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Capillary liquid chromatography–microchip atmospheric pressure chemical ionization–mass spectrometry

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A miniaturized nebulizer chip for capillary liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (capillary LC–microchip APCI-MS) is presented. The APCI chip consists of two wafers, a silicon wafer and a Pyrex glass wafer. The silicon wafer has a DRIE etched through-wafer nebulizer gas inlet, an edge capillary insertion channel, a stopper, a vaporizer channel and a nozzle. The platinum heater electrode and pads for electrical connection were patterned on to the Pyrex glass wafer. The two wafers were joined by anodic bonding, creating a microchip version of an APCI-source. The sample inlet capillary from an LC column is directly connected to the vaporizer channel of the APCI chip. The etched nozzle in the microchip forms a narrow sample plume, which is ionized by an external corona needle, and the formed ions are analyzed by a mass spectrometer. The nebulizer chip enables for the first time the use of low flow rate separation techniques with APCI-MS. The performance of capillary LC–microchip APCI-MS was tested with selected neurosteroids. The capillary LC–microchip APCI-MS provides quantitative repeatability and good linearity. The limits of detection (LOD) with a signal-to-noise ratio (S/N) of 3 in MS/MS mode for the selected neurosteroids were 20–1000 fmol (10–500 nmol l^{-1}). LODs (S/N = 3) with commercial macro APCI with the same compounds using the same MS were about 10 times higher. Fast heat transfer allows the use of the optimized temperature for each compound during an LC run. The microchip APCI-source provides a convenient and easy method to combine capillary LC to any API-MS equipped with an APCI source. The advantages and potentials of the microchip APCI also make it a very attractive interface in microfluidic APCI-MS.

Introduction

Liquid chromatography–mass spectrometry (LC–MS) is a powerful analytical technique widely applied in bioanalysis, life sciences, drug research, and environmental research. From the different interfacing techniques developed for LC–MS, the most viable have been the atmospheric pressure ionization (API) techniques: electrospray ionization (ESI),[1,2] atmospheric pressure chemical ionization (APCI),[3,4] and atmospheric pressure photoionization (APPI).[5,6] These API techniques provide an efficient ionization for a wide variety of molecules. At present ESI is the most common ionization method, and until now it has been the only ion source available for capillary LC applications and microfluidics. The flow rates used with commercial APCI and APPI ion sources are high, typically above 50 μl min^{-1}, making them incompatible for low flow rate separations.

With the pressing need for analytical methods faster than capillary LC–MS still having the same sensitivity of detection, microfluidic separation systems combined with MS have been intensively studied. Research into these microchip–MS devices has been concentrated almost totally on microchip ESI techniques. ESI can be easily incorporated into microfluidic systems,[7–10] and the flow rates used in microfluidic separations (sub-μl min^{-1}) are ideal for ESI-MS.

ESI is an excellent technique for ionic and polar compounds, but only polar solvents can be used and the ionization efficiency for neutral and non-polar compounds may be poor. In contrast, APCI provides high ionization efficiency for polar and neutral compounds,[3,4] allows use of polar and non-polar solvents and tolerates higher electrolyte concentrations. Additionally, suppression of ionization by the co-eluting compounds is significantly less with APCI than with ESI.[11]

We have earlier presented a miniaturized ion source, a microchip heated nebulizer for microchip APCI-MS.[12] The microchip APCI provides flow rates down to 50 nl min^{-1}, excellent sensitivity, robust analysis and efficient ionization for

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neutral and non-polar compounds in addition to polar and ionic compounds. The microchip APCI is directly compatible with microfluidic systems as well as with nano- and capillary LC. Furthermore, Kauppila *et al.* demonstrated that the microchip heated nebulizer can be used for microchip APPI-MS,[13] and Östman *et al.* demonstrated its use in GC–microchip APCI-MS.[14] Here we present a miniaturized heated nebulizer for coupling of capillary LC to MS. The feasibility of new microchip APCI for quantitative LC–MS work was investigated with a set of neurosteroids.

Experimental

Fabrication of the miniaturized heated nebulizer

Double-side polished 100-oriented silicon wafers (380 μm thick, high resistivity >500 Ω cm) were used as substrates, and 500 μm thick Pyrex glass wafers (Corning #7740) as channel cover plates. Three photomasks were required for chip fabrication. The first mask defined the capillary insertion channel, vaporizer channel, and the nozzle on the silicon wafer top side, mask two was for inlet holes etching from the wafer backside (for nebulizer gas), and mask three defined the platinum heater on a glass wafer. Channel dimensions and shapes were designed to take advantage of the excellent dimensional control in deep reactive ion etching (DRIE).

Silicon wafers were cleaned in ammonia–peroxide and hydrogen chloride–peroxide mixtures (RCA-1 and RCA-2), followed by thermal oxidation at 1000 °C, which resulted in 1000 nm thick oxide. Photoresist (AZ 5214 , 1.4 μm thick) was applied on the top side, and the capillary insertion channel, vaporizer channel, and nozzle patterns were lithographically defined. After exposure and development, the process was repeated on the wafer backside for the inlet hole using double sided alignment (Electronic Visions AL6-2). However, the alignment was not critically sensitive as the fluidic inlet was very wide. Silicon dioxide etching was done in three steps: reactive ion etching (RIE) was used to etch about 900 nm (90%) of oxide, first on the top side and then on the back, and the final 100 nm was removed in buffered hydrofluoric acid (BHF) solution (etching both sides simultaneously). Photoresist was not removed after this step, but instead the double layer of photoresist/oxide was used as a mask during the deep reactive ion etching (DRIE) of silicon.

DRIE was done using a pulsed (Bosch) process. Top side feature shapes were etched first, and after turning the wafer over, inlets were etched from the backside. A carrier wafer was used in the second DRIE step so that the reactor electrode was not exposed to plasma after breakthrough. SF_6 etch cycles had additional 10 sccm oxygen flow and one second overlaps with C_4F_8 passivation steps. The average etch rate was *ca.* 4.8 μm min^{-1}. Capillary insertion channel width and depth were 250 μm and 230 μm, respectively. Vaporizer channel width and depth were 800 μm and 240 μm, respectively (Fig. 1A).

Pyrex glass wafers were cleaned in acetone and isopropanol. The heater patterning (Fig. 1B) was done by a lift-off method. Initially a 5 μm thick layer of AZ 4562 photoresist was spin-coated, exposed through a photomask and developed. A chromium adhesion promotion layer (10 nm) was sputtered first, followed by a 100 nm thick platinum layer, without vacuum break. Platinum was sputtered at 3.3 mTorr, 500 W power and 70 sccm argon flow. Deposition rate was *ca.* 180 nm min^{-1}. Platinum was sputtered in two steps to reduce thermal cracking of the photoresist. Finally, the photoresist was removed in acetone and isopropanol baths with additional ultrasound agitation. The resistance of the heater was *ca.* 250 Ω.

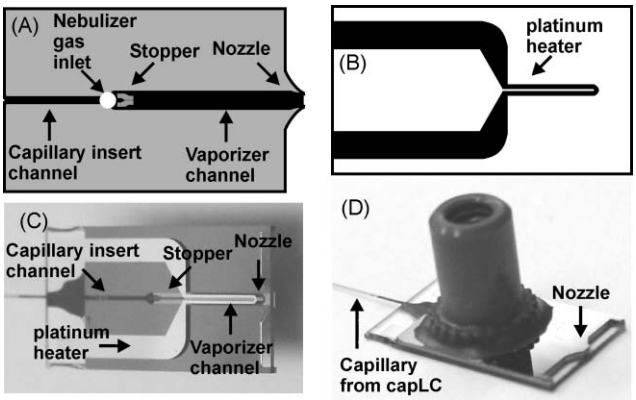

Fig. 1 (A) Silicon wafer feature shapes. (B) Platinum heater on the glass wafer. (C) Bottom view of the microchip APCI with the sample inlet capillary present. (D) Top view of the microchip APCI with the nebulizer gas inlet and sample inlet capillary present. The size of the microchip is 18 × 10 mm.

The remaining photoresist after DRIE was removed in acetone and isopropanol with ultrasound agitation, and the silicon dioxide mask was removed completely in BHF solution. Both silicon and glass wafer surfaces were then hydrophilized in ammonia–peroxide solution (RCA-1). After alignment the silicon and glass wafers were joined together by anodic bonding. The wafer stack was heated to 320 °C, and a voltage of 500 V was applied for 15 minutes, with the current reaching a maximum of 15 mA. After dicing the bonded wafer stack, Nanoport™ fluidic connectors were glued (Fig. 1C and 1D) with high temperature epoxy (Cotronics 4703, Cotronics Corp., New York, USA). A more detailed account of the fabrication process has been presented earlier by Franssila *et al.*[15]

Chemicals

All the neurosteroid standards were obtained from Sigma–Aldrich (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Structures of the compounds studied are presented in Fig. 2. Stock solutions of the sample compounds were prepared by first dissolving the compounds in methanol (J. T. Baker, Mallinckrodt Baker B.V, Deventer, Holland) to a concentration of 10 mmol l^{-1} and then diluting the solutions with methanol–water (50 : 50 v/v) to a concentration of 1 mmol l^{-1} for dehydroisoandrosterone (DHEA) and 100 μmol l^{-1} for testosterone (TEST), progesterone (PROG) and pregnenolone (PREG). Final working solutions of the analytes were prepared by diluting stock solutions further with methanol–water (20 : 80 v/v).

Liquid chromatography

The capillary LC used was an 1100 Series Capillary LC system (Agilent Technologies, Waldbronn, Germany) using a

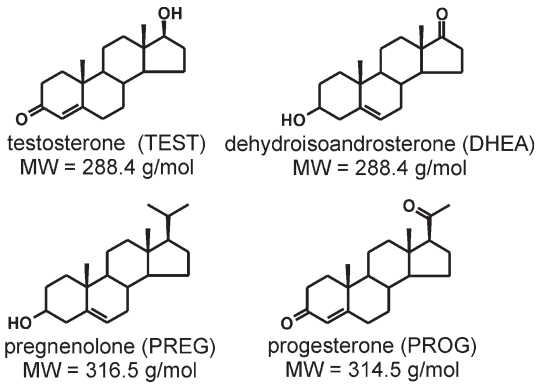

Fig. 2 Structures of the compounds studied.

SymmetryShield RP18-capillary column (0.32 mm i.d. × 100 mm, particle size 3.5 μm) from Waters (Milford, USA). The column oven temperature was set to 50 °C. Samples (2 μl) were injected by an autosampler with an 8 μl loop. The flow rate was 10 μl min^{-1}. The sample inlet capillary (ID 50 μm, o.d. 220 μm, deactivated fused silica capillary, SGE) was pushed into the microchip through the insertion channel until it was firmly fixed in the stopper, and glued with high temperature epoxy (Cotronics 4703) to the rear end of the microchip. The other end of the inlet capillary was fixed to the column with NanoTight Fitting (Upchurch Scientific, Oak Harbor, USA). The mobile phase was composed of H_2O (A) and H_2O–MeOH–ACN 1 : 3 : 6 (B) with gradient elution as follows: 20% B from 0 to 0.1 min, 20–40% B from 0.1 to 7 min, 40–100% B from 7 to 12 min, and 100–20% B from 12 to 13 min. This was followed by a 15 min re-equilibrium period under the initial conditions (20% B).

The reference LC–APCI-MS system consisted of an HP1050 (Hewlett-Packard GmbH, Waldbronn, Germany) using a XTerra MS C-18 (2.1 mm id × 100 mm, particle size 3.5 μm) from Waters (Milford, USA). Flow rate was 300 μl min^{-1}. The mobile phase was composed of H_2O (A) and H_2O–MeOH–ACN 1 : 3 : 6 (B) with gradient elution as follows: 20–55% B from 0 to 1 min, 55–65% B from 1 to 6 min, 65–100% B from 6 to 7 min, 100% B from 7 to 11.5 min, and 100–20% from 11.5 to 12 min. This was followed by an 8 min re-equilibrium period at the initial conditions (20% B). The injection volume was 2 μl.

Mass spectrometry

The mass spectrometer was a PE Sciex API-300 triple quadrupole (Perkin–Elmer Sciex, Concord, Canada). Nitrogen produced by a Whatman 75–720 nitrogen generator (Whatman Inc., Haverhill, MA) was used as the nebulizer gas. Nebulizer gas was introduced to the microchip APCI by 510 μm i.d. PEEK tubing (Upchurch Scientific, Oak Harbor, USA) through the Nanoport™ with a back-pressure of 0.35 bar. The external corona discharge needle was a darning needle (John James size 3/9, Entaco Limited, Warwickshire, England) with a current of 1 μA. The corona discharge needle was 0.5 cm from the MS orifice and the microchip APCI was positioned 1.0 cm away from the mass spectrometer orifice in an orthogonal position, as in our earlier work.[14] The external power supply (EPS EP-6515, EPS Stromversorgung GmbH, Augsburg, Germany) was used to adjust the temperature of the microchip APCI, measured with a Fluke 54 Series II thermometer (Fluke Corporation, USA) with a K-type thermoelement outside the chip. The chip temperatures were taken from the silicon surface above the stopper and were in the range 110–170 °C.

Data for the reference LC–APCI-MS system was measured using the same MS. The ion source was a commercial heated nebulizer for APCI (Perkin–Elmer Sciex, Toronto, Canada) with corona discharge needle current set to 1 μA. Compressed air filtered by an Atlas Copco air-dryer (Wilrijk, Belgium) was used as the auxiliary gas. The temperature of the heated nebulizer was optimized by running a mixture of the sample compounds (10 μM TEST, PROG and PREG; 100 μM DHEA, injection volume of 2 μl) in MRM mode with the temperature ranging from 300 °C to 500 °C in steps of 50 °C. The optimal temperature was found to be 500 °C.

The mass spectrometer was operated in a positive ion mode. The mass spectrometer voltages were optimized for each analyte, both for MS and tandem mass spectrometric (MS/MS) measurements, with the controlling software program. For mass spectrum measurements the scan range was *m*/*z* 50–400 (1.0 s per scan with a step size of 0.1 *m*/*z*). Measurements of limit of detection (LOD), linearity, and repeatability were done in multiple ion monitoring mode (MRM) with two ion pairs of each neurosteroid standard (dwell time 200 ms). Data was acquired with MassCrom 1.1.1 software (PE Sciex).

Deactivation of the microchip

The surface of the microchip heated nebulizer was deactivated with chlorotrimethylsilane (Aldrich, Sigma–Aldrich GmbH, Steinheim, Germany). Undiluted chlorotrimethylsilane was introduced to the chip at a flow rate of 50 μl min^{-1} *via* the nebulizer gas inlet by direct infusion with a micro syringe pump (Harvard PHD 2000 Advanced Syringe Pump, Harvard Apparatus, Holliston, MA, USA). The heating power was set to 1.2 W during the chip deactivation. A total of 700 μl of chlorotrimethylsilane was introduced to the chip. At the same time acetonitrile (Rathburn, Rathburn Chemicals Ltd, Scotland) was introduced *via* the inlet capillary at a flow rate of 500 nl min^{-1} by a Nanopump (Upchurch Scientific, Oak Harbor, USA) to prevent the sample inlet capillary becoming clogged up with silane. After derivatization, the microchip nebulizer was washed with 10 ml of acetonitrile at a flow rate of 200 μl min^{-1}, which was introduced to the chip *via* the nebulizer gas inlet. The eluent flow from the Nanopump was kept constant during washing but the heater was turned off.

Results and discussion

The microchip APCI is a two-dimensional version of a commercial APCI source, in which a nebulizer gas flows co-axially to the liquid sample flow. The microchip APCI consists of an insert channel for sample inlet, a nebulizer gas inlet, a stopper, a vaporizer channel and an exit nozzle fabricated on a silicon wafer, and an integrated platinum heater sputtered on a Pyrex glass wafer. The DRIE process enables shape freedom and better dimensional control, without the restrictions of

crystal orientation or need for large corner compensation structures associated with wet etching, especially in the stopper and nozzle.

The deactivated fused silica capillary from the separation system is inserted from the rear edge through an insert channel, past the nebulizer gas inlet, until the tip of the capillary meets the stopper, and is then fixed by gluing it to the rear end of the microchip. The stopper is a conical narrowing fabricated in front of the vaporizer channel. The stopper provides reproducible insertion of the sample inlet capillary and minimization of the dead volumes. The nebulizer gas, brought into the chip from behind the stopper, flushes the eluted compounds efficiently from the fused silica capillary into the heated vaporizer channel and towards the exit nozzle minimizing memory effects and peak tailing. Deactivation of the microchip APCI inner surface was done to minimize adsorptions and chromatographic peak broadening (Fig. 3).

It can be presumed that the microfabricated nozzle produces a very narrow plume towards the corona needle. Since the initial ionization in corona discharge takes place in a very small volume near the needle tip,[16] it can be expected that a confined plume results in a larger fraction of ionized analytes, leading to improved sensitivity. Positioning of the microchip APCI towards the corona discharge needle was found to have a significant effect on signal intensity, implicating a very confined plume. When the position of the microchip APCI was varied only a few millimetres the MS signal disappeared altogether.

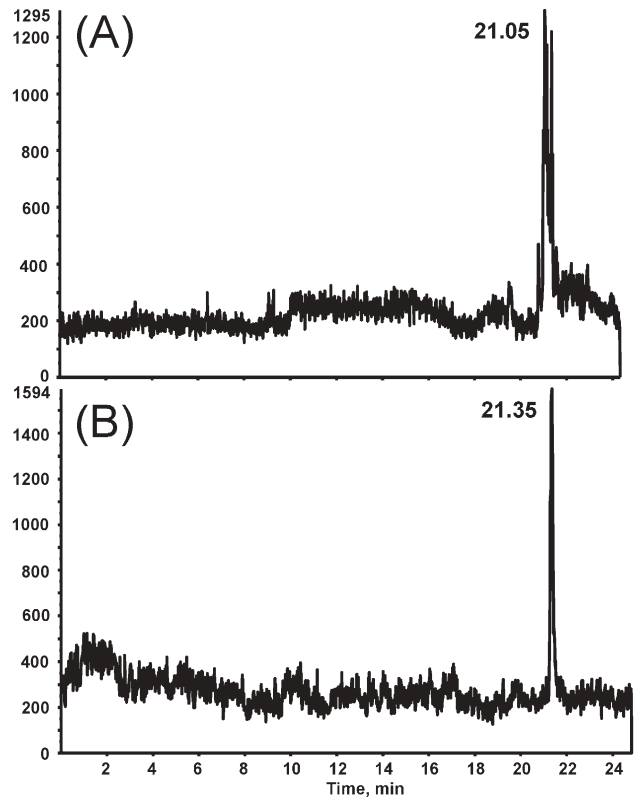

Fig. 3 Merged MRM ion chromatogram of two ion pairs of TEST with (A) undeactivated microchip APCI and (B) deactivated microchip APCI. The concentrations were 100 nmol l^{-1}. The ion pairs were *m*/*z* 289.1 → 97.2 and *m*/*z* 289.1 → 109.2.

While the temperature of the platinum heater is able to reach the glass softening temperature of *ca*. 600 °C,[15] the maximum operational surface temperature limit of *ca*. 300 °C was set by the epoxy glue used in connecting the Nanoport™ assemblies to the microchip. As shown in our earlier work the temperature of the microchip APCI can be ramped from room temperature to 300 °C in less than one minute.[15] The fast heat transfer enables changes of temperature of the microchip APCI during a LC run, and therefore optimal conditions for each compound in a sample mixture. The temperature optimization was done by adjusting the heating power in the range 2 W to 3.5 W in steps of 0.5 W (up to the maximum APCI chip temperature) and running 10 μmol l^{-1} DHEA and 1 μmol l^{-1} TEST, PREG and PROG with an injection volume of 2 μl and a flow rate of 10 μl min^{-1} in MRM mode, and calculating the signal intensity of individual ion pairs. Maximal signal intensity was recorded at a heater power of 3.0 W for TEST and DHEA and at 3.5 W for PROG and PREG.

The capillary LC–microchip APCI-MS produces high quality mass spectra (Fig. 4A), very similar to those measured with a commercial APCI source (Fig. 4B). Each compound shows an abundant protonated molecule with very little fragmentation. However, since the microchip APCI is not

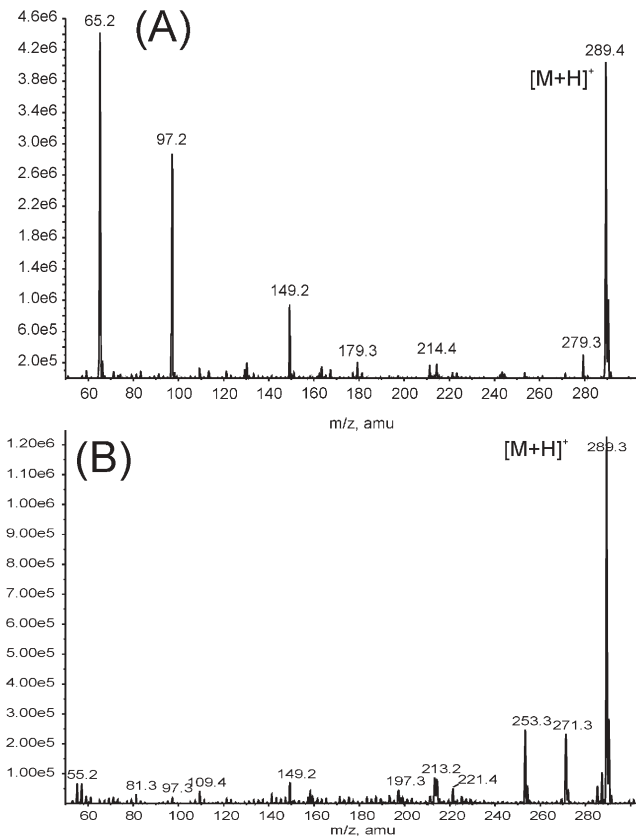

Fig. 4 (A) Mass spectrum of TEST produced by the microchip APCI heated nebulizer by direct infusion of 100 μmol l^{-1} TEST with water–methanol (20 : 80 v/v) solution at a flow rate of 2 μl min^{-1}. (B) Mass spectrum of TEST produced by commercial APCI source by direct infusion of 100 μmol l^{-1} TEST with water–methanol (20 : 80 v/v) solution at a flow rate of 10 μl min^{-1}.

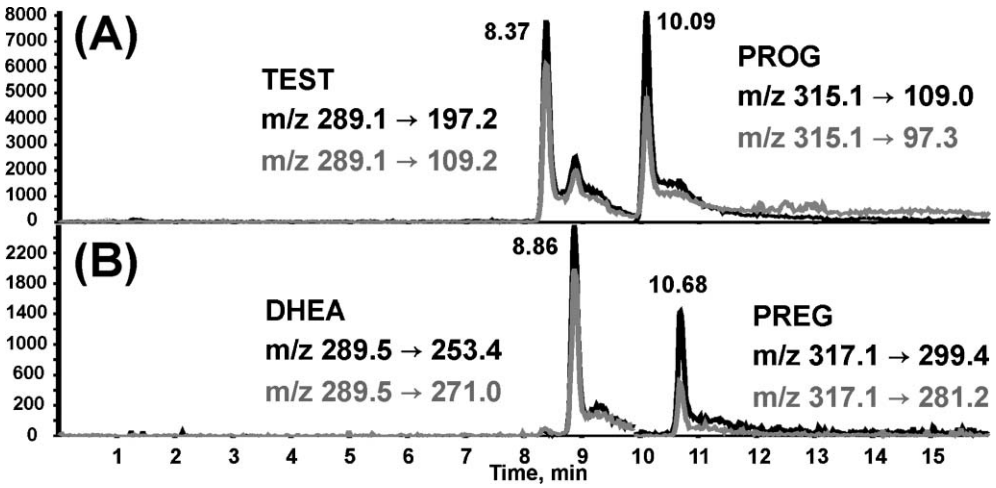

Fig. 5 Overlay of extracted ion chromatograms of (A) TEST and PROG and (B) DHEA and PREG. The concentrations were 1 μmol l^{-1} for TEST, PREG and PROG, and 10 μmol l^{-1} for DHEA.

enclosed, more background ions from the laboratory air are visible (in Fig. 4A) at *m/z* 149.2, at *m/z* 179.3, at *m/z* 211.3, at *m/z* 214.5, and at *m/z* 279.3 and reagent ions at *m/z* 65.2 and at *m/z* 97.2 corresponding to $[(CH_3OH)_2H]^+$ and $[(CH_3OH)_3H]^+$.

Separation efficiency of the capillary LC–microchip APCI-MS (Fig. 5) is comparable to that of capillary LC–ESI-MS.[17] Peak widths at half height and peak base widths are 0.1–0.12 min and 0.3–0.4 min, respectively. All this indicates that the dead volume of the microchip APCI is minimal and APCI analysis can be carried out with low flow rate separation systems.

The feasibility of the microchip APCI for quantitative work was tested by determining the linearity of response, repeatability, and limits of detection for the set of neurosteroids. Linearity of response was measured using MRM in the range of 200 nmol l^{-1} to 20 μmol l^{-1} for TEST, PREG, and PROG, and 2 μmol l^{-1} to 200 μmol l^{-1} for DHEA. Good linearity was observed, with a regression coefficient (r^2) of area varying between 0.9919 and 0.9994 (Table 1). Repeatability was measured in MRM mode with 10 μmol l^{-1} DHEA and 1 μmol l^{-1} TEST, PREG and PROG, an injection volume of 2 μl and a flow rate of 10 μl min^{-1}. The relative standard deviations (in six measurements) of the peak areas of the most abundant fragment ion of each neurosteroid varied between 12.7% and 18.9% (Table 1), indicating reasonable repeatability at this stage of the device development. Limits of detection were measured in MRM mode and compared to LODs obtained with the commercial APCI source. LODs with a signal-to-noise ratio of 3 (Table 1) were between 10 and 500 nmol l^{-1} for the microchip APCI, and between 500 nmol l^{-1} and 5 μmol l^{-1} for the commercial APCI source when the injection volume in both experiments was the same (2 μl). Since the initial ionization occurs in a minute volume close to the corona needle tip,[15] a very narrow and confined plume generated by the DRIE etched nozzle[14] is likely to cause ionization of a larger fraction of the neutral analytes resulting in improved sensitivity.

Conclusions

The microchip APCI provides a very interesting alternative interface to microchip ESI for microfluidic and capillary LC separation systems. The use of an external corona discharge needle provides easy connection of the microchip APCI to any mass spectrometer equipped with an atmospheric pressure ionization source. The limits of detection demonstrated here can be easily improved by using a more sensitive mass spectrometer than used in this study. The results in this work show that the microchip APCI also has potential as an interface in microfluidic-APCI-MS.

The microchip APCI provides high ionization efficiency for neutral and non-polar compounds with detection limits down to the fmol range, temperature optimization for individual analytes, good linearity, reasonable reproducibility, and cost-efficient manufacturing. This opens up new possibilities to analyze small non-polar and neutral compounds in addition to ionic compounds in biological and environmental systems with high sensitivity from minimal sample volumes.

Table 1 Linearity of response and repeatability of the capillary LC–microchip APCI-MS system, and limits of detection (S/N = 3) measured both with the capillary LC–microchip APCI-MS and LC–APCI-MS with the commercial APCI source

			LOD (S/N = 3)	
Compound	Linearity of response (r^2)	Repeatability (STD%, n = 6)	Microchip APCI	Commercial APCI
TEST	0.9994	12.7%	0.01 μmol l^{-1} (20 fmol[a])	0.2 μmol l^{-1} (400 fmol[a])
DHEA	0.9946	14.9%	0.5 μmol l^{-1} (1 pmol[a])	5 μmol l^{-1} (10 pmol[a])
PREG	0.9928	15.7%	0.02 μmol l^{-1} (40 fmol[a])	0.2 μmol l^{-1} (400 fmol[a])
PROG	0.9919	18.9%	0.15 μmol l^{-1} (300 fmol[a])	2 μmol l^{-1} (4 pmol[a])

[a] Injected amount.

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