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# Mass Spectrometry of New Growth Promoting Drugs: Hydantoin-Derived Selective Androgen Receptor Modulators and Growth Hormone Secretagogues

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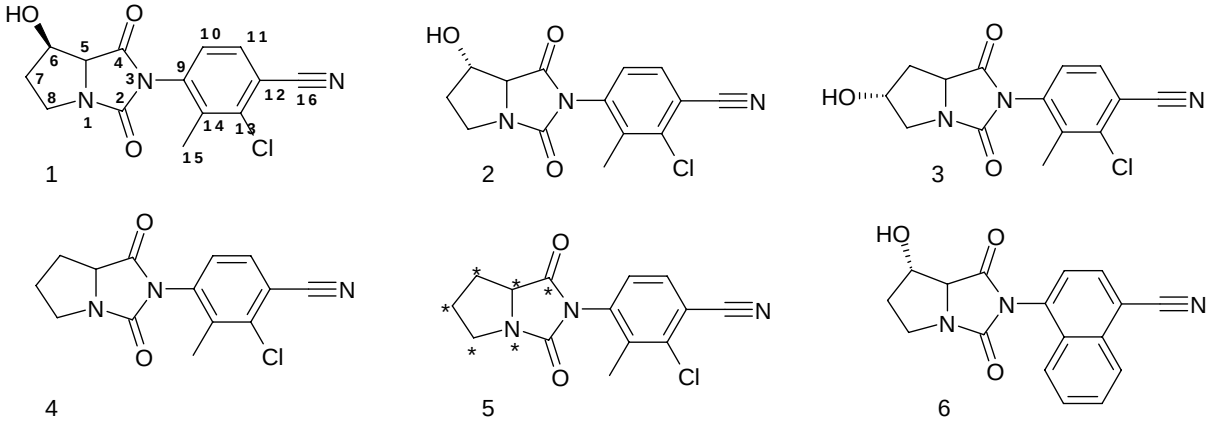
## Introduction

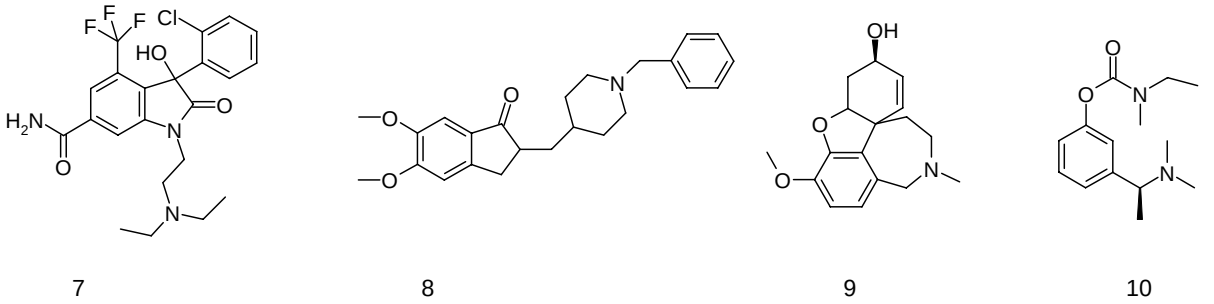
Novel classes of therapeutics with growth promoting properties are so-called selective androgen receptor modulators (SARMs) and orally active growth hormone secretagogues. The first mentioned group has gained much attention due to its considerable anabolic properties with significantly reduced side effects due to tissue selectivity, and potential clinical applications range from the treatment and prevention of osteoporosis, frailty, and wasting diseases[1]. Moreover, the use as contraceptive agent for males has been discussed recently. SARMs comprise a very heterogeneous group of compounds, and bicyclic hydantoins (Scheme 1) were subject of the present investigation in terms of their mass spectrometric behaviour and the option to analyze these compounds from urinary matrix.

The endogenous growth hormone (hGH) production decreases from the early-to-mid adulthood by about 14% per decade[2], which is, in combination with the concomitant decline in IGF-1, considered a major reason for age-related decrease of physical performance and increased frailty. Orally active growth hormone secretagogues (GHS) are intended to complement/substitute hGH therapies, and numerous drug candidates were introduced by pharmaceutical companies including peptidyl and non-peptidyl candidates[3; 4]. The peptides including GHRP-6, GHRP-1, GHRP-2, hexarelin and alexamorelin as well as non-peptidyl secretagogues such as MK-0677 (Ibutamoren) or SM-130686 (Scheme 2) promote the release of GH in humans via a mechanism that differs from that known from the endogenous GH releasing hormone. Despite promising pre-clinical and clinical test results, none of the synthetic GHS has been introduced as therapeutic agent that would allow the substitution of injections of recombinant GH for disease treatments such as GH deficiencies, but one drug (Ibutamoren) has recently finished phase-II clinical studies and SM-130686 has demonstrated excellent *in vivo* activitiy after oral administration. In addition to intended developments of GHS, the potential of two cerebral selective acetylcholine esterase inhibitors (AChEI) to act

as GHS was described in the literature recently. The drugs donepezil as well as rivastigmine (Scheme 2) are commonly used for the treatment of symptoms arising from Alzheimer's disease by extending the half-life of the neurotransmitter acetylcholine by inhibition of its hydrolysis mediated by acetylcholine esterase. The increased concentration of hypothalamic acetylcholine results in a suppression of somatostatin, a fact which is assumed to cause an improved availability of endogenously produced GH. A screening and confirmation assay for donepezil, rivastigmine, galantamine, as well as some major metabolites and a structural analogue to SM-130686 in human urine was developed based on mass spectrometric studies using LC-ESI-MS/MS.




Scheme 1: Chemical structures of the drug candidate BMS564929 (**1**) and structural (**2**-**4** and **6**) as well as a stable isotope labeled (**5**) analogues. The isotopically modified positions are marked with an asterisk.




Scheme 2: Chemical structures of the drug candidate SM130686 (**7**) and donepezil (**8**), galantamine (**9**) and rivastigmine (**10**).

## Materials and methods

Bicyclic hydantoins such as BMS-564929 (Scheme 1) and analogues were prepared according to a method published by Ostrowski and co-workers[5], and a structural analogue to SM-

130686 (**1**, Scheme 2), a promising candidate of GH secretagogues, was prepared according to procedures published by Tokunaga et al[6]. Here, the ortho-positioned chlorine atom was located in para-position, and the analogue is referred to as compound **1** in the following. The drugs donepezil-HCl (Aricept), galantamine-HBr (Reminyl), and rivastigmine hydrogen tartrate (Exelon), were from Pfizer (Karlsruhe, Germany), Janssen-Cilag (Neuss, Germany) and Novartis (Horsham, UK), respectively.

*Mass spectrometry*

ESI-MS(/MS) was performed on a Thermo LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) employing positive as well as negative ionization. The instrument was calibrated using the manufacturer's calibration mixture allowing for mass accuracies < 3 ppm. Analytes were dissolved in acetonitrile/water (1:1, v:v) at concentrations of 2 µg/mL and introduced into the mass spectrometer using a syringe pump at a flow rate of 5 µL/min. The ionization voltage was 4.5 kV. The capillary temperature was set to 275 °C, protonated precursor ions were isolated at isolation widths of 2 Da and dissociated using normalized collision energies (arbitrary units) between 15 and 35 such that the relative abundance of the precursor ion was ca. 10% in product ion spectra. Damping gas in the linear ion trap was helium 5.0, and gas supplied to the curved linear ion trap (CLT) was nitrogen obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany).

*Liquid chromatography – tandem mass spectrometry*

All analyses were performed using an Agilent 1100 Series liquid chromatograph (Waldbronn, Germany) coupled to an Applied Biosystems API2000 Qtrap mass spectrometer (Darmstadt, Germany) with electrospray ionization. The LC was equipped with a Phenomenex Synergi Polar RP column (2 x 50 mm, 2 µm particle size), and the eluents used were 5 mM ammonium acetate containing 0.1% acetic acid (mobile phase A) and acetonitrile/methanol (1:1, v:v, mobile phase B). A gradient was employed starting at 1% B increasing to 100% B within 6 min followed by re-equilibration at 10% B for 10 min. The flow rate was set to 250 µL/min. The ion source was operated in the positive mode for GH secretagogues and negative mode for bicyclic hydantoins using spray voltages of 5500 V and -4500 V, respectively. All analytes were detected by means of characteristic product ions formed from protonated molecules or deprotonated and adduct ion species by collision-induced dissociation (CID) utilizing the multiple reaction monitoring mode (MRM, Table 1).

*Sample preparation*

SARMs and GH secretagogues were extracted from urine specimens using established SPE and LLE procedures[7; 8].

*Assay validations*

The qualitative determination of donepezil, galantamine, rivastigmine, and compound **1** in human urine was validated regarding specificity, recovery, lower limit of detection (LLOD), intraday and interday precision according to ICH guidelines as summarized in Table 2. For bicyclic hydantoins only the LLOD was estimated as no data on the metabolic fate are currently available.

Table 1: Mass spectrometric parameters for donepezil, galantamine, rivastigmine, compound **1**, BMS-564929 and selected analogues

| Compound | Ionization | Precursor ion | Declustering potential (V) | Ion transition (*m/z*) | Collision offset voltage (V) | Dwell time (ms) |
|---|---|---|---|---|---|---|
| donepezil | positive | $[M+H]^+$ | 66 | 380 – 288 | 25 | 40 |
| | | | 66 | 380 – 243 | 31 | 40 |
| | | | 66 | 380 – 91 | 55 | 40 |
| galantamine | positive | $[M+H]^+$ | 36 | 288 – 231 | 21 | 40 |
| | | | 36 | 288 – 225 | 30 | 40 |
| | | | 36 | 288 – 213 | 27 | 40 |
| rivastigmine | positive | $[M+H]^+$ | 21 | 251 – 206 | 19 | 40 |
| | | | 21 | 251 – 121 | 40 | 40 |
| | | | 21 | 251 – 86 | 31 | 40 |
| compound **1** | positive | $[M+H]^+$ | 76 | 470 – 397 | 29 | 40 |
| | | | 76 | 470 – 100 | 47 | 40 |
| | | | 76 | 470 – 86 | 45 | 40 |
| BMS-564929 | negative | $[M-H+CH_3OH]^-$ | -50 | 336 – 304 | -8 | 50 |
| and | | | -50 | 336 – 248 | -18 | 50 |
| | | | -50 | 336 – 165 | -36 | 50 |
| isomer (2) of BMS-564929 | negative | $[M-H]^-$ | -60 | 304 – 248 | -18 | 50 |
| analogue (6) of BMS-564929 | negative | $[M-H+CH_3OH]^-$ | -100 | 306 – 288 | -14 | 50 |
| | | | -100 | 306 – 250 | -14 | 50 |
| | | | -100 | 306 – 195 | -20 | 50 |

## Results and discussion

*Mass spectrometry of bicyclic hydantoins*

Using positive electrospray ionization, the protonated precursor of **BMS-564929** is generated at *m/z* 306 (Figure 1a) that gives rise to a number of characteristic product ions upon CID. Protonation of hydantoins and related structures most likely occurs at either one of the two

nitrogens or the carbonyl oxygens while an initial O-protonation is considered thermodynamically more favorable. However, the mobile nature of the introduced proton also allows its subsequent location at nitrogen and, thus, dissociation routes starting from both scenarios. An initial loss of water gives rise to the product ion at *m/z* 288 that subsequently eliminates CO (-28 Da) yielding the ion at *m/z* 260. The exclusive elimination of the carbonyl residue including C-4 was demonstrated using a $^{13}C_5{}^{15}N$-labeled analogue that solely lost 29 Da proving the participation of $^{13}C$ in the leaving group (Figure 1d). In addition, the elimination of acetaldehyde from the protonated molecule was observed generating a product ion at *m/z* 262 presumably comprising a three-member ring structure.

Additional product ions of BMS-564929 with considerably lower abundance but nevertheless structural significance were observed at *m/z* 193, 96 and 86 (Figure 1a). The first mentioned ions represent counterparts in the dissociation of the intact and protonated molecule (Scheme 3) that either retains the charge within the former hydroxylated proline residue or the isocyanate structure. The product ion at *m/z* 86 is proposed to result from a protonated molecule bearing the charge at N-1, which simultaneously eliminates CO (-28 Da) and 2-chloro-4-isocyanato-3-methyl benzonitrile (- 192 Da) forming *m/z* 86 with a proposed structure of protonated 4,5-dihydro-3H-pyrrol-3-ol. Also here, substantiating information was obtained from stable isotope labeling.

*Mass spectrometry of GH secretagogue SM-130686*

Compound **1**, which was synthesized to mimic physicochemical properties of SM-130686, gave rise to the product ion mass spectrum depicted in Figure 2d. Its major fragmentation pathway leads to *m/z* 397 by the loss of diethylamine (-73 u) that subsequently eliminates chlorobenzene (-112 u) and carbon monoxide (-28 u) generating *m/z* 285 and 257, respectively. The initial loss of water from $(M+H)^+$ at *m/z* 470 produces *m/z* 452, which additionally releases ethylvinylamine (-71 u) and ethene (- 28 u) or $C_3H_5NO$ (-71 u) yielding *m/z* 381 and 353 or 310, respectively. Furthermore, abundant product ions are observed at *m/z* 86 and 100 (Figure 2d) that are composed by $C_5H_{12}N$ and $C_6H_{14}N$, respectively.

*Mass spectrometry of acetylcholine esterase inhibitors*

The protonated molecule of donepezil $(M+H)^+$ at *m/z* 380 dissociates efficiently under CID conditions as illustrated in Figure 2a. The precursor ion predominantly yields the product ion

at *m/z* 91, the composition of which represents a typical tropylium ion as substantiated with deuterium labeling[9].

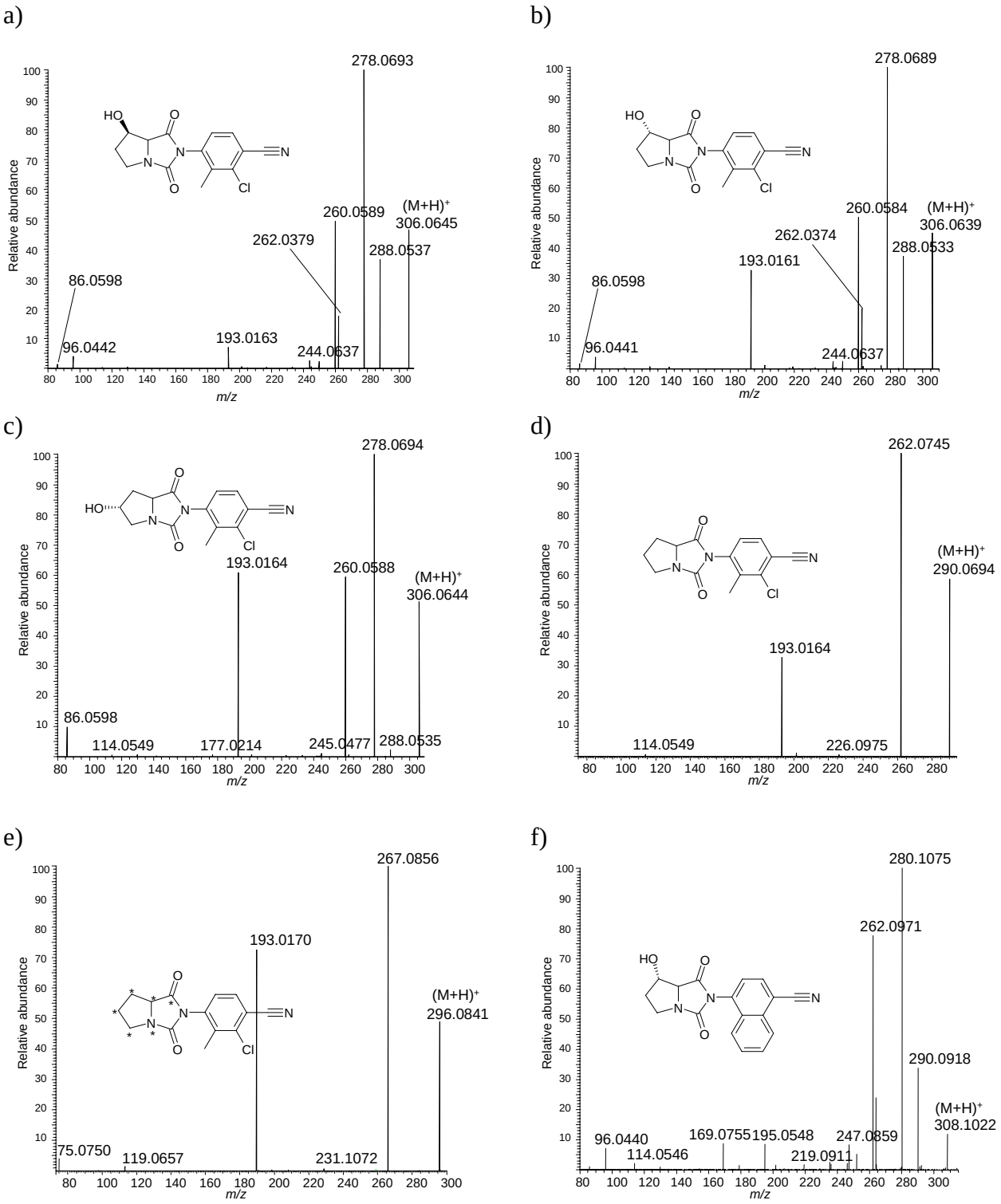



Figure 1: Positive ESI product ion spectra of the synthesized drug candidate BMS564929 (a) and its structural as well as isotope-labeled analogues (b-f). All spectra were recorded on a Thermo LTQ Orbitrap at arbitrary collision energy values between 30 and 35.

Locating the initial charge at the keto function of donepezil a neutral loss of toluene is observed (-92 u) that yields an abundant signal at *m/z* 288 that subsequently generates numerous additional product ions as demonstrated in $MS^3$ studies (data not shown). The loss of water (- 18 u) gives rise to *m/z* 270 that showed considerable stability in $MS^4$ experiments yielding no evaluable product ions. Hence, the generation of *m/z* 243 is suggested to result from a simultaneous elimination of water (- 18 u) and HCN (- 27 u).

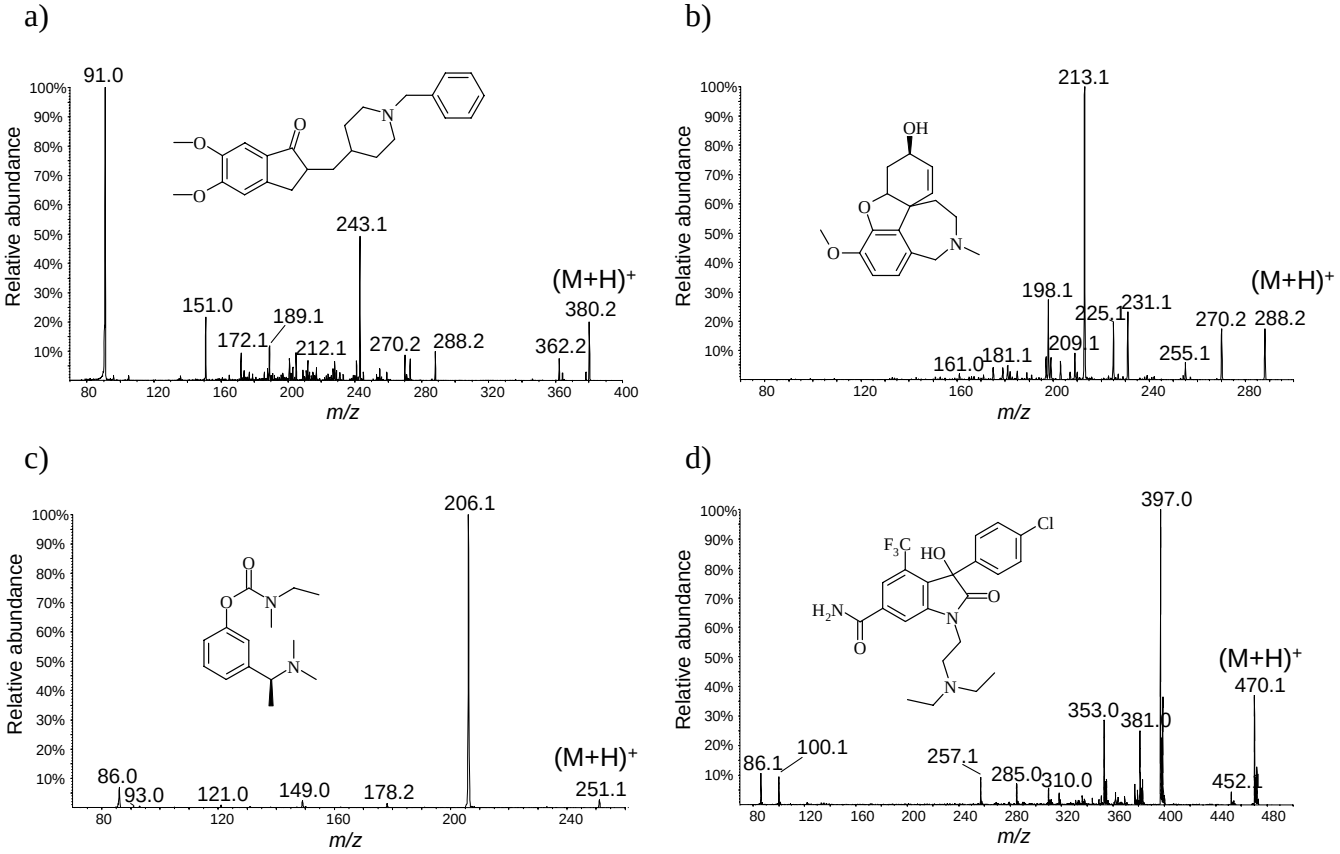



Figure 2: ESI product ion spectra of protonated molecules $(M+H)^+$ of a) donepezil (collision offset voltage = 45 V), b) galantamine (collision offset voltage = 30 V), c) rivastigmine (collision offset voltage = 25 V), and d) compound **1** (collision offset voltage = 40 V). All spectra were recorded on an Applied Biosystems 2000 Qtrap using nitrogen as collision gas.

The dissociation pathway of galantamine (Figure 2b) after positive electrospray ionization was studied a few years ago providing suggestions for structure and composition of selected gas phase product ions [10; 11]. Using high resolution/high accuracy $MS^n$ measurements, several postulations were substantiated. The precursor ion at *m/z* 288 protonated presumably at the tertiary amino function that bears the highest proton affinity in the present molecule (approximately 952 kJ/mol)[12], yields the product ion at *m/z* 231 due to a loss of methylvinylamine (-57 u), and the subsequent elimination of water gives rise to *m/z* 213 as

proposed also by Verhaeghe *et al.*[11] However, the fragmentation pathway leading to *m/z* 225 and 197 as determined in our study is not in accordance with the postulated structure made in the above mentioned article. $MS^3$ experiments demonstrated that the generation of *m/z* 225 occurs via *m/z* 257. The release of methylamine from *m/z* 288 yields the ion at *m/z* 257, which presumably requires the initial protonation of the precursor ion at the methoxy residue although it bears a considerably weaker proton affinity (approximately 840 kJ/mol)[13; 14] than the tertiary amino function. The subsequent loss of methanol (-32 u) gives rise to *m/z* 225, which eliminates 28 u to yield *m/z* 197. High accuracy/high resolution mass spectra provided evidence for the loss of carbon monoxide.

The product ion spectrum of the protonated molecule $(M+H)^+$ at *m/z* 251 of rivastigmine is presented in Figure 1c. The most intense product ion of rivastigmine is observed at *m/z* 206 resulting from the elimination of dimethylamine (-45 u), which subsequently dissociates into numerous additional product ions. The loss of carbon monoxide (- 28 u) gives rise to *m/z* 178, presumably accompanied by a rearrangement shifting the amino function to the phenolic oxygen. The removal of ethylidene-methylamine (-57 u) yields the product ion at *m/z* 149 that consecutively eliminates carbon monoxide (-28 u) to *m/z* 121 as proved using $MS^3$ experiments. The ion at *m/z* 86 is suggested to consist of *N*-ethyl-*N*-methyl-formamide resulting from the loss of 4-ethylidene-cyclohexa-2,5-dienone from *m/z* 206.

*Assay validation*

The GH secretatgogue SM-130686 as well as the Alzeimer drugs donepezil, galantamine and rivastigmine undervent validation yielding the results summarized in Table 2. All drugs were recovered from spiked urine specimens between 55 and 97%, and LLODs were accomplished between 15 and 20 ng/mL. Intraday and interday precisions ranged from 5.2 to 21.6% for all compounds.

## Conclusion

New growth promoting drugs based on either GH secretagogue or SARM activity were studied using synthesized and commercially available reference material and high resolution/high accuracy $MS^n$. Based on characteristic product ions drugs, were measured from urine specimens employing LC-MS/MS instruments that shall allow their detection in doping control samples. Neither bicyclic hydantoins nor orally active GH secretagogues such as compound **1** have received clinical approval yet, but their potential for being misused in sports is very high and test methods might be necessary very soon.

**Acknowledgments**

The authors thank the Manfred Donike Institute for Doping Analysis for supporting the presented work.

Table 2: Assay validation results

| Compound | | | Intraday precision (n=30) | | Interday precision (n=90) | |
|---|---|---|---|---|---|---|
| | LLOD (ng/mL) | Recovery (%) at 100ng/mL | Concentration (ng/mL) | CV (%) | Concentration (ng/mL) | CV (%) |
| donepezil | 20 | 92 | 50 | 14.3 | 50 | 15.1 |
| | | | 250 | 10.5 | 250 | 15.8 |
| | | | 500 | 5.2 | 500 | 12.4 |
| galantamine | 20 | 55 | 50 | 11.0 | 50 | 13.5 |
| | | | 250 | 15.8 | 250 | 17.4 |
| | | | 500 | 11.1 | 500 | 19.1 |
| rivastigmine | 20 | 97 | 50 | 11.3 | 50 | 14.8 |
| | | | 250 | 9.3 | 250 | 15.2 |
| | | | 500 | 7.3 | 500 | 10.2 |
| compound **1** | 15 | 95 | 50 | 8.7 | 50 | 17.1 |
| | | | 250 | 9.2 | 250 | 21.2 |
| | | | 500 | 8.2 | 500 | 21.6 |
| BMS-564929 | 20 | n.d. | - | n.d. | - | n.d. |

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