Biosensors and Bioelectronics 26 (2010) 1521–1527

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Electrochemistry-on-chip for on-line conversions in drug metabolism studies

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ARTICLE INFO

Article history:
Received 14 April 2010
Received in revised form 23 July 2010
Accepted 26 July 2010
Available online 3 August 2010

Keywords:
Electrochemistry
Lab-on-chip
Drug metabolism
High-turnover
Lab-in-a-Briefcase
Procainamide
IrOx

ABSTRACT

We have designed an integrated 3-electrode electrochemical cell on-chip with high analyte conversion rates for use in drug metabolism studies. The electrochemical cell contains platinum working and counter electrodes and an iridium oxide pseudo-reference electrode. The pseudo-reference electrode has a pH sensitivity of −52 mV/s, and thus will provide a constant potential in solutions with known and constant pH. The average drift of the iridium oxide electrode is below 5 mV for a typical 15 min conversion experiment. We have been able to mimic the oxidative drug metabolism reactions catalysed by enzymes of the cytochrome P-450 family, normally occurring in the human body. With the chip, the different reaction products of both rat liver cell microsome and human liver cell microsome incubations have been observed.

1. Introduction

Electrochemistry (EC) is recently gaining more attention as a tool in rapid, on-line drug screening as it can be used to mimic oxidation reactions occurring in the human body. Oxidation reactions play a crucial role in the biotransformation of drugs. By introducing polar functional groups, like hydroxyl groups, into the drug molecule, the organism facilitates the detoxification and excretion of xenobiotics (Guengerich, 2007). However, from the biotransformation of specific compounds, metabolites may arise, which bear an increased toxicity (bioactivation). One typical example are quinones and quinone imines, which are very often highly reactive, electrophilic compounds, formed upon oxidative metabolism (Park et al., 2000). During drug development processes, drug candidates, potentially undergoing bioactivation into toxic metabolites, have to be excluded from the further development process as early as possible. Conventional drug metabolism studies include in vitro tests, utilizing liver cells and liver cell extracts (Iyer and Zhang, 2007) as well as in vivo tests with laboratory animals. Most frequently used cell extracts to study oxidative metabolism reactions are liver microsomes from humans and rats (HLM and RLM). Liver microsomes contain a high enzyme concentration of the cytochrome P-450 group, which catalyses the majority of oxidative metabolism reaction (Brandon et al., 2003).

As a purely instrumental alternative to investigate drug oxidation reactions, the electrochemical simulation has been developed. Potential oxidative metabolites are generated in an electrochemical cell and are subsequently identified by on-line liquid chromatography/mass spectrometry (EC/LC/MS). The major benefit of this instrumental approach, compared to metabolism studies based on hepatic cell matrices, is that reactive metabolites can be directly detected. In vitro, those reactive metabolites frequently remain unidentified, because the compounds bind to proteins in the cell matrix (Lohmann and Karst, 2007). Moreover, besides being a rapid screening tool for oxidative metabolites, EC/LC/MS can be used to study the reactivity of metabolites towards cellular compounds like proteins or DNA and thus allows the evaluation of a potential liver toxicity (Lohmann et al., 2009).

Examples of the electrochemistry-based drug screening in comparison to traditional techniques are described in literature (Baumann et al., 2009; van Leeuwen et al., 2005) where the metabolic pathway of tetrazepam and clozapine is studied. Tetrazepam is a benzodiazepine derivative and mainly used as muscle relaxant, while clozapine is an anti-psychotic drug. Also reported is the electrochemical oxidation of metoprolol (Johansson et al., 2007) a selective β_1 receptor blocker. In that paper, it is described to which extent electrochemical oxidation can mimic typical metabolism reactions. Current developments and applications of the electrochemical metabolism simulation have been summarized in three reviews (Baumann and Karst, 2010; Lohmann and Karst, 2008; Permentier et al., 2008).

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doi:10.1016/j.bios.2010.07.102

Recently, a paper has been published where electrochemical oxidation of a drug is studied to mimic the phase-1 metabolites of Verapamil, a known antiarrythmic drug (Nissila et al., 2009). The chip fabricated in this study was using the photo-catalytic effect of titanium dioxide to oxidise the introduced species. This chip also contained an electrospray ionisation needle to directly introduce the metabolites into the MS, thus minimizing the volume between the reaction area and the ionisation interface.

We have already shown that a microfluidic electrochemical cell can be used successfully (Odijk et al., 2009) to study, e.g., the metabolic pathway of amodiaquine, a known antimalarial agent. The major benefits of using a microscale electrochemical cell is that diffusion lengths are greatly reduced. Therefore, reaction rates are no longer determined by diffusive mass transport and we have observed total conversion of electrochemically active species for small and fast reacting ions. Moreover, the required sample volumes are reduced, since the internal volume of the chip is typically in the nanoliter range. The major advantage of our chip over the photo-catalytic chip (Nissila et al., 2009) is that we have more control over the electrochemical reactions taking place. Moreover, we can also measure the oxidation current. Finally, we can achieve higher oxidation potentials than possible with the photo-catalytic chip. Therefore, a wider range of oxidation reactions mimicked by the electrochemical cell can be observed, which is especially important in (partly) organic solutions were the conductivity is lower.

Here we present an improved version of the electrochemical 3-electrode cell on-chip. The previous chip (Odijk et al., 2009) contained a palladium pseudo-reference electrode. Using this metal, the fabrication process is limited to temperatures up to 500 °C. At temperatures above 500 °C the palladium thin-film starts to agglomerate. Therefore, the bonding of glass during the chip fabrication was not as good as desired. As a result, the previous chip could not withstand fluidic pressures above approximately 500 kPa. The mechanical stability of the palladium film was inferior if the chip was used for longer periods of time. Especially in strong acidic solutions the film detached after a few minutes, probably caused by intrinsic stress induced by hydrogen uptake into the palladium (Ives and Janz, 1961). In the improved version of this chip, the palladium electrode is replaced by an iridium oxide (IrOx) film. IrOx is mainly known as pH electrode (Carroll and Baldwin, 2010; Wang et al., 2002; Yao et al., 2001). However, in solutions with known pH it can be used as pseudo-reference electrode (Li et al., 2009; Schabmueller et al., 2006; Yang et al., 2004). The major benefit of using IrOx is that it can withstand much higher temperatures (Midgley, 1990), which is especially important for chip fabrication.

We have tested both the mechanical and electrochemical stability of the IrOx pseudo-reference electrode, of which the results are included in this paper. Moreover, the metabolism pathway of procainamide is studied on-chip. Procainamide is a known antiarrythmic drug (Ha and Follath, 2004). In this paper mass spectrometry (MS) measurements of the different metabolites separated by liquid chromatography (LC) are reported. We compare MS results from liver cell microsome incubations to MS results from electrochemical oxidation on-chip, to show that the chip is a feasible alternative to microsomal studies.

2. Materials and methods

2.1. Chip fabrication

The chip design is aimed at high conversion of introduced analyte. In our previous paper, it is explained how the geometry of electrodes and fluidic channels is optimized towards high-turnover rates (Odijk et al., 2009). The chip layout presented here is designed following the same design principles as described earlier.

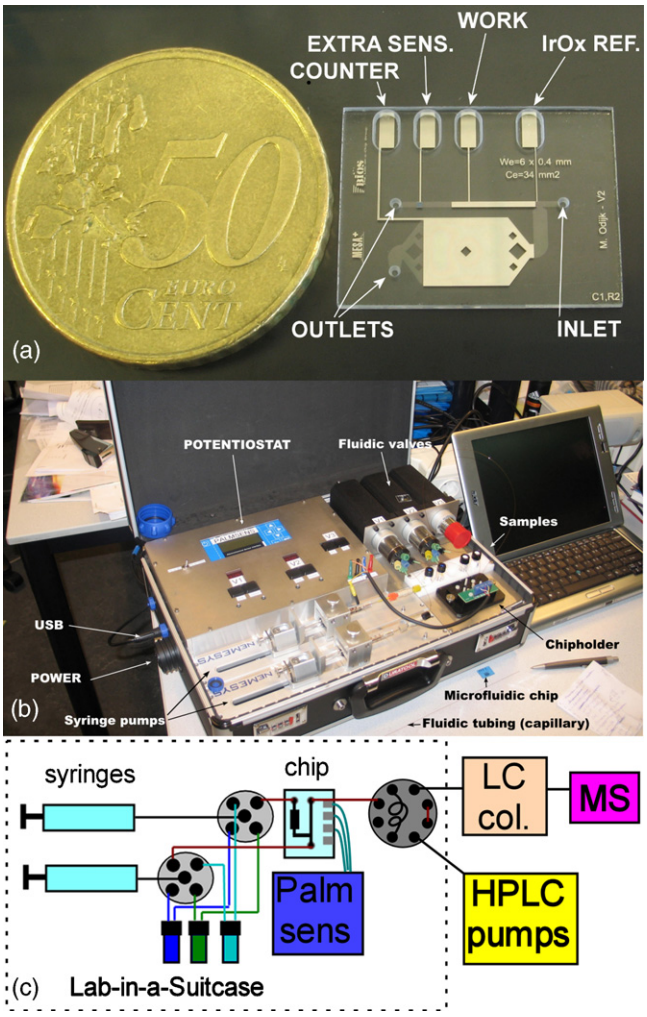

Fig. 1. (a) Photo of the electrochemical cell on-chip. In the picture, fluidic inlet and outlets and the working, reference and counter electrode are indicated. (b) Photo of the Lab-in-a-Briefcase setup. In the picture, chipholder, syringe pumps, fluidic switching and injection valves, sample vials and the portable potentiostat are indicated. (c) Schematic overview of the total setup including the Lab-in-a-Briefcase, LC pumps and mass spectrometer.

In Fig. 1a, a photo of a finished chip is shown. The fluidic port at the right side is used as main inlet. The fluidic port at the left side is connected to the external LC and MS devices. The fluidic port on the lower left side is used as outlet during measurements, to extract a fraction of the total injected sample volume. Only during cleaning, this port is used as a second inlet. The platinum working electrode is located in the main fluidic channel. This channel is 4 μm high, 400 μm wide and 10 mm long. On the right side of the working electrode, the IrOx reference electrode is visible. The platinum counter electrode is placed in a separate side-channel to prevent unwanted species to appear in the measured mass spectra. This channel is much larger to contain the larger counter electrode. The counter electrode is made this large to prevent gas formation inside the chip. The main channel volume is only 16 nL, while the total chip volume is 180 nL.

The chip is made of two glass borofloat wafers. On the first wafer, wells are etched using buffered HF to adjust the electrode surface 10 nm below the glass surface. Next, electrodes are patterned by lithography using lift-off techniques. A thin film of 135 nm platinum is deposited onto an adhesion layer of 10 nm tantalum. For the IrOx, the same etching and lift-off steps are repeated. A thin film of 73 nm IrOx is sputtered on a sandwich of 10 nm titanium,

107 nm platinum and 10 nm titanium, where titanium is acting as adhesion layer and platinum as conducting layer. The iridium oxide is sputtered using an iridium 4 inch target (obtained from Kurt J. Lesker). The sputtering power applied was 46 W DC, with 20 sccm Argon and 20 sccm oxygen gas flow into the sputtering chamber. This sputtering is conducted using an in-house developed sputtering machine, making it possible to keep the wafer in vacuum for the deposition of the whole IrOx/Ti/Pt/Ti sandwich layer.

On the second wafer, a layer of 10 nm chromium and 100 nm gold is sputtered, which acts as etching mask for the fluidic channels. These 4 μm deep channels are etched into the glass with 25% HF. Afterwards, Ordyl BF410 foil is laminated onto the other side of the top wafer and patterned with lithography. Fluidic inlets and contact holes for the electrode pads are made using powderblasting. Finally, the foil and the chromium/gold layer is removed and the two wafers are directly bonded at a temperature of 600 °C. In a last step, the combined wafers are diced into individual chips of 15 mm × 20 mm.

2.2. Chemicals

For the pH-response measurement a linear pH buffer was used containing 0.033 M boric acid, 0.033 M citric acid, 0.033 M potassium dihydrogenphosphate. This buffer was titrated during the experiment with 0.1 M potassium hydroxide. For the open-circuit measurements, a 0.1 M potassium dihydrogenphospate solution was used with pH adjusted to 7.4.

For the electrochemical metabolism study, 100 μM procainamide was dissolved in a solution containing 1 mM ammonium acetate. Between each experiment, the chip was flushed with a 50% acetonitrile/50% water solution containing 0.1 vol.% acetic acid.

Chemicals used for the microsomal incubation: 50 mM phosphate buffer solution (potassium dihydrogenphosphate, dipotassium hydrogenphosphate, adjusted to pH 7.4), magnesium chloride hexahydrate and NADPH (AppliChem, Darmstadt, Germany). Pooled male rat liver microsomes (RLM) (Sprague Dawley) and pooled human liver microsomes (HLM) both with a protein concentration of 20 mg/mL, were delivered by BD Bioscience (Woburn, MA, USA). Unless otherwise specified, all chemicals were obtained from Sigma–Aldrich.

2.3. Setup

The drug metabolism measurements were conducted with the in-house developed Lab-in-a-Briefcase. A photo of this setup is shown in Fig. 1b. An overview of this total setup is given in Fig. 1c.

In the lower right side of Fig. 1b, the chip is placed inside an in-house developed chipholder. In this holder, fluidic connections are provided by commercially available connectors (nanoports, Upchurch scientific), while electrical connections are provided by spring metal contact probes (ISI Interconnect Specialities Inc.). The flows over the chip are regulated in such a way that the generated products at either the working or counter electrode cannot reach the iridium oxide pseudo-reference electrode. This is done using an inward flow of 0.25 μL/min at the inlet port (visible on the right hand side of Fig. 1a) and a syringe pump connected to the side-channel pumping at an outward flow of 0.125 μL/min, which is exactly half of the rate of the inward flow. As a result, the flow rate over the main channel and into the MS is equal to 0.125 μL/min. Since, at these flow rates, mass transport due to convection is more dominant compared to mass transport by diffusion, generated products cannot reach and disturb the potential of the pseudo-reference electrode. These flow rates have been tested and determined to give the most oxidation products.

The chipholder is connected to two switching valves and one injection valve using fused silica glass capillary (Valco type C75X-6694EMH and C72MX-4698ED, Vici Valco Instruments Co. Inc.). The switching valves are connected to several sample vials and to two syringe pumps (Nemesys, Cetoni GmbH, Germany). The total volume of all the tubing, valves and connectors is calculated to be approximately 5 μL, excluding two 50 μL syringes (Type 1705N, Hamilton Bonaduz AG).

Potentials are applied to the chip using a portable potentiostat (Palmsens, Palm Instruments BV, the Netherlands). The whole briefcase is controlled by a laptop running a single home-build labview program (Labview, National Instruments Corporation).

The injection valve was connected to a sample loop of 5 μL. After filling the loop with the analyte, the valve was switched and a sample plug was flushed with 500 μL/min onto a LC column. The LC separation was performed on a Nucleodur Sphinx RP column (Macherey-Nagel, Düren, Germany) with the dimension 150 mm × 3 mm, particle size 5 μm and pore size of 10 nm. The mobile phase consists of 10 mM aqueous ammonium formate buffer with 0.02% formic acid as eluent A and acetonitrile as eluent B. For the separation, the following gradient programme was used: 0–2 min isocratic at 15% B, 2–10 min gradient from 10% to 50% B, 10–11 min gradient from 50% to 95% B, 11–13 min isocratic at 95% B, 13–16 min equilibration at 15% B.

A LC system was used from Shimadzu (Duisburg, Germany) coupled to a QTRAP mass spectrometer with an electrospray ionisation (ESI) source (Applied Biosystems, Darmstadt, Germany), working in positive ion mode. The LC system consists of two LC pumps (LC-10ADVP), a degasser (DGU-14A), an autosampler (SIL-HTA), a column oven (CTO-10AVP) and an UV-detector (SPD-10AVVP) operating at 254 nm. Control of the LC/MS system and data handling was carried out by the software Analyst 1.4.1 (Applied Biosystems). For the determination of exact masses and molecular formulas, single measurements were repeated using an LC system from Antec Leyden (Zoeterwoude, The Netherlands) coupled to a microTOF mass spectrometer, which was equipped with an ESI source (Bruker Daltonics, Bremen).

The pH response and potential stability measurements of the IrOx pseudo-reference electrode were conducted off-chip, using a processed wafer which is not bonded to the wafer containing the fluidic channels and inlets. These measurements are recorded with a VSP potentiostat (Bio-Logic SAS, France) using a silver/silverchloride saturated KCl reference electrode (Radiometer Analytical) and platinum counter electrode. The pH was measured using a battery-powered portable pH meter (CyberScan pH 310, Eutech Instruments). During the determination of the pH, the IrOx electrode was removed temporarily from the solution to prevent interference with the pH meter.

2.4. Methods

2.4.1. Electrochemical metabolism simulation

For the on-chip drug metabolite study, a series of experiments was conducted to determine the optimal potential. The optimal potential for oxidation was determined to be 1.75 V, since it gave the most oxidation products without generating compliance errors on the potentiostat. This potential was applied for 60 min before injecting the sample plug onto the LC column, to make sure that the sample loop was completely filled with the generated oxidation products.

2.4.2. Microsomal incubations

A mixture of microsomal protein and procainamide, dissolved in phosphate buffer solution was preincubated for 5 min at 37 °C. Magnesium chloride and NADPH were added to the incubation mixture, which was then further incubated at 37 °C for 90 min.

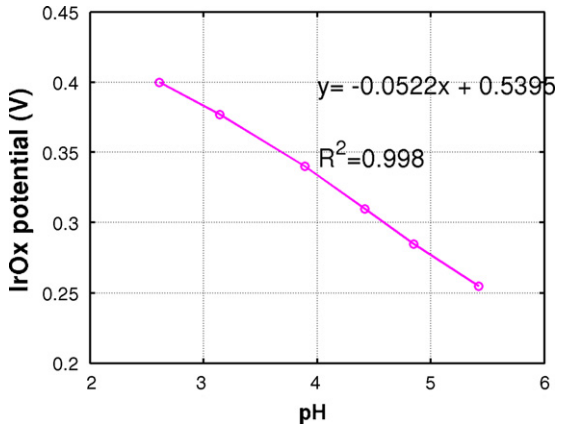

Fig. 2. pH sensitivity of the IrOx electrode. Potential measured vs. Ag/AgCl sat. KCl reference electrode. The IrOx electrode shows a pH sensitivity of −52 mV/pH.

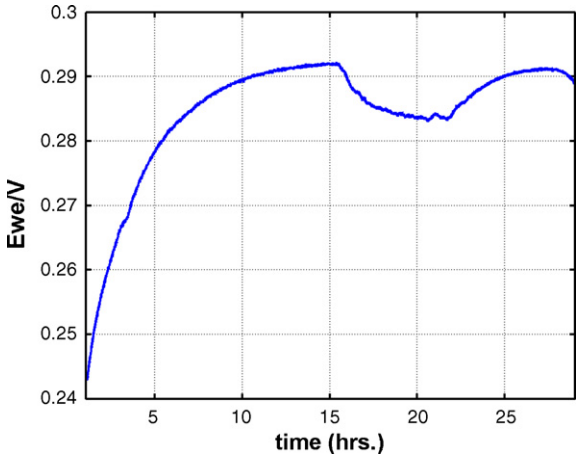

Fig. 3. Potential stability measurements of the IrOx electrode. Potential measured vs. Ag/AgCl sat. KCl reference electrode. An initial drift of 50 mV and a long-term drift within a range of 8 mV is observed.

The total volume of the incubation mixture was 500 μL for RLM incubation mixtures and 250 μL for HLM incubation mixtures. The final concentrations were as follows: 1.3 mg/mL microsomal protein, 50 μM procainamide, 0.5 mM magnesium chloride, 1.2 mM NADPH. Subsequent to the incubation, proteins were precipitated by adding 500 μL acetonitrile (250 μL for HLM). After centrifugation, the supernatant was analysed by LC/MS. The LC/MS conditions are the same as described for the electrochemical simulation, only the injection volume was increased to 10 μL. As negative control, a second incubation without NADPH was performed.

3. Results and discussion

3.1. IrOx pH response

In Fig. 2, the potential of the IrOx electrode is shown in the pH range of 2.7–5.5, measured versus an Ag/AgCl sat. KCl reference electrode. The measured data points are fitted to a linear response of −52 mV/pH, which is slightly lower than the theoretically expected value of −59 mV/pH (Bard and Faulkner, 2001). The pH response strongly depends on the way it is fabricated as shown in literature in a nice summary (Yao et al., 2001). In this summary the pH response for sputtered IrOx films (SIROFs) varies between −54 and −60 mV at room temperature, which is slightly higher than our measured value.

3.2. IrOx stability

The potential of the IrOx is also observed for a period of 29 h in a phosphate buffer solution with pH adjusted to pH 7.4. The results are shown in Fig. 3. This experiment was carried out at ambient temperature. Also, the solution was exposed to air to get a similar environment to the drug metabolism studies. Exposure to air can possibly influence the potential of the IrOx electrode, because of interaction with dissolved oxygen in the solution (Olthuis et al., 1990).

The initial potential shift of the IrOx electrode was 50 mV over a period of 15 h after the start of the experiment, followed by a long-term drift within a range of 8 mV. The initial drift is known to result from hydration effects after the initial film fabrication (Marzouk et al., 1998; Yao et al., 2001). The maximum drift was approximately 10 mV/h during the initial phase. The long-term drift was below 2 mV/h.

In other literature describing IrOx pseudo-reference stability measurements (Yang et al., 2004), an initial drift of 120 mV over 1 day and a long-term drift less than 20 mV over 9 days is reported using anodically grown IrOx films (AIROFs). Compared to the drift values reported in literature, our IrOx electrode performs better, both in terms of initial and long-term drift.

Compared to the large overpotentials applied during drug metabolite studies, this drift is well within acceptable limits. A typical drug conversion experiment takes approximately 15–20 min. Therefore, the expected drift of the pseudo-reference is estimated to be below 5 mV for a single experiment.

Over a period of 8 months of continuous exposure of IrOx to both neutral and acidic solutions we have not observed any detachment or mechanical deformation, thus indicating the excellent long-term stability of the electrodes.

3.3. Procainamide metabolism study

Procainamide (p-amino-N-[2-(diethylamino)ethyl]benzamide, PA, *m*/*z* 236), is given for the treatment of both ventricular and supra-ventricular arrhythmias. It is especially useful in the case of lidocaine-resistant ventricular arrhythmias during acute myocardial infarction (Ha and Follath, 2004). The metabolism mechanism is indicated in Fig. 4. During biotransformation of procainamide, conjugation reactions take place, which result in the formation of N-acetylprocainamide. Upon oxidative metabolism N-monodealkylprocainamide (DEPA, *m*/*z* 208), N-hydroxylamine procaineamide (NOH-PA, *m*/*z* 252) and the N-oxide derivative of procainamide (NO-PA, *m*/*z* 250) are formed as major metabolites. The latter metabolite is highly reactive and may bind to macromolecules, leading to the drug-induced lupus erythematosus syndrome observed during chronic therapy with procainamide (Ha and Follath, 2004). The goal of our study is to simulate the formation of the main oxidative metabolites of PA with the on-chip electrochemical cell and to compare it with the results of the conventional microsomal studies.

Fundamentally, there are differences between direct electrochemical and enzymatic oxidation as the underlying mechanisms are different. However, a large number of reactions can be simulated by EC/MS, while only a few of these reactions cannot be simulated as described in several review articles (Baumann and Karst, 2010; Lohmann and Karst, 2008). The most precise data are generated from isolated enzymes of the CYP-450 family. However in the organism, there is a strong variation in kind and concentration of the individual enzymes of this group. Therefore, data obtained using individual enzymes are scientifically well defined, but not always the best way of predicting toxic effects on a complex organism. EC/MS certainly will never replace the use of CYP-450

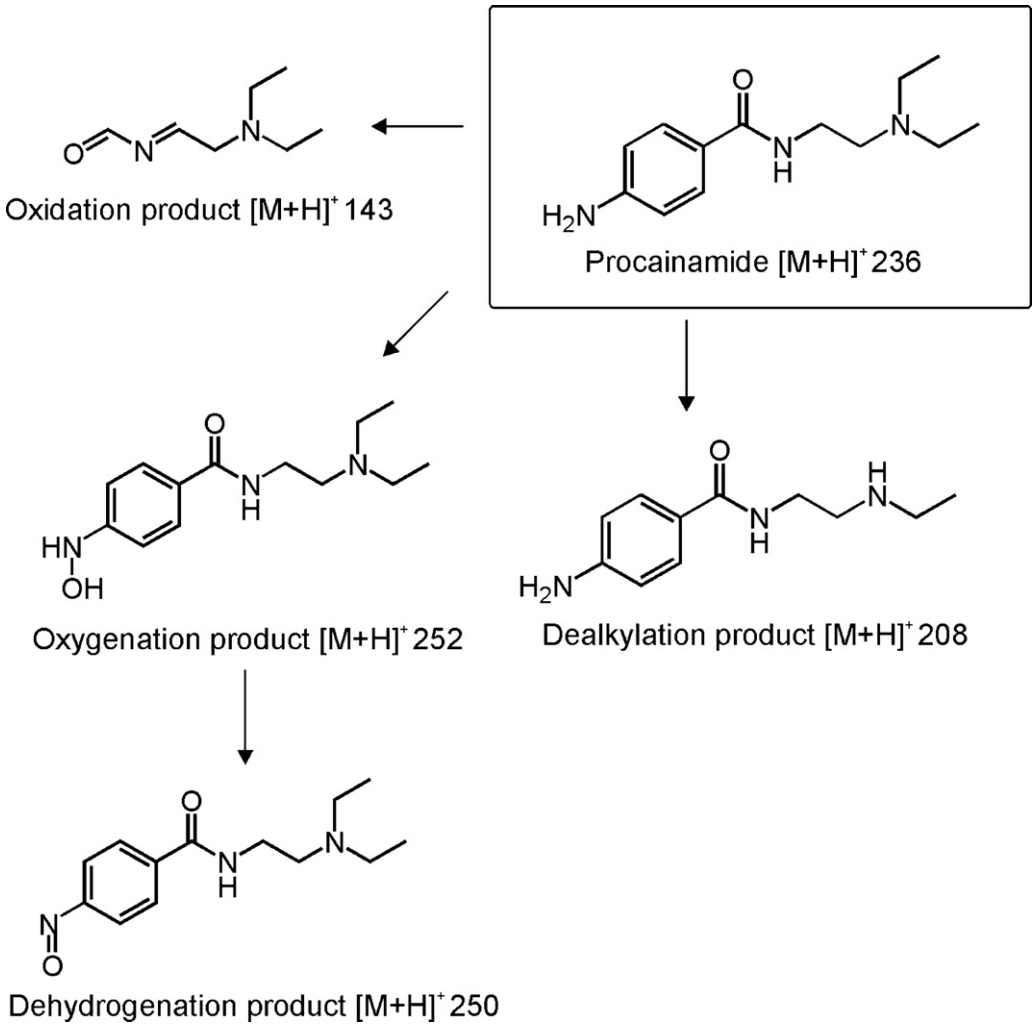

Fig. 4. Oxidative metabolites of procainamide (*m*/*z* 236). Upon oxidative metabolism N-monodealkylprocainamide (DEPA, *m*/*z* 208), N-hydroxylamine procaineamide (NOH-PA, *m*/*z* 252) and the N-oxide derivative of procainamide (NO-PA, *m*/*z* 250) are formed as major metabolites.

enzymes (isolated or in microsomes), but it can be used as complementary method.

In vitro microsomal incubations have been conducted with RLM and HLM, respectively. The incubation mixtures have been analysed by LC/MS measurements. Extracted ion traces of PA and its metabolites are shown in Fig. 5a and b. The structures of the metabolites are presented in Fig. 4. In the RLM incubation mixture, the metabolites NOH-PA (*m*/*z* 252) and NO-PA (*m*/*z* 250) were identified. In contrast, in the HLM incubation mixture the dealkylation product DEPA (*m*/*z* 208) and the oxygenation product NOH-PA were detected. These results are in agreement with the metabolites of PA described in the literature (Ha and Follath, 2004). The differences between the RLM and the HLM incubation mixtures are due to the varying distribution of isoforms of the enzym group cytochrome P-450 in each organism. Besides that, the absence of the reactive metabolite NO-PA (*m*/*z* 250) in the HLM incubation mixture may result from a protein binding of NO-PA to cellular proteins.

Using the on-line system consisting of the electrochemical cell, integrated on chip, the LC separation and subsequent MS detection, the metabolism of PA was simulated. Fig. 5c shows the extracted ion traces of PA and its oxidation products, formed at a potential of 1750 mV (vs. IrOx pseudo-reference) applied to the working electrode. The used flow rate was 0.125 μL/min over the main channel of the chip. The structures of the oxidation products are shown in Fig. 4. As can be seen from Fig. 5c, the formation of

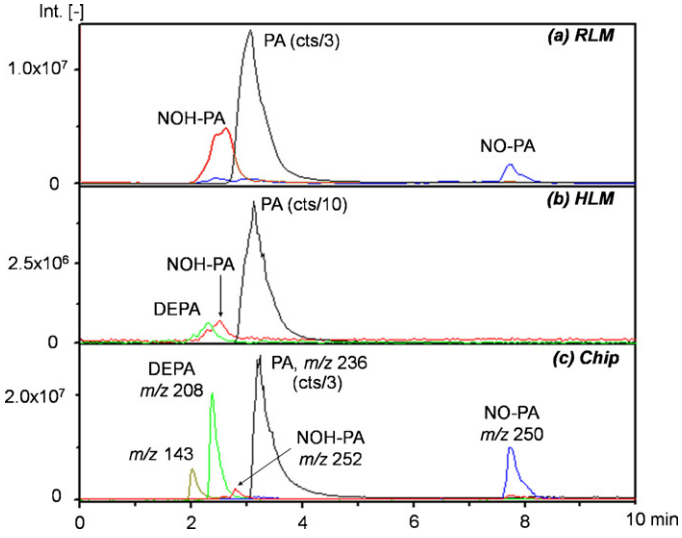

Fig. 5. Extracted ion traces of PA and its oxidative metabolites DEPA, NO-PA, NOH-PA. (a) LC/MS analysis of the RLM incubation mixture of PA. (b) LC/MS analysis of the HLM incubation mixture of PA. (c) EC on-chip oxidation of the metabolism of PA (1750 mV). Structures of the oxidation products are shown in Fig. 4.

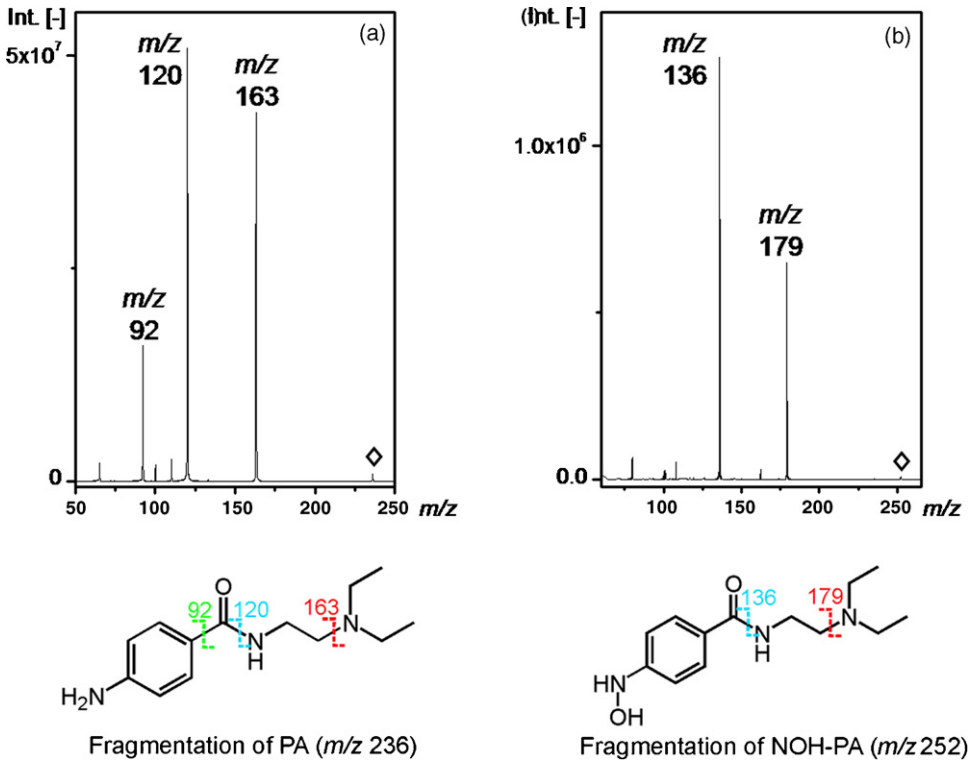

Fig. 6. Product ion spectra of (a) PA and (b) NOH-PA, obtained by chip/LC/MS/MS measurements. The fragmentation patterns correlate to the structures shown below the spectra. NOH-PA was formed on chip at a potential of 1750 mV vs. IrOx pseudo-reference.

all three metabolites occurring in RLM and HLM (DEPA, NOH-PA, NO-PA) was successfully mimicked on-chip. Besides that, the oxidation product *m*/*z* 143 was formed. The proposed structure of this compound, shown in Fig. 6, indicates an oxidative cleavage in the benzylic position.

In order to ensure that the identified products derive from the oxidation on chip, blank measurements without applied potentials were performed. The dealkylation product DEPA was also present in the blank measurement but the intensities were approximately 10 times lower than at 1750 mV. The presence of DEPA in the blank sample might result from PA impurities or from air oxidation in the sample.

For identification of the oxidation products the exact masses were determined by time-of-flight mass spectrometry (ToF/MS). The deviation between the calculated and the measured *m*/*z* was below 10 ppm for all compounds. Moreover, fragmentation experiments (LC/MS/MS) of PA and its oxidation products have been performed. Fig. 6 shows the fragmentation of PA and NOH-PA. All fragmentation patterns (product ion spectra) correlate to the structures shown in Fig. 4.

4. Conclusion

An on-chip electrochemical cell was fabricated for routine use in drug metabolism studies. The electrochemical cell contains an integrated 3-electrode electrochemical cell with platinum working and counter electrodes and an IrOx pseudo-reference electrode. The internal volume of the main channel of this chip is 16 nL and the total internal volume of the chip is 180 nL. The pseudo-reference electrode has a potential drift lower than 5 mV during a typical drug conversion experiment (15–20 min). Therefore, the potential of the pseudo-reference electrode provides a stable operation for solutions with known and constant pH. During the entire series of experiments no mechanical problems have been observed with the chip, even at high flow rates or acidic conditions.

The chip has been used routinely in our Lab-in-a-Briefcase instrument over the last year for the drug metabolism study of procainamide, a known antiarrythmic drug. The chip is used to mimic the oxidative mechanisms of the enzymes of the cytochrome P-450 family. The generated metabolites of rat and human liver microsomes are compared to the generated metabolites on-chip. The chip was able to generate all the known metabolites of both types of microsomes in a single experiment. Therefore, we have successfully mimicked the drug metabolism of procainamide in our electrochemical cell on-chip. In the case of procainamide, on-chip oxidation actually proves to give more information on the generated metabolites in a single experiment compared to microsomal studies.

Acknowledgements

Hans de Boer is gratefully acknowledged for the mechanical work on the Lab-in-a-Briefcase. We would also like to thank Jan van Nieuwkasteele and Johan Bomer for the assistance during the chip fabrication. The authors would like to thank STW (Stichting Technische Wetenschappen) and the DFG (Deutsche Forschungsgemeinschaft, Bonn, Germany) for financial support of this project.

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