controlled treatment are therefore essential to minimize the impact of hyperammonemia on neurological functions. This is done by restriction of proteins intake, using drugs to enhance ammonium elimination or carrying out dialysis or hemofiltration in acute cases [8,11,16].

There are different commercial clinical equipment and kits to determine ammonium ion mainly in plasma samples. Among them, those that are based on enzymatic (using the glutamate dehydrogenase-catalyzed reaction) and spectrophotometric methods are the most commonly used [17]. [–] [21] However, despite they meet some of the requirements to take adequate medical decisions, they still show some of the following different constraints: lack of portability (large, bulky and heavy instrumentation), high cost, use only by skilled professionals in 3rd level Hospitals or limited working range and precision. This issue makes these analyzers not suitable for regular and routine control of ammonium in blood to diagnose or follow-up diseases associated with hyperammonemia episodes out of reference hospitals (for instance in secondary hospitals or health centers). Moreover it cannot be implemented in developing countries, where even analytical kits are inaccessible due to their high price. For these reasons, portable, low cost, easy-to-use and automatic Point-of-Care (POC) analyzers with high robustness, high throughput sample analysis and low sample and reagents consumption are needed with great immediacy to allow precise bedside ammonium determination in plasma or whole blood.

Among the different analytical techniques to determine ammonium ion, potentiometry and especially ion selective electrodes (ISEs) are a good option to develop POC microanalyzers due to their robustness, good selectivity, wide working range and compatibility with flow techniques in which a high level of integration, automation, autonomy and high speed of analysis are achieved, without the need of using unstable enzymes or colorimetric reagents. In addition, the utmost selectivity can be reached by the use of gas-diffusion membranes to separate the analyte from the complex blood matrix [22–24].

In this work, a cyclic olefin copolymer (COC)-based continuous flow potentiometric POC microanalyzer is presented to monitor the amount of ammonium ion in plasma and blood of patients diagnosed with IEM or other diseases causing hyperammonemia. The microanalyzer integrates monolithically microfluidics, a gas-diffusion module and a potentiometric detection system in a credit-card size single substrate.

2. Experimental

2.1. Reagents and materials

POC microanalyzer prototypes were fabricated using layers of COC from Tekni-Plex (Erembodegem, Belgium) in different grades and thicknesses: COC 6013 foils of 400 μm-thick and COC 8007 foils of 25 and 50 μm-thick.

A hydrophobic gas-diffusion membrane made of polyvinylidene fluoride (PVDF) with a 0.45 μm-pore diameter (Millipore, USA) was used in order to separate the analyte from blood or plasma matrix.

A hydrophilic dialysis membrane made of polycarbonate (PC) with a 0.05 μm-pore diameter (Whatman, USA) was used in order to protect the gas-diffusion membrane from adsorption of proteins and other lipophilic compounds present in blood matrix.

A carbon-based ink (ELECTRODAG PF-407A, Acheson, France) was used as a conductive support for the ISE. An Ag/AgCl paste (C2030812P3, Gwent Group, UK) was used as reference electrode.

For the optimization and evaluation of the POC microanalyzer, analytical grade reagents were used. All solutions were prepared by weighing out and dissolving the corresponding salt or compound in double distilled water.

Standard solutions of ammonium chloride (Acros Organics, Belgium) were prepared by successive dilutions of a 100 mmol L^{-1} stock. A KCl (Sigma Aldrich, Barcelona, Spain) solution (0.1 M) was used to keep constant the potential of the reference electrode. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Fisher Scientific, Spain) buffering solution (1 mM) adjusted to pH 7 with barium hydroxide (Sigma Aldrich, Spain) was used as acceptor solution. NaOH (0.1 M) (Sigma Aldrich, Spain) with ethylenediamine tetraacetic acid (EDTA) (10^{-3} M) (Panreac, Spain) were used as a basic solution that once mixed with samples or standards, acted as donor solution.

ISE-membrane reagents were nonactine, bis(1-butylpentyl) adipate (BBPA), polyvinyl chloride (PVC) and tetrahydrofuran (THF), all of them obtained from Fluka (Barcelona, Spain).

2.2. Fabrication of POC microanalyzer

Modular and monolithic prototypes were developed during the optimization stage for different purposes. The first was used to evaluate the influence of the sample matrix on the integrity of different elements of the microsystem, so that if it was necessary to replace any (malfunction or configuration changes) this could be done easily without discarding the rest. The later was the final version of the POC microsystem, used once the compatibility of all elements with the sample matrix was verified.

A multilayer approach was employed for the manufacture of prototypes. It consists in the lamination of different machined COC layers with different glass transition temperatures (Tg). COC 8007 foils with Tg = 75 °C were used as sealing layers. Blocks of two COC 5013 foils with Tg = 130 °C, bonded previously using a sealing layer between them, were used as fluidic structural layers, where all the designed patterns were machined. The fabrication process is described in detail elsewhere [25–28]. It briefly consists of four main steps: prototype design using CAD software, pattern machining using a computer numerically controlled (CNC)

micromilling machine (Protomat C100/HF, LPKF, Spain), integration of different elements (such as electrodes and PVDF and PC membranes) and final lamination at 102 °C and 4 bar to seal the microanalyzer using a thermo-compression press (Francisco Camps, Granollers, Spain).

The modular prototype consisted of three separated components (Fig. 1): a micromixing, a gas-diffusion and a detection module. The size of each one was 27.0 × 34.0 × 1.5 mm, 32.0 × 56.0 × 6.0 mm and 26.5 × 34.0 × 2.0 mm, respectively. The dimensions of the microfluidic channels both for micromixing module and detection module were 0.4 mm-wide and 0.3 mm-height. The dimensions of the meander in the gas-diffusion module (Fig. 1.C) were 1.0 mm-wide and 0.1 mm-height, in order to maximize the contact area between the ammonia generated and the gas-diffusion membrane. With the aim to allow an easy exchange of the PVDF/PC membranes when necessary, an aluminum holder was fabricated (Fig. 1.B and C). The diameter of the detection chamber (Fig. 1.D c) was 3.3 mm and the dead volume of the detection module was 22 μL.

The monolithic prototype of the microanalyzer had the same elements as the modular version but in a single integrated device (Fig. 2). Its size was 50.8 × 66.6 × 4.5 mm and it weighed 14 g. The dimensions of the microfluidic channels, where the gas-diffusion takes place and the detection chamber were the same as in the modular prototype. The dead volume of the detection chamber was 15 μL and the total dead volume of the monolithic microanalyzer was 110 μL.

The principle of operation of the POC microanalyzer is very simple. A carrier solution (Fig. 3 A or B channel d), where the sample/standard solution containing ammonium is injected, converges with a NaOH solution (c) in a Y-shape confluence point. They get mixed along a serpentine micromixer obtaining ammonia. The mixed stream reaches the gas-diffusion module, where a bottom meander-shape channel allows ammonia to diffuse, through the binomial formed by the PC and PVDF membranes, to an acceptor solution on the upper side. This acceptor solution (b), composed by HEPES at pH 7, collects and converts ammonia to ammonium. Finally, a volume of ammonium solution arrives to the detector, causing a signal variation on the measured potential difference, which is proportional to its concentration. To keep constant the potential of the reference electrode, an auxiliary KCl solution (0.1 M) is continuously pumped at 0.1 mL min^{-1} over the reference

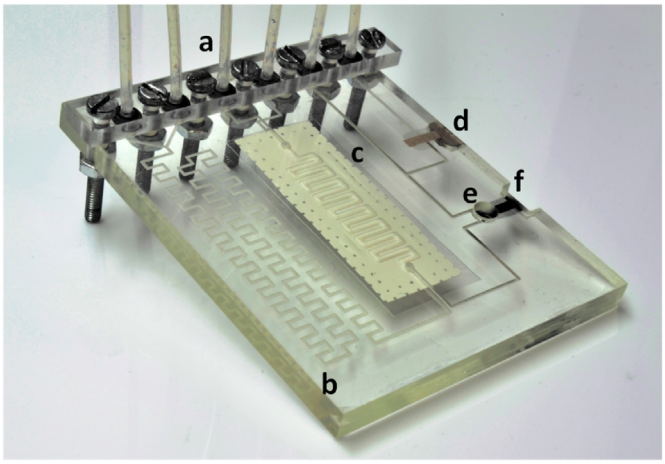

Fig. 2. Picture of the monolithic POC microanalyzer; a) fluidic connections; b) microfluidics; c) PVDF (above) + PC (below) membranes; d) reference electrode; e) detection chamber; f) ISE.

electrode during operation [29]. This reference channel acts as a flowing liquid junction by merging with the main channel after the detection chamber.

The solid inner support for the ISE, made of a carbon-based ink, was placed by filling a bas-relief machined onto the COC layer. It was cured at 80 °C for 30 min. The Ag/AgCl paste, acting as the reference electrode, was screen-printed in a specific place over the reference channel using a screen-printer machine (DEK 248, DEK, Spain) and then it was cured at 80 °C for 30 min.

The PC dialysis and PVDF gas-diffusion membranes were cut, washed with deionized water, dried and placed correctly in the gas-diffusion chamber.

The ammonium selective polymeric membrane was prepared by weighing out, mixing and dissolving 1% nonactin, 65.5% BBPA and 33.5% PVC in THF (3 mL) [30]. This cocktail was deposited dropwise on the surface of the conductive support of a carbon-based ink inside the detection chamber [25,27], which was defined between the ISE membrane surface and the microchannels. The protocol consisted in the repeated deposition of 1 μL of membrane cocktail and evaporation for 5 min until the cavity of the detection chamber was filled.

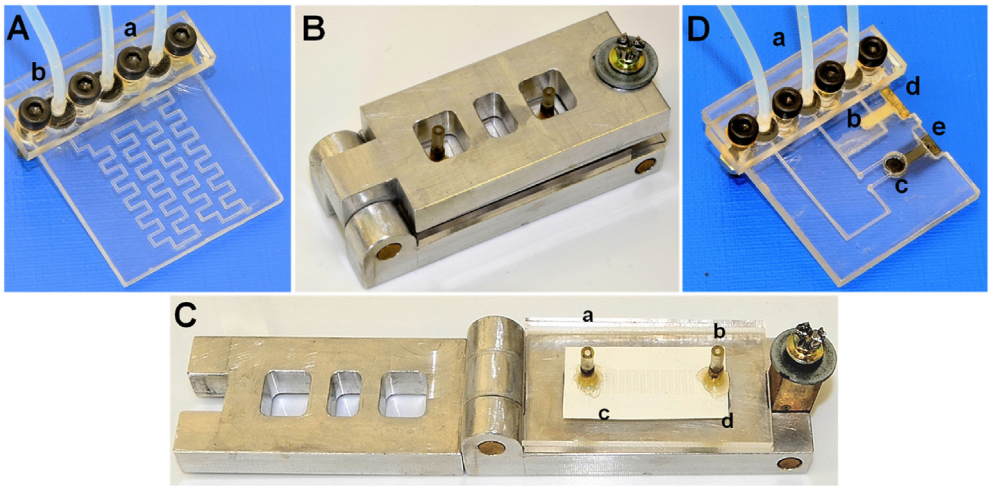

Fig. 1. Modular microsystem prototype. A) Micromixing module: a) inlets and b) outlet; B and C) gas-diffusion module: a) inlet, b) outlet, c) microfluidic channels and d) PVDF/PC membranes; D) Detection module: a) inlets/outlet, b) reference electrode, d) reference electrode connection, c) Ammonium ion selective electrode (ISE) and e) ISE electric connection.

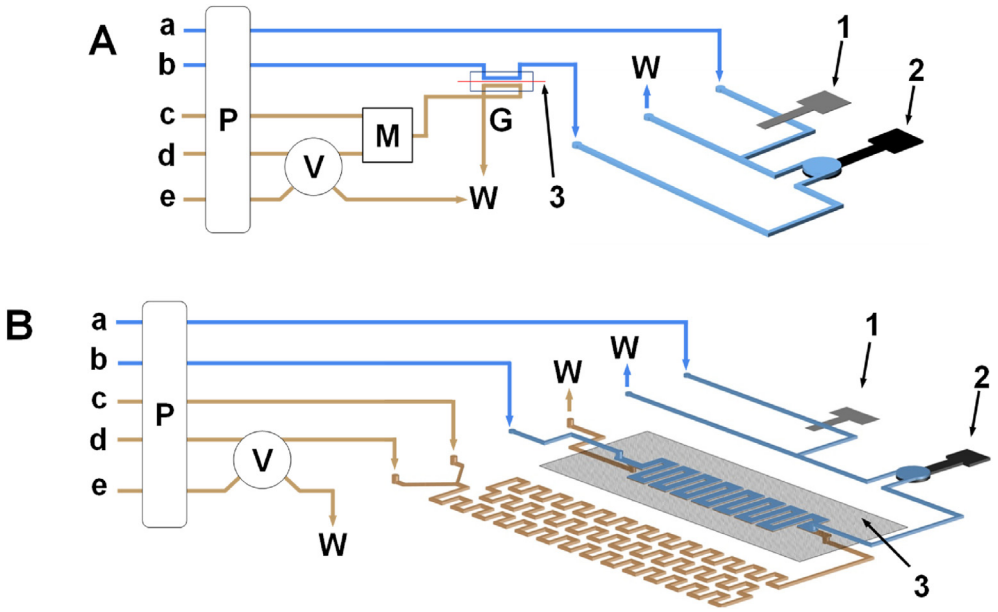

Fig. 3. A) Experimental setup for the modular POC microanalyzer. B) Experimental setup for the monolithic POC microanalyzer. In both cases: (a) KCl (0.1 M); (b) HEPES (1 mM) at pH 7; (c) NaOH (0.1 M) + EDTA (10^{-3} M); (d) H_2O; (e) NH_4^+ standard solution or sample; (P) peristaltic pump; (V) six-way injection valve; (M) micromixing module; (G) gas-diffusion module; (W) waste; (1) reference electrode; (2) ISE; (3) gas-diffusion membrane. Brownish color: microchannels under the PVDF membrane; Blue color: microchannels over the PVDF membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.3. Experimental setup

Fig. 3.A shows the experimental assembly used with the modular prototype. Once the proper functioning of the analytical system was verified with a complex matrix, the monolithic prototype was developed and tested using the experimental setup shown in Fig. 3.B. In both cases, the continuous flow system setup consists of an external peristaltic pump (Minipuls 3, Gilson, Wisconsin, US) with Tygon® tubing (Ismatec, Wertheim, Germany) of 1.14 mm internal diameter and a six-way injection valve (Hamilton MVP, Reno, US). Teflon tubing (Scharlab, S. L., Cambridge, England) of 0.8 mm internal diameter was used to connect the external elements to the microsystem. A customized potentiometer (6016 4-electrodes, TMI, Barcelona, Spain) and its software were used to signal acquisition and data processing.

2.4. Plasma and blood samples collection and preparation

Before starting this study, the permission from the Ethics Committee of HSJD was obtained in order to validate the POC system using patient's samples. These were remnant blood specimens from routine determinations of ammonium performed by the reference method in the HSJD laboratory. Once ammonium is analyzed, theses samples are usually discarded after 1 week. We collected an aliquot of 500 μL in an anonymized way. Thus, no sensitive information about patients or their identity was stored, all the experiments were conducted in the HSJD laboratory, and no-data were transferred to external Hospital data repositories.

77 venous blood samples were collected in tubes containing heparin or EDTA (three of them in both tubes to compare the anticoagulant influence over the ammonium determination), as indicated in protocols of HSJD for routine ammonium analysis. In 26 of these blood samples, ammonium was immediately measured by the developed POC microanalyzer in whole blood and in parallel in plasma samples by the reference method, which is done only in plasma, to obtain contrast results. Plasma fraction was obtained from the resulting supernatant after centrifugation of blood samples for 10 min at 3000 rpm at 10 °C. The remaining 54 blood samples were centrifuged by the same way to obtain the plasma fraction. These were analyzed in parallel using both methods.

Results obtained by the potentiometric POC microanalyzer were validated by comparison with those obtained by the reference method implemented in the clinical laboratory of HSJD. The later is based on an enzymatic method by means of an automated spectrophotometric procedure in an Architect ci8200 automated analyzer (ABBOT, Park, Illinois, EE.UU) [31]. The analysis of the samples by both methods was simultaneously done. The reference method is accredited by ENAC agency following the ISO 15189 norm, and it is subjected to external and internal quality control schemes. These data are available on request.

3. Results and discussion

3.1. Optimization and analytical features of the potentiometric POC microanalyzer

The main goal of this work was the development of a simple, selective, robust, portable and low cost POC microanalyzer for the bedside monitoring of ammonium ion in plasma or whole blood of patients with hyperammonemia. This application sets out some specific requirements such as enough working range to cover the ammonium physiological and pathological intervals, good baseline stability, limited sample and reagents consumption, high automation potential and avoiding sample pretreatments and operational skills. Keeping all this in mind, different parameters and variables that could influence the microsystem performance were evaluated and optimized.

The composition of the polymeric membrane used for the ammonium selective electrode was based on a previous work [25]. The fabrication process of the carbon conductive support for the ammonium ISE was improved regarding epoxy-graphite paste-based electrodes used in other works [25,32]. Herein, a commercial screen-printable carbon ink was employed. A bas-relief was mechanized onto the COC substrate to enhance adhesion between

materials and simplify the deposition process, which will allow an easier future industrialization of the microsystem manufacturing process. Carbon ink was cured at 80 °C for 30 min to ensure the development of proper electrical properties by removing the organic components. Since the thickness of the conductive support layer was 300 μm, the conductive material was not damaged, when the THF-sensor cocktail was deposited dropwise. Analytical features such as sensitivity, baseline stability and detection limits obtained with ISEs fabricated with this procedure were comparable to those obtained using epoxy-graphite paste.

Regarding lifetime of the microsystem with whole blood samples, previous works revealed a progressive deterioration of the PVDF gas-diffusion membrane with successive injections of the same whole blood sample [32]. A peak decrease and a positive drift of the baseline potential were continuously observed until total signal loss, even with standard materials. This phenomenon could be related to the adsorption of lipophilic compounds present in the whole blood into the surface of the hydrophobic PVDF membrane. This fact, eventually leads to a series of surface modifications that trigger a change in the membrane permeability, allowing NaOH to permeate to the acceptor channel. In order to protect the gas-diffusion membrane from this undesired event, different hydrophilic dialysis membranes, regenerated cellulose (RC) and polycarbonate (PC), with different pore size were tested. These membranes were integrated into the gas-diffusion module between the donor microchannel and the PVDF membrane. In this way, cross of the lipophilic compounds through the hydrophilic dialysis membrane was considerably minimized, while ammonia pass was not blocked. With this configuration, signal peaks decreased a bit but were big enough to comply with the required limit of detection and PVDF membrane lifetime, and thus, the whole microsystem lifetime was enhanced dramatically. Best results in terms of microsystem lifetime, easy integration, detection limit and analysis time were provided by a PC dialysis membrane with 0.05 μm pore size.

A countercurrent flow mode and a ratio 2:1 between donor and acceptor streams inside the gas-diffusion module were used as previously optimized [25,32].

Chemical and hydrodynamic parameters were also evaluated using a univariate optimization procedure in order to achieve a compromise between baseline signal stability, expected sensitivity, proper working range, maximum analysis throughput and minimum reagents and sample consumption. All optimized parameters, including tested intervals and optimal values, are shown in Table 1.

Other improvements in lifetime were directed to the buffering solution. A TRIS solution is commonly used in ammonium determination procedures using ISEs [22,23,25]. However, as a primary ammine in solution, it decomposes easily to ammonium as a result of bacterial degradation and thermal instability [33,34]. In order to prevent the use of biocides or other preservatives and to simplify the required chemistry, HEPES, phosphate or sulfate buffer solutions were tested. Best analytical features were obtained using HEPES (1 mM) at pH 7, being even slightly better than the ones obtained with TRIS.

Table 1
Optimization of chemical and hydrodynamic parameters.

Parameter	**Tested interval**	**Optimal value**
Injection volume (μL)	25–500	100
Channel flow rate (μL min^{-1})	300–800	650
Buffer solution:		
[HEPES] (mM)	0.5–50	1
pH	7–8	7
[NaOH] (M)	$1 \cdot 10^{-4}$ – 0.1	0.1

A basic solution composed by NaOH (0.1 M) + EDTA (10^{-3} M) was used as donor solution to generate ammonia from ammonium. EDTA was added to avoid the precipitation in form of hydroxide of some metals presents in blood matrix, a fact that could block or damage the microfluidic channels or the PC and PVDF membranes.

Regarding the main interfering compounds of the ammonium ISEs present in plasma or blood, such as sodium or potassium ions [35], none of them are capable of diffusing through the PVDF gas-diffusion membrane, therefore the proposed analytical microsystem performs the highest selectivity versus ammonium ion.

Flow rate of the carrier, donor and acceptor streams were tested from 300 to 800 μL min^{-1} and the sample injection volume was varied from 25 to 500 μL. Best results, as a compromise between the expected analytical and operational features stated before, were obtained using a flow rate of 650 μL min^{-1} for carrier, donor and acceptor streams, and a sample injection volume of 100 μL.

Fig. 4 shows, as example, the recording of the signal and the corresponding curve for one calibration. The obtained Nernst equation (n = 7; 95% confidence) was E = 297 (±3) + 63 (±1) log [NH_4^+] with r^2 = 0.9993. Linear range was from 30 to 1000 μmol L^{-1} NH_4^+ and the limit of detection, according to the IUPAC [36], was 18 μmol L^{-1} NH_4^+. As it can be seen, the obtained linear range included the target concentrations corresponding to normal values of ammonium ion in blood, the cut-off value to identify hyperammonemia in adults (50 μmol L^{-1}) and in children (75 μmol L^{-1}), and typical values of acute cases of hyperammonemia (>200 μmol L^{-1}), so that the analytical features of the microanalyzer were ideal for the desired application. Moreover, it is worth noting that the upper limit of the linear range is defined by the highest concentrated standard solution used during the calibration, which was chosen according to the concentrations most commonly present by patients. In the event that an ammonium concentration in a sample was punctually higher than the maximum established in the calibration curve, the POC analyzer would provide valid results because the ISE presents a linearity two orders of magnitude above this limit.

Repeatability studies were performed by successive injections (n = 10) of three standard solutions of 30, 100 and 1000 μM NH_4^+, in order to test the performance of the microdevice in the lower, medium and upper part of the expected range of ammonium in real samples. RSD of the signals were 4, 2 and 1%, respectively. Control materials and calibration solutions provided by Sant Joan de Déu Hospital (HSJD), were tested to determine method accuracy obtaining differences smaller than 6%. Reproducibility was also tested from 5 calibration experiments performed along 1 month. Sensitivity determined as the mean slope of the 5 calibration curves was 57 mV with RSD value of 8% and the mean of the y-intercept for the Nernst equation was calculated as 333 mV with a RSD of 8%, thereby demonstrating the intra and inter-day precision of the whole system. Reproducibility between POC devices was calculated as the difference in the analytical response of 8 different ISEs. RSD below 2% were obtained in both the mean of y-intercept and slope. A maximum sampling rate of 10 samples h^{-1} was obtained, taking as a reference the most concentrated standard solution (1000 μM), which takes longer to analyze. As the lower the ammonium concentration in the sample the higher the sampling rate, improved sampling rates are foreseen when real samples are analyzed in an automated POC, since not extremely high values are expected in the routine follow-up of the associated diseases. Microsystem lifetime was calculated to be about 3–4 weeks, depending on the number of samples to be analyzed and the matrix (plasma or blood).

All results showed the potentiality and robustness of the whole experimental setup for the proposed application.

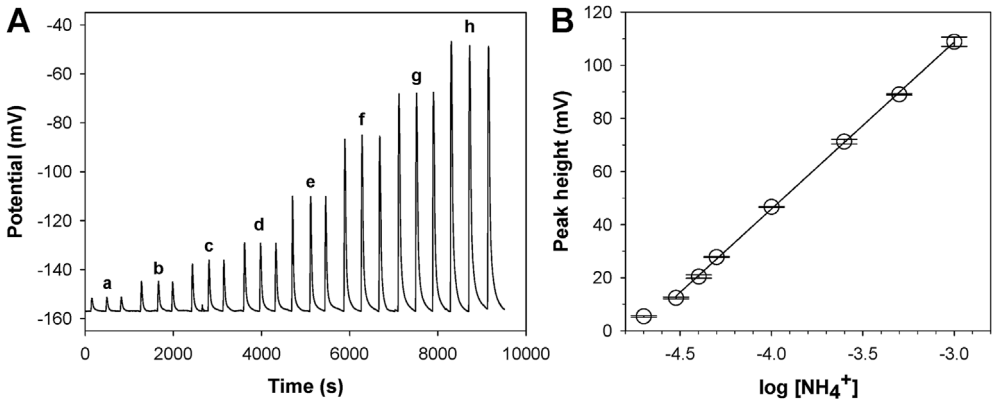

Fig. 4. Signal recording (A) and calibration curve (B) for a POC microanalyzer calibration using standard solutions of NH_4Cl with NH_4^+ concentrations of 20 μmol L^{-1} (a), 30 μmol L^{-1} (b), 40 μmol L^{-1} (c), 50 μmol L^{-1} (d), 100 μmol L^{-1} (e), 250 μmol L^{-1} (f), 500 μmol L^{-1} (g) and 1000 μmol L^{-1} (h).

3.2. Analysis of plasma and blood samples

Different samples of plasma and whole blood from patients with different degrees of hyperammonemia, provided by HSJD, were analyzed using the proposed POC microanalyzer.

First of all, plasma samples with a simpler matrix than that of whole blood were analyzed. Once verified the technical feasibility of the POC microsystem, we proceed with whole blood samples analysis.

Three plasma samples were pretreated with heparin and EDTA as anticoagulant. Differences between results obtained with the POC analyzer and those obtained with the reference method were less than 3% in all cases, thus demonstrating that it is indistinct to use samples pretreated with heparin or EDTA.

Fig. 5 shows the comparative test of both the developed POC microanalyzer and the reference method for 80 plasma samples. From the statistical data treatment, it can be concluded that results obtained with the proposed POC microanalyzer were not significantly different from the ones measured with the reference method according to the paired *t*-test ($t_{calc} = 1.372$, $t_{tab\ two\text{-}sided} = 2.000$, $t_{calc} < t_{tab}$) and to the graphical representation of the results of each method, obtaining a regression equation (n = 80; 95% confidence)

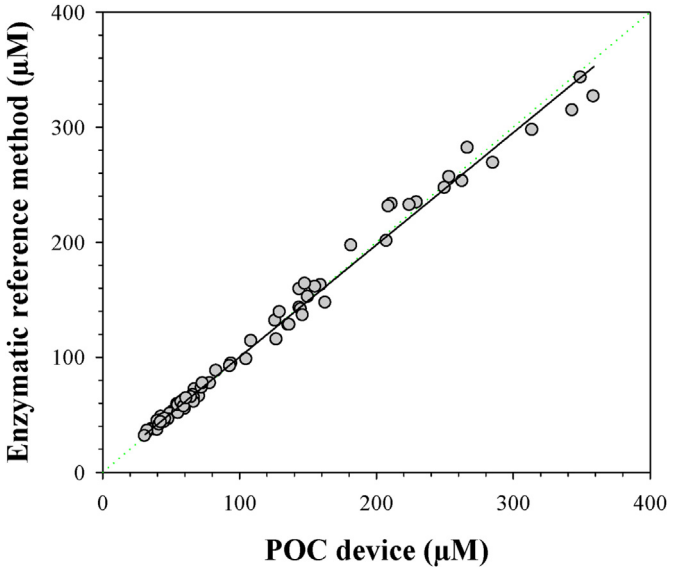

Fig. 5. Comparative study between the results for the analysis of plasma samples (n = 80) obtained by the developed POC microanalyzer and the reference method.

with a intercept of a = 3 ± 3, a slope of b = 0.98 ± 0.02 and a correlation coefficient of 0.9900.

These data show that the plasma matrix do not alter or damage the analytical microdevice nor any of its parts, demonstrating thus the viability of the POC microanalyzer presented for the analysis of ammonium in plasma.

Fig. 6 shows the results obtained from the analysis of 26 whole blood samples. From the statistical data treatment, a systematic error of approximately 12% underestimation was obtained with the POC in comparison with the reference method. This could be explained for two reasons. 1) On the one hand, as the reference method is only prepared for plasma samples, whole blood centrifugation must be previously done to obtain the plasma. This process is likely to release ammonium from the red blood cells to the plasma, which could justify the higher concentration values found. 2) On the other hand, the complex whole blood matrix could alter the ammonium diffusion pattern with respect to the one existing when performing the calibration process with aqueous standard solutions. This fact would cause a lower percentage of diffusion of ammonia through the PVDF membrane, which would lead to slightly lower concentrations than expected. It is worth mentioning that despite these slight differences do not alter the medical decision related with blood ammonium concentrations (since the pathologic threshold range applied in practice is set to avoid these problems and to prevent possible false negatives), we further studied this phenomenon to improve the POC performance.

To evaluate the first hypothesis, two aliquots of the same blood sample were centrifuged. The one to be measured as plasma was analyzed by the reference method analogously to the rest of the samples, and the one to be measured with the POC microanalyzer was vortexed before being analyzed as whole blood. The results obtained with 3 samples did not show significant differences between analyzing whole blood directly or whole blood previously centrifuged and vortexed, so this hypothesis was discarded. Taking into account the results obtained, we can conclude that matrix affects the diffusion profile temporarily, consequently reducing the amount of diffused analyte. In order to avoid this situation, blood matrix standard solutions could be used instead of aqueous ones. However, this would undoubtedly increase the cost per analysis, limit the reagents stability and cause an overestimation of 12% in the results of plasma samples analyzed. With a view of obtaining simple and low-cost microanalyzers to be able to be used in secondary hospitals, health centers or in developing countries, and taking the underestimation in blood samples results as a systematic error, we considered to apply a signal correction by adding the average of the differences between both methods (12% for the 26

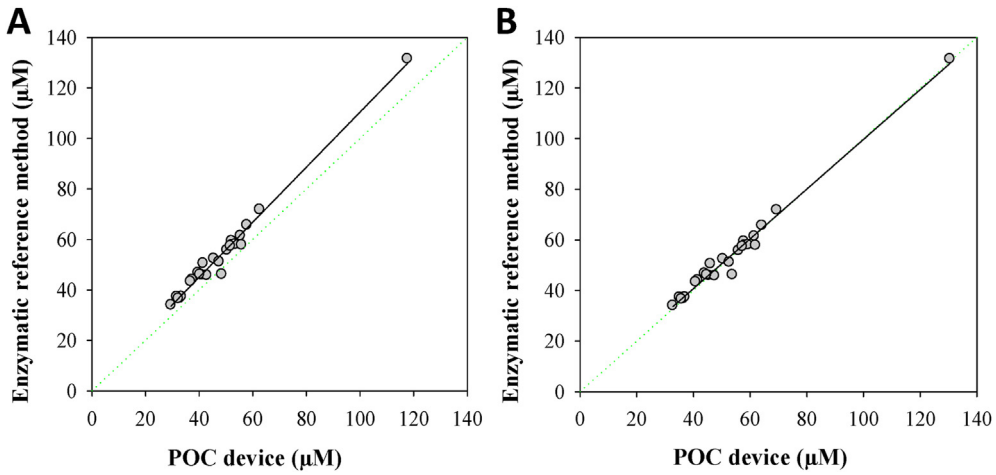

Fig. 6. A) Comparative study between the results for the analysis of whole blood samples (n = 26) obtained by the developed POC microanalyzer and the reference method. **B)** The same comparative study with the correction factor applied to the POC microanalyzer results.

analyzed blood samples), even if its origin is not currently exactly known. Corrected results showed no significant differences according to the paired *t*-test (t_{calc} = 1.152, $t_{tab\ two\text{-}sided}$ = 2.064, $t_{calc} < t_{tab}$) and to the graphical representation of the results of each method, obtaining the following regression equation (n = 26; 95% confidence): $[NH_4^+]_{HSJD}$ = 0.97 (±0.05) $[NH_4^+]_{POC}$ + 1 (±3); R^2: 0.9840. In order to obtain a highly robust and accurate method, a much more extensive study will be carried out in the near future to obtain a statistically representative correction factor with a large enough sample population.

In order to test the repeatability of the microanalyzer with samples, a plasma sample and three whole blood samples were analyzed per quintuplicate. The results show satisfactory results even with complex matrices, with a mean ammonium concentration of 108 ± 5, 44 ± 4, 66 ± 3 and 68 ± 6 μM and RSD values of 2, 3, 2 and 4% respectively.

To sum up, obtained results showed that the proposed analytical POC microsystem is suitable for the determination of ammonium in plasma and is analogous to the reference method in terms of quality of the results and time of analysis, but in addition with a simple and robust operation. For direct blood analysis, attained results were as good as for plasma analysis, if a correction factor was applied. In this case, the advantages are clearly more significant since centrifugation process to obtain plasma is not necessary, which means saving time and resources, and allowing bedside measurements practically immediately.

4. Conclusions

A miniaturized and low-cost POC microanalyzer for direct determination of ammonium ion in whole blood and plasma is developed and successfully applied in samples from patients presenting different plasma ammonium values. In comparison with other previously reported POC analyzers [21], we improve analytical characteristics in terms of working range and limit of detection, and regarding the operational mode, it results in simplicity and ease of automation in continuous mode, with good precision, repeatability and reproducibility. In addition, it is a much smaller and cheaper analytical system than common clinical laboratory equipment. Moreover, thanks to its reduced dimensions, it consumes few amounts of reagents (which are common reagents indeed) and sample and shows a relatively fast analysis time. Above all, the fact that direct whole blood can be analyzed, allows avoiding the centrifugation process, thus saving time, which makes the proposed device much more competitive and attractive than current ammonium analytical systems.

Results and analytical features show that after an industrialization stage, the potentiometric POC microanalyzer assesses the commercialization potential in clinical applications for the bedside determination of ammonium in blood and plasma as well in primary or secondary care centers as in health centers in developing countries, as alternative of current more expensive equipment.

CRediT authorship contribution statement

Antonio Calvo-López: Conceptualization, Microsystem design and fabrication, Methodology, development, Investigation, Raw data analysis, Formal analysis, Validation, and, Writing – original draft. **Beatriz Rebollo-Calderon:** Microsystem fabrication, Investigation, Raw data analysis, Formal analysis, and, Writing – review & editing. **Aida Ormazábal:** Samples preparation, Validation, and, Writing – review & editing. **Rafael Artuch:** Conceptualization, Resources, Supervision, Project administration, and, Funding acquisition, Writing – review & editing. **Javier Rosell-Ferrer:** Electronics integration, Writing – review & editing. **Julián Alonso-Chamarro:** Conceptualization, Resources, Supervision, Project administration, and, Funding acquisition, Writing – review & editing. **Mar Puyol:** Conceptualization, Resources, Supervision, Project administration, and, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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