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Feasibility of gas chromatography–microchip atmospheric pressure photoionization-mass spectrometry in analysis of anabolic steroids

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ABSTRACT

Mass spectrometers equipped with atmospheric pressure ion sources (API-MS) have been designed to be interfaced with liquid chromatographs (LC) and have rarely been connected to gas chromatographs (GC). Recently, we introduced a heated nebulizer microchip and showed its potential to interface liquid microseparation techniques and GC with API-MS. This study demonstrates the feasibility of GC–microchip atmospheric pressure photoionization-tandem mass spectrometry (GC–μAPPI-MS/MS) in the analysis of underivatized anabolic steroids in urine. The APPI microchip provides high ionization efficiency and produces abundant protonated molecules or molecular ions with minimal fragmentation. The feasibility of GC–μAPPI-MS/MS in the analysis of six selected anabolic steroids in urine samples was studied with respect to intra-batch repeatability, linearity, linear range, and limit of detection (LOD). The method showed good sensitivity (LODs 0.2–1 ng/mL), repeatability (relative standard deviation < 10%), and linearity (regression coefficient ≥ 0.9995) and, therefore, high potential for the analysis of anabolic steroids. Quantitative performance of the method was tested with two authentic urine samples, and the results were in good agreement with those obtained with conventional GC–electron ionization-MS after derivatization.

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1. Introduction

Anabolic–androgenic steroids (AAS) represent an important class of abused drugs in sport, and the World Anti-Doping Agency classifies them as prohibited substances in sports [1]. The attractiveness of these testosterone-derived compounds is their ability to improve physical performance of skeletal muscle and reduce the catabolic response of the body to stress [2–4]. In addition, supraphysiologic doses of AAS increase muscle size [5,6]. These effects are also sought after by non-athletes and AAS abuse among adolescents is increasing rapidly. However, mediated mainly by their androgenic activity, AAS also can have serious side effects, such as cardiovascular or liver diseases [3].

Within the human body, nonpolar AAS are extensively biotransformed by metabolic reactions, and are excreted in urine mainly as glucuronide conjugates [7]. AAS in biological samples are typically analyzed by gas chromatography–electron ionization-mass spectrometry (GC–EI-MS) after hydrolysis of the conjugates and trimethylsilyl (TMS)-derivatization [8,9]. However, the high energy involved in the EI process causes extensive fragmentation of AAS derivatives and the intensity of the molecular ion may be low, with consequent poor sensitivity and selectivity in selected ion monitoring.

Liquid chromatography–mass spectrometry (LC–MS) by using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) is extensively used in doping control [10–16], since AAS or their glucuronide conjugates can then be analyzed without hydrolysis or derivatization, and analysis is faster than with GC–MS. Although ESI and APCI are the most common ionization techniques in LC–MS, neither is effective in ionizing low-polar or nonpolar compounds. To overcome that problem, in the year 2000, Bruins and co-workers [17] and Syage and Evans [18] introduced atmospheric pressure photoionization (APPI), a new ionization method for LC–MS. APPI is capable of ionizing both polar and nonpolar compounds via proton transfer or charge exchange reactions [19,20]. In comparison with ESI and APCI, APPI provides better sensitivity for steroids [21–24], wider linear dynamic range [25] and less matrix effects [26] which means that it should also perform well in the analysis of AAS [27,28]. Although LC–MS is increasingly being applied, it has several drawbacks: the system is more complicated than GC–MS, and often the resolving power is poorer than with GC separation. The solvent composition in LC–MS affects

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Table 1
Nomenclature, precursors, and structures of the anabolic androgenic steroids.

Abbreviation	Compound	Structure	Mw
NANm	5α-Estran-3α-ol-17-one		276
MDNm	17β-Methyl-1-ene-5β-androstane-3α,17α-diol		304
MTm	17α-Methyl-5β-androstane-3α,17β-diol		306
NAN	Nandrolone		274
DNZm	Ethisterone		312
17MDN	17-Epimethandienone		300
MTS	Methyltestosterone (ISTD)		302

the sensitivity, and LC–MS is always a compromise between chromatographic performance and MS sensitivity. For all these reasons GC–MS is still widely used in the analysis of AAS.

Conventional GC–EI-MS is preferred in the analysis of volatile or semivolatile organic compounds, but GC can also be connected to mass spectrometers equipped with an atmospheric pressure ion (API) source. The advantage is that expensive mass spectrometers, designed to be interfaced with LCs, can also be exploited with GC separation and separate GC–MS are not necessarily needed. GC–APCI-MS was introduced in 1976 [29] and GC–APPI-MS 10 years later [30]. However, neither technique has received much attention over the years [31–33], mainly because commercial interfaces to couple GC to API-MS have only recently become available [34,35]. Present commercial interfaces are based on APCI, but atmospheric pressure laser ionization is also available.

We recently described a heated nebulizer microchip [36] that provides easy interfacing of liquid microseparation techniques and GC to API-MS and can be operated in all API modes: APCI [37–41], APPI [39–42], sonic spray ionization [43], ionspray [44] and thermospray ionization [45]. The performance of the heated nebulizer microchip in APCI (μAPCI) and APPI (μAPPI) has been demonstrated in GC–API-MS analysis of volatile organic compounds [38], drugs [39,41], polyaromatic hydrocarbons [39], and polychlorinated biphenyls [40]. Both GC–μAPCI-MS and GC–μAPPI-MS provide efficient and soft ionization, high sensitivity, and good quantitative performance [38–41] and represent therefore, a promising alternative to conventional GC–EI-MS.

The aim of this study was to develop an GC–μAPPI-MS/MS method for the determination of selected anabolic steroids in human urine. The method was validated with respect to detection limit, repeatability, linearity and linear range. Performance of the method was tested with two authentic urine samples collected after single a dose of methandienone. Results are compared with those obtained with conventional GC–EI-MS after derivatization.

Table 2
Precursor-product ion pairs of AAS in SRM method. See Table 1 for analyte nomenclature. CE = collision energy, expressed as offset voltage.

Compound	Precursor		Product	CE (V)	Period
	277	$[M+H]^+$	241	20	1
NANm	277	$[M+H]^+$	259	12	1
	277	$[M+H]^+$	145	30	1
	303	$[M-1]^+$	201	25	2
MDNm	286	$M^{+\bullet}-H_2O$	228	15	2
	286	$M^{+v}-H_2O$	150	15	2
	305	$[M-1]^+$	269	18	2
MTm	306	$M^{+\bullet}$	230	15	2
	306	$M^{+\bullet}$	270	15	2
	275	$[M+H]^+$	109	37	3
NAN	275	$[M+H]^+$	257	24	3
	275	$[M+H]^+$	239	25	3
MTS	303	$[M+H]^+$	109	37	4
ISTD	303	$[M+H]^+$	285	24	4
	303	$[M+H]^+$	97	30	4
	313	$[M+H]^+$	109	37	4
DNZm	313	$[M+H]^+$	295	22	4
	313	$[M+H]^+$	97	33	4
	301	$[M+H]^+$	121	30	4
17MDN	301	$[M+H]^+$	283	17	4
	301	$[M+H]^+$	149	23	4

2. Experimental

2.1. Reagents and samples

The selected anabolic steroids (Table 1) were from the National Measurement Institute (NMI, Australia), except for nandrolone (NAN), which was purchased from Diosynth (Oss, The Netherlands). LC–MS grade ethyl acetate was from Riedel-de Haën (Seelze, Germany), and analytical reagent grade pentane from Lab-Scan (Dublin, Ireland). Potassium carbonate (K_2CO_3) and sodium bicarbonate ($NaHCO_3$) were purchased from J.T. Baker (Deventer, The Netherlands), and β-glucuronidase (Type HP-2 Helix pomatia), sodium acetate, formic acid, and toluene (≥99.9% pure) from Sigma–Aldrich (Steinheim, Germany).

A urine sample (2 mL) was buffered with 1.5 mL of 0.2 M sodium acetate and pH was adjusted to 5.0 with formic acid. β-Glucuronidase (40 μL) from Helix pomatia, 0.2–1000 ng of AAS, and 50 ng of MTS as internal standard (ISTD) were added to the sample, which was mixed carefully and incubated at 55 °C for 2 h. After cooling to room temperature, 0.2 g of a mixture of $NaHCO_3$ and K_2CO_3 (2:1, w/w) was added to the hydrolyzed urine samples to adjust the pH to 9.2. After 3 mL of *n*-pentane was added, the mixture was shaken gently and centrifuged (3000 rpm/min for 5 min). The pentane layer was transferred to an eppendorf tube and evaporated to dryness (assisted with N_2). Before GC–μAPPI-MS/MS analysis, 100 μL of ethyl acetate was added to the dry residue, and thus the concentration coefficient was 20.

For the method validation, a drug-free pool of blank urine from five individuals was spiked with a mixture of anabolic steroids at levels 0.1–500 ng/mL per analyte. Linearity, linear range, detection limit, and repeatability of injection (six injections at the level of 25 ng/mL) were investigated.

Performance of the method was tested with two urine samples collected after oral administration of methandienone to two healthy male volunteers (30 mg p.o., urine collected between 18 and 48 h; 20 mg p.o., urine collected between 0 and 48 h). The samples were from previous studies carried out in United Medix Laboratories Ltd. (Helsinki, Finland) and were stabilized with sodium azide and stored frozen. Sample preparation was as described above. Reagent and urine blanks were prepared together with the excretion study and calibration curve samples (from 0.5 ng/mL to 100 ng/mL).

2.2. Instrumentation

The heated nebulizer microchip consists of a silicon wafer and a Pyrex glass cover and features a sample introduction channel, an auxiliary gas inlet, a vaporizer channel, a heater, and a nozzle. The channels and gas inlet were deep reactive ion etched to the silicon wafer and the integrated platinum heater was sputtered onto the Pyrex glass cover. The wafer and plate were then anodically bonded together. A deactivated silica capillary (SGE, Victoria, Australia) for sample introduction and a Nanoport connector (Upchurch Scientific Inc., Oak Harbour, WA) for connecting the auxiliary gas tubing to the chip were glued to the silicon wafer with high-temperature-resistant epoxy. A detailed description of the fabrication process has been presented earlier [46].

An HP 5890 II gas chromatograph (Hewlett–Packard, Waldbronn, Germany) equipped with a split/splitless injector, a column transfer line (an MSD transfer line), and a CTC-A200S autosampler (CTC Analytics, Zwingen, Switzerland) was used for chromatographic separation of the analytes. The column was forte BPX 5 (length 15 m, i.d. 0.25 mm, 5% phenyl 95% dimethylpolysiloxane, film thickness 0.25 μm) (SGE Europe Ltd., Milton Keynes, UK). A methyl-deactivated fused-silica precolumn (1.5 m × 0.25 mm i.d.) was connected in front of the analytical column. The analytical column was connected to a methyl deactivated transfer capillary with a deactivated press-fit connector. A heated transfer line was used to attach the GC system to the microchip. The transfer line consisted of the original GC–MS transfer line and of a self-made resistance wire heater, which were set to 300 °C and 280 °C, respectively. Injector temperature was 280 °C. Samples (1 μL) were injected with 1 min splitless injection. The carrier gas was 99.996% pure helium (AGA, Espoo, Finland) with 95 kPa column pressure and a flow rate of 1.7 mL/min (at 100 °C oven temperature). The analytical run was initiated with 1 min isothermal period (180 °C), after which the oven temperature was raised to 320 °C at 20 °C/min and the column was cleaned by raising the temperature to 350 °C at 25 °C/min (hold 5 min).

The mass spectrometer was an API 3000 (MDS SCIEX, Concord, ON, Canada) triple quadrupole instrument. The conventional ion source was replaced by a nanospray stand (Proxeon Biosystems A/S, Odense, Denmark) to enable positioning of the microchip about 1 cm distance from the MS orifice. Ionizing photons with 10 eV energy were generated by a krypton discharge UV lamp (Heraeus Noblelight, Cambridge, UK). The dopant (toluene) was delivered to the auxiliary gas line at a flow rate of 3.5 μL/min with a syringe pump (PHD 2000; Harvard Apparatus, Hollston, MA).

Table 3
μAPPI mass spectra of anabolic androgenic steroids; *m*/*z* (rel. abund.).

	$[M+H]^+$	$M^{+\bullet}$	$[M-H]^+$	$[M+H-H_2O]^+$	$[M-H_2O]^+$	$[M-H-H_2O]^+$	$[M+H-2{\bullet}H_2O]^+$	$[M-2{\bullet}H_2O]^+$
NANm	277 (100)			259 (76)				
MDNm		304 (47)	303 (97)	287 (45)	286 (100)	285 (98)	269 (79)	268 (34)
MTm		306 (35)	305 (83)	289 (64)	288 (100)	287 (38)	271 (83)	270 (30)
NAN	275 (100)							
MTS	303 (100)							
DNZm	313 (100)							
17MDN	301 (100)			283 (23)				

Nitrogen (generated with a Whatman 75–72 nitrogen generator, Whatman Inc., Haverhill, MA) was used as auxiliary, curtain, and collision gas. The auxiliary gas flow (80 mL/min) to the microchip was controlled with a mass flow controller (model GCF17; Aalborg, Orangeburg, NY). The microchip was heated with a dc power supply (2.2 W). The system setup has been described in more detail in our earlier work [38].

Analyses were carried out in positive ion mode APPI. The declustering voltage was 20 V. MS and MS/MS spectra were measured from *m/z* 50 to 350 with a speed of 1 scan/s. The MS/MS collision energies were optimized separately for each analyte in toluene solution by direct injection with a syringe pump (PHD 2000; Harward Apparatus, Hollston, MA, USA). The collision energies and the product ions monitored in selected reaction monitoring (SRM) mode are presented in Table 2. The measurement was divided into four detection periods. The first period (0–6.70 min) included characteristic SRM ions for NANm. The second period (6.70–7.20 min) included ions for MDNm and MTm, NAN was measured in a third period (7.20–7.52 min), and MTS, DNZm and 17MDN were measured in the last period (7.52–11.20 min). Total scan time was 0.33–0.36 s, and dwell times varied from 35 to 110 ms depending on the number of ion pairs measured in each period. Analyst 1.4.2. software (MDS SCIEX) was applied to data processing throughout the method validation.

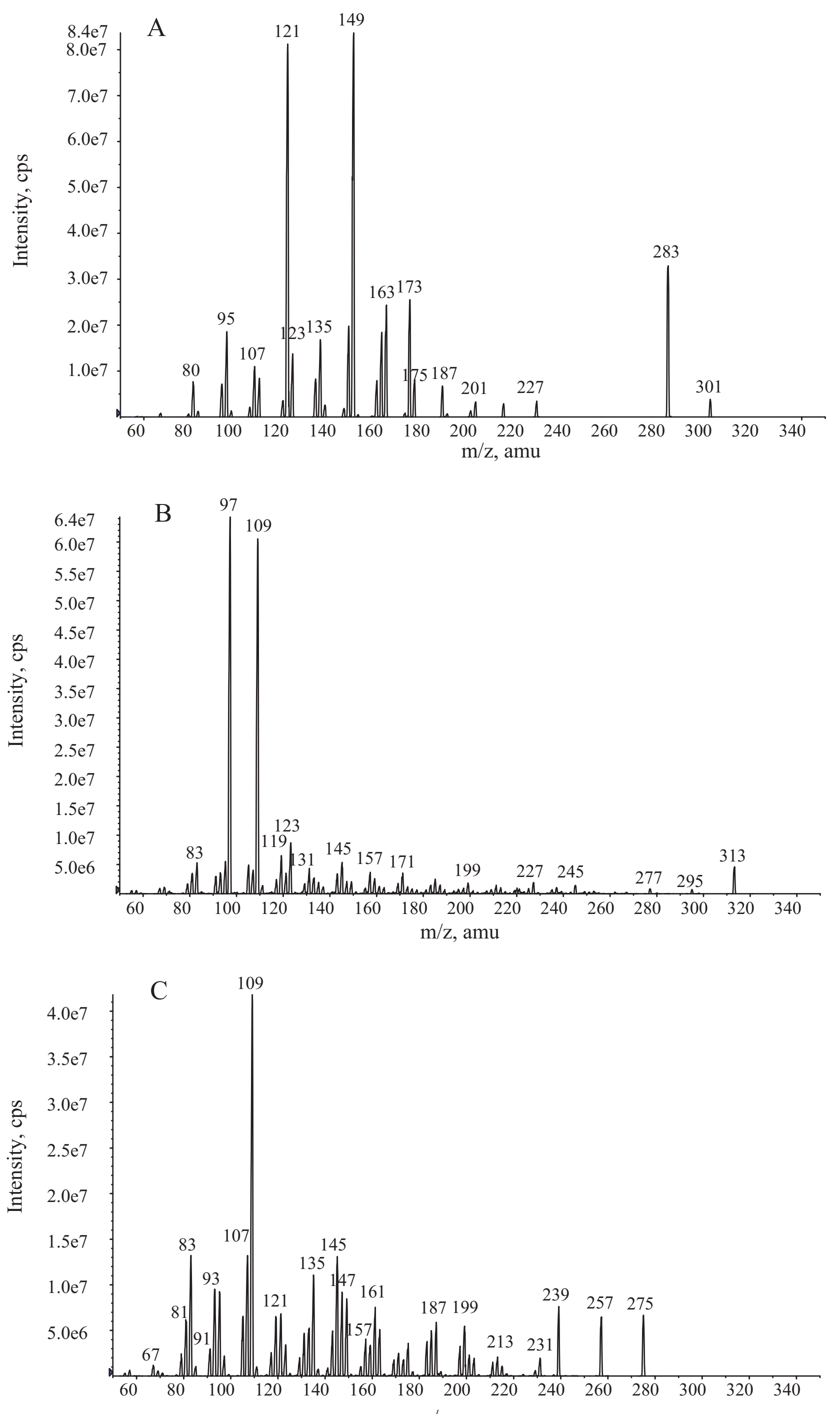
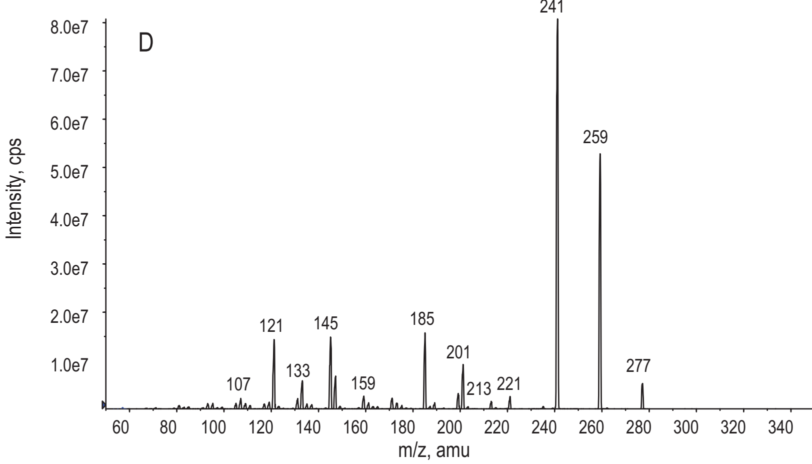


Fig. 1. MS/MS spectra of $[M+H]^+$ (A) 17MDN CE = 25 V, (B) DNZm CE = 32 V, (C) NAN CE = 32 V, (D) NANm CE = 35 V and (E) MTS (ISTD) CE = 33 V.



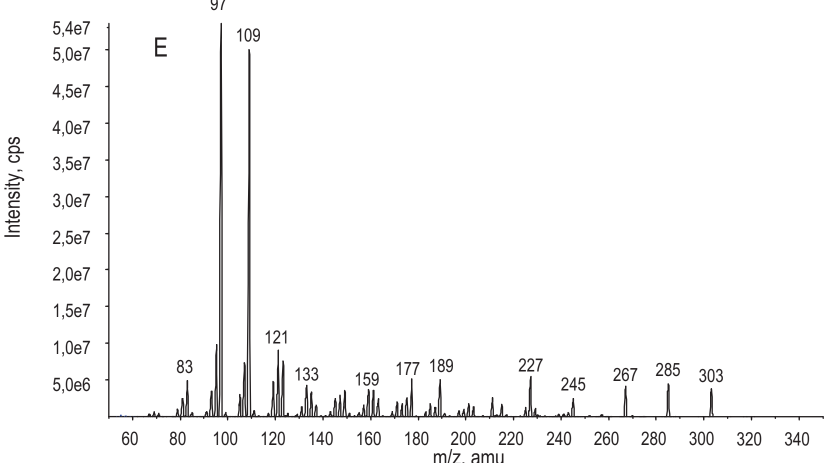


Fig. 1. *Continue*

3. Results and discussion

The positioning of the microchip and the krypton discharge lamp and the flow rates of the nebulizer gas and dopant all had an effect on the ionization efficiency. The optimal position for the lamp was 5–10 mm from the curtain plate (orifice) and at an angle of about 45° to the longitudinal axis of the MS. The microchip was set perpendicularly and about 1 mm from the lamp so that the nozzle was 1–3 mm from the photon beam and 4–9 mm from the orifice. To achieve good stability of the signal, the chip was positioned to the right side of the orifice and the spray from the nozzle was directed 1 mm left of the orifice. Sensitivity and stability were optimal with dopant and nebulizer gas flow rates of 3.5 μL/min and 80 mL/min, respectively. The heating power of the nebulizer chip was selected so that the temperature was about 300 °C in order to prevent adsorption and peak broadening. Toluene was chosen as dopant, since it enables efficient proton transfer reaction for high proton affinity steroids and efficient ionization via charge exchange reaction for low proton affinity steroids which cannot be ionized by proton transfer.

The positive ion APPI spectra of the steroids including a carbonyl and hydroxyl groups (NAN, NANm, DNZm, 17MDN, and MTS) showed an abundant $[M+H]^+$ ion, whereas the steroids with only hydroxyl functionality (MDNm and MTm) showed $[M-H]^+$ ion and fragments formed by the loss of one or two water molecules (Table 3). Proton transfer from toluene radical cation ($D^{+\bullet}$) to analyte can occur if proton affinity (PA) of the analyte is higher than PA of the deprotonated $D^{+\bullet}$ ($[D-H]^\bullet$), which is 831.4 kJ/mol [47]. In compounds with a carbonyl group, the PA is high enough for the proton transfer from $D^{+\bullet}$ to occur, but for the compounds possessing only hydroxyl functionality this is not a possible route. In this study, abundant $[M-H]^+$ ion was formed from the two AAS with hydroxyl groups (MDNm and MTm), evidently via hydride abstraction. However, it cannot be excluded that the $[M-H]^+$ ion is partly formed by oxidation of a hydroxyl group to a carbonyl group (i.e., via loss of H_2) followed by proton transfer reaction. Moreover, although the formation of $[M-H]^+$ ion is favored with MDNm and MTm, an intense radical cation ($M^{+\bullet}$) was observed as well. The ratio of relative abundances of $[M-H]^+$ to $M^{+\bullet}$ was dependent on the experimental conditions and varied from day to day. This variation will be studied systematically in future work.

The most abundant ion, or ions, were selected as precursor ion for the GC–μAPPI-MS/MS analysis. Orifice (declustering), focusing, and entrance potentials were optimized to maximize the intensity of potential precursor ions, and they were fairly universal for all compounds (20, 200, and 10 V respectively). All the MS/MS spectra of the AAS showed loss of one or two water molecules and numerous characteristic product ions depending on the structure of the steroid (Figs. 1 and 2). The fragmentation patterns of the protonated molecules of DNZm and MTS, which have 4-ene-3-one structure and angular methyl group (C-19) showed the same features: the product ions at m/z 109 and m/z 97 were characteristic and the protonated molecule was relatively stable. A detailed fragmentation mechanism for these ions has been presented [48]. The protonated

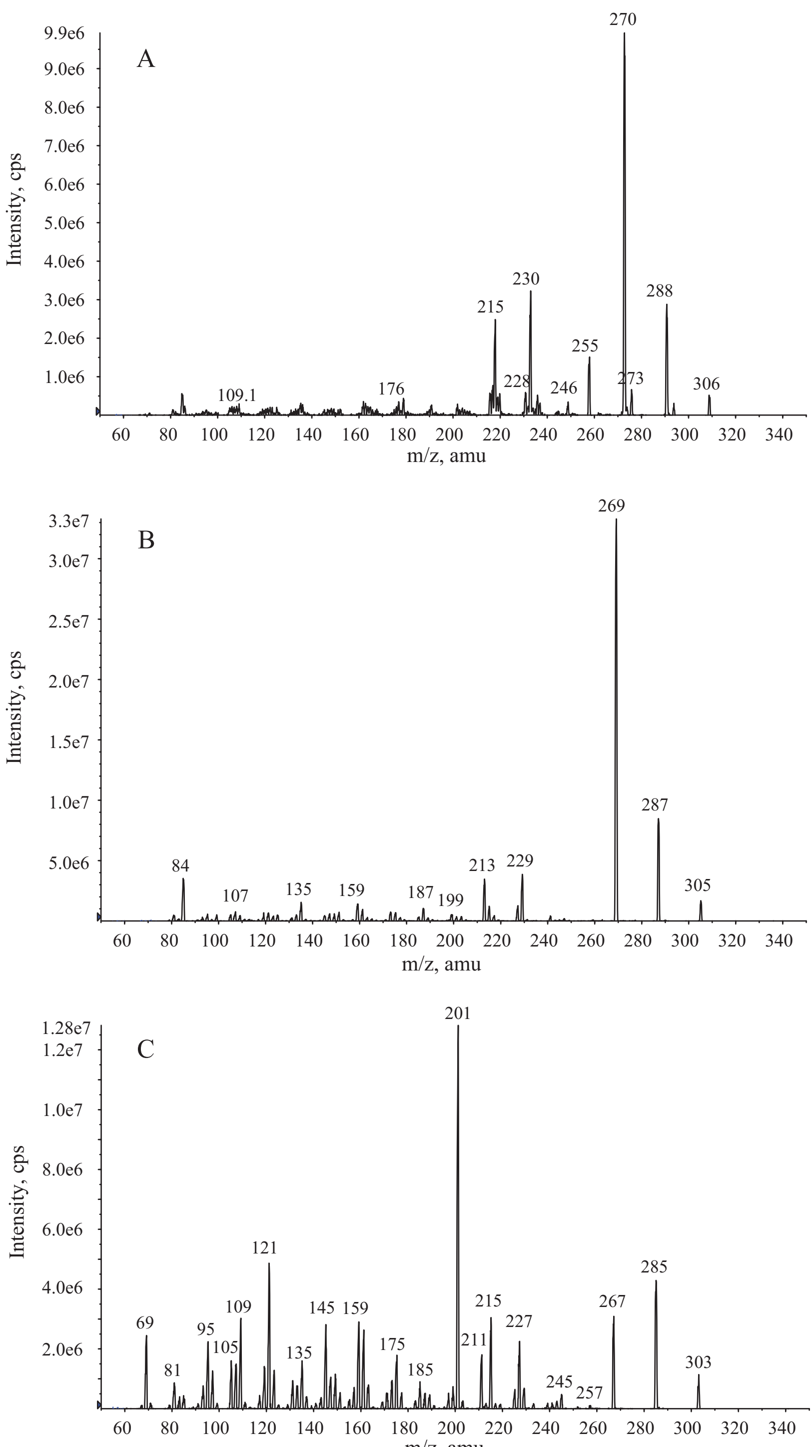


Fig. 2. MS/MS spectra of (A) MTm $M^{+\bullet}$ = 306 CE = 20 V, (B) MTm $[M-H]^+$ = 305 CE = 20 V and (C) MDNm $M^{+\bullet}$ = 303 CE = 27 V.

molecule of NAN, with 4-ene-3-one structure but without a methyl group at C-10, was also relatively stable, and the MS/MS spectra showed an abundant ion at *m/z* 109. The product ion spectrum of 17MDN was exceptional as it did not show an abundant ion at either *m/z* 97 or *m/z* 109 even though it contains both 4-ene-3-one structure and angular methyl group (C-19). The most abundant fragments for 17MDN were *m/z* 121 and *m/z* 149, the structures of which have been presented earlier [49,50]. The $[M-H]^+$ ion of the steroids containing 3α-hydroxyl group (MDNm and MTm) instead of 4-ene-3-one structure was relatively unstable and the product ions spectra showed abundant ions formed by loss of one or two water molecules. In addition, numerous product ions were observed, formed by dissociation of the ring structure. The three most abundant and structure-specific product ions (target ion and two qualifier ions) were selected for the final GC–μAPPI-MS/MS method, and the collision energies were optimized separately for each product ion to achieve maximum sensitivity. Optimal collision energies for the selected ions were 12–37 V. MS measurement was divided into four periods, and 3–12 SRM pairs were measured at the same time (Table 2).

The GC temperature program was optimized for fast separation. The total GC analysis time was 11.20 min. In general the retention

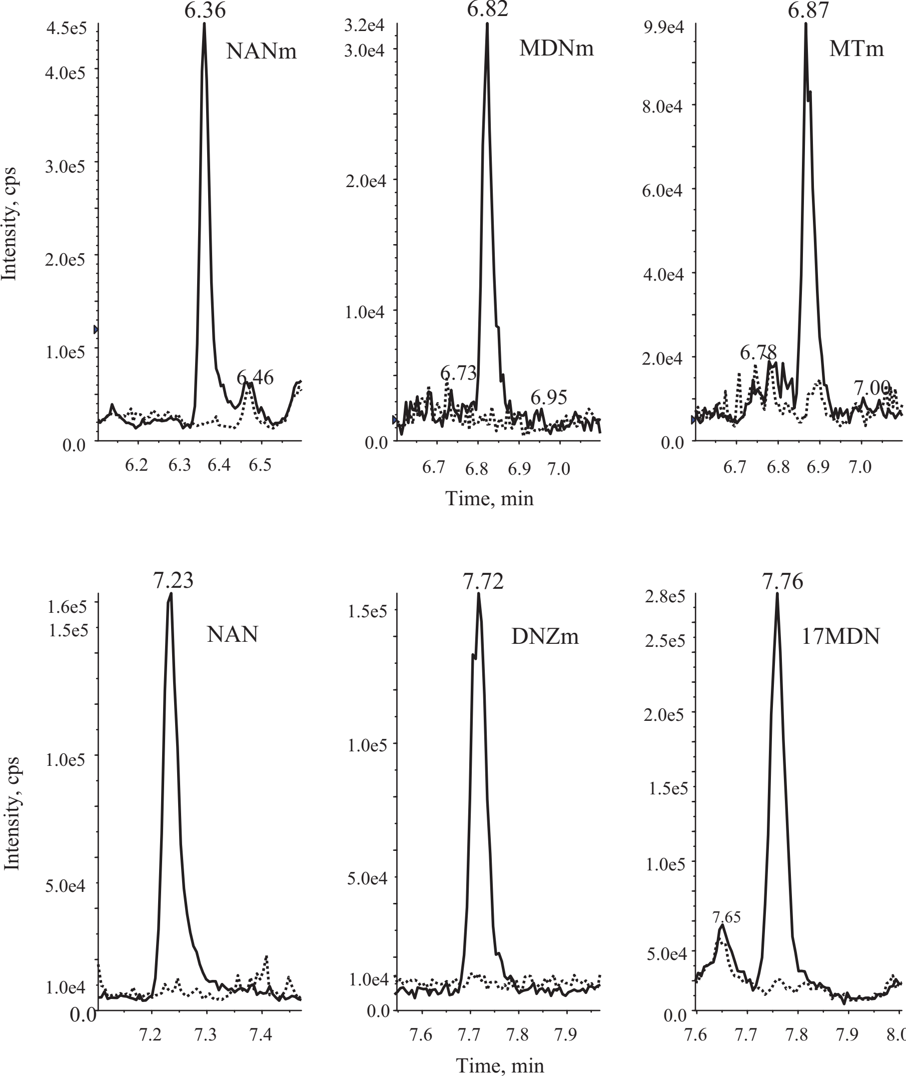


Fig. 3. Chromatograms from a urine sample spiked (5 ng/mL) with anabolic steroids (solid line) and urine blank (dotted line).

times were proportional to the polar moieties in the chemical structure of the steroid and the size of the molecule. The steroids without conjugated double bond eluted first and then the steroids with 4-ene-3-one structure starting from nandrolone (Fig. 3). Although the peaks of MTm/MDNm and DNZm/17MDN were partly overlapping, the unique fragmentation of the compounds in MS/MS allowed their specific analysis. The retention times of analyte peaks were repeatable (RSDs 0.06–0.08%), and the peak widths at half height (0.0141–0.0283 s) and the peak symmetry factors (0.74–1.81) were acceptable for all compounds (Table 4). These results indicate the proper functioning of the chromatography and a minimal dead volume in the microchip. The specificity of the method was good: the product ion chromatogram of urine blank samples did not show any interfering peaks at the retention times of the analytes (Fig. 3).

In doping analysis, qualitative determination is sufficient for exogenous anabolic steroids, but quantification is required for endogenous AAS (e.g., nandrolone and its metabolites). The feasibility of GC–μAPPI–MS/MS in quantitative analysis of anabolic steroids in urine was therefore validated with respect to limit of detection (LOD), linearity, linear range, and repeatability. LODs determined at signal-to-noise ratio of three, using standard deviation of the analyte peak height *vs.* background noise, were 0.2–1 ng/mL (Table 4). Since the minimum required performance limits are 2 or 10 ng/mL (depending on the compound) sensitivity of the method is easily sufficient for doping control [51]. Our detection limits for underivatized AAS from urine matrix are similar to those obtained by LC–MS/MS methods [52]. Linearity, linear range, and repeatability were examined with respect to peak area ratio of analyte to ISTD. Repeatability of injection (conc. 25 ng/mL; $n = 6$) was 2.5–4.2% for all compounds except MDNm and MTm, where values were higher but still under 10%. The poorer repeatability for MDNm and MTm may originate from instability of the ion–molecule reaction in the ionization process (see discussion above). Linearity determined in the range of 1–250 ng/mL was very good as the regression coefficient (r) was over 0.9995 for all compounds.

Performance of the GC–μAPPI-MS/MS method was tested with two authentic urine samples collected from two male subjects after a single dose of methandienone. Results were compared with those obtained from a doping control laboratory (United Medix Laboratories Ltd.) applying the conventional GC–EI-MS method to TMS derivatives. Three metabolites of methandienone (17MDN, MTm and MDNm) were quantified. Concentrations in sample 1 were high and the sample was diluted before analysis in order to reach the calibration range. Concentrations of 17MDN obtained by GC–μAPPI-MS/MS method were in good agreement with results obtained by conventional GC–EI-MS method (Table 5).

Table 4
Repeatability, and detection limits of the GC–μAPPI-MS/MS method. Peak widths at half height, peak symmetry factors, and regression coefficients for concentration versus peak area. See Table 1 for analyte nomenclature.

Compound	Repeatability of injection (RSD%)	Repeatability of retention time (RSD%)	LOD (ng mL^{-1})	W_h	Peak symmetry factor	R (weighting 1/x)
NANm	3.2	0.07	0.2	0.0223	0.95	0.9995
MDNm	9.6	0.08	1.0	0.0283	1.10	0.9997
MTm	9.4	0.08	0.5	0.0141	1.07	0.9997
NAN	4.2	0.06	0.2	0.0283	1.81	0.9999
DNZm	2.7	0.07	0.3	0.0245	0.74	0.9998
17MDN	2.5	0.08	0.3	0.0272	1.36	0.9996

Table 5
Analysis of authentic urine samples: quantitative results obtained in two laboratories, one using GC–EI-MS method and the other GC–μAPPI-MS/MS.

Compound	GC–EI-MS sample 1	GC–μAPPI-MS/MS sample 1	GC–EI-MS sample 2	GC–μAPPI-MS/MS sample 2
MDNm	388	579	86	151
MTm	373	461	171	212
17MDN	303	328	33	32

4. Conclusions

The heated nebulizer microchip operated in APPI mode (μAPPI) provides efficient ionization of anabolic steroids and an easy way to couple GC to API-MS. The GC–μAPPI-MS/MS method was developed and its quantitative performance was validated in the analysis of underivatized anabolic steroids in urine samples. The method showed good sensitivity and quantitative performance, demonstrating its potential for the analysis of biological samples. The advantage of GC–μAPPI-MS/MS is soft and efficient ionization that produces abundant protonated molecule or molecular ion with only slight fragmentation, ensuring good selectivity and sensitivity.

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