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Complementary use of ion trap/time-of-flight mass spectrometry in combination with capillary high-pressure liquid chromatography: early characterization of in vivo metabolites of the cathepsin K inhibitor NVP-AAV490 in rat

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Abstract

Cathepsin K is a cysteine proteinase, primarily expressed in osteoclasts, which has a strong collagenolytic activity and plays an essential role involved in bone matrix degradation. Its inhibition could provide a novel approach to the treatment and prevention of osteoporosis. One structural class of lead compounds in our cathepsin K inhibitors program is based on an arylaminoethyl amide scaffold, which has potential metabolic weak points that might be stabilized by appropriate chemical modification(s). For the identification of potential metabolic ''soft spots'' and the rational design of improved derivatives, early biotransformation of a potent arylaminoethyl amide cathepsin K inhibitor (NVP-AAV490-NX) was investigated in plasma, urine and liver homogenates of rats after intravenous bolus administration of 10 mg/kg. The detection and identification of metabolites was achieved by high-resolution mass spectrometry (time-of-flight MS) and multi-dimensional mass spectrometry (ion trap MS). Both mass spectrometers were combined with reversed-phase capillary high-performance liquid chromatography columns. It was demonstrated that both mass analyzers complement each other and that, even in the sub-nanogram range, the resulting set of MS data can be successfully used to elucidate most of the metabolic changes unambiguously, solely by mass spectrometric techniques. The proposed metabolite structures were additionally corroborated by exact mass measurement of the protonated molecular ions to confirm the predicted elemental composition, by determination of the number of the exchangeable hydrogen atoms replacing water against deuterium oxide as mobile phase and, in one case, by an MS^3 product ion experiment in order to elucidate the site of conjugation.
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1. Introduction

The accelerated pace in drug development has generated an increasing demand for early information on the biotransformation of drug candidates during the optimization process in order to improve

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the pharmacokinetic parameters and/or to extrapolate metabolic data and risk assessment from animals to humans and to support the selection of new compounds for clinical evaluation.

Since in metabolites, the parent structure is normally modified in its periphery rather than in its structural core, mass spectrometric techniques provide an excellent means for revealing most of the metabolic modifications. Today, the most sensitive and popular analytical approach for early metabolite characterization is based, nearly exclusively, on mass spectrometric techniques (MS) in combination with high-performance liquid chromatography (HPLC).

However, the direct on-line recognition and characterization of sub-nanogram amounts of potential in vivo metabolites in blood, urine, liver extracts etc. in the early phase of compound development is almost always a challenge. The mixtures to be analyzed can be rather complex, and in most of the cases the target compounds are covered by huge amounts of endogenous matrix compounds or vehicles used in experimental formulations. In the early stage of compound development, unlabelled drug candidates are administered and no radioactive detection is paving the way. Moreover, the concentration of the potential metabolites can be very low, their polarity can vary over a wide range, and if small animal models are used, which is very often the case, only some microlitres of plasma or organ extracts are available.

In a recent report we pointed out that none of the available mass analyzers recommended for metabolite identification, such as time-of-flight-MS (TOF), triple stage quadrupole MS (triple-quad), ion-trap MS (IT), or hybrid instruments such as quadrupole/time-of-flight MS (Q-TOF) etc., fulfils all technical prerequisites for sensitive and comprehensive metabolite characterization [1]. Each of these instruments has its particular merits and limitations and, in order to use the entire potential of mass spectrometric techniques, it is unavoidable to perform the investigation on various complementary mass analyzers.

In this context, we reported on the advantages of the complementary use of time-of-flight-MS and triple stage quadrupole-MS in combination with capillary HPLC [1]. In this work we discuss a variation of this procedure, the in vivo metabolite characterization by means of the complementary use of capillary HPLC–TOF-MS and capillary HPLC–IT-MS. Although TOF and IT mass spectrometers are based on fundamentally different mass analyzer designs, both have an extraordinary high detection sensitivity in full-scan mode in common.

The single analyzing TOF has its strength in a relatively high resolution, resulting in more selective ion chromatograms detected within the range of some millidaltons, and on its extremely high acquisition speed as one prerequisite of exact mass measurements with an acceptable accuracy ($<$10 ppm).

When comparing the multidimensional MS performance of the ion trap with the triple quadrupole mass spectrometer, the IT-MS demonstrates two advantages, higher full-scan product ion spectra sensitivity and last but not least MS^n capability [2].

Our current area of research, osteoporosis, comprises several different disorders associated with decreased bone mass in the absence of a mineralization defect, resulting in a higher rate of bone resorption than bone formation. In this context, osteoclasts have been established as the cells responsible for bone resorption [3]. The cysteine proteinase cathepsin K, predominantly and highly expressed in osteoclasts, might serve as a novel target to inhibit osteoclastic bone resorption [4–6]. The internal lead structures in the current cathepsin K inhibition program are based on an arylaminoethyl amide scaffold [7]. In order to optimize metabolic stability of this structural class, the in vivo metabolism of the cathepsin K inhibitor NVP-AAV490 (see Fig. 1) was investigated by capillary HPLC–MS(MS^n) in an early phase of compound development in plasma, urine and liver homogenates of rats, after intravenous administration of 10 mg/kg of parent compound.

It has been demonstrated that TOF- and IT mass spectrometers complement each other, and that the resulting set of MS data can be successfully used to elucidate most of the metabolic changes, even in the sub-nanogram range and even in the case of co-

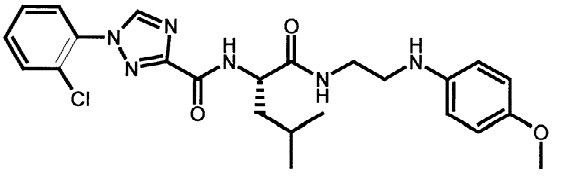


Fig. 1. Structural formula of NVP-AAV490.

eluting compounds, solely by mass spectrometric techniques.

Results of such investigations provide an early insight into metabolic weak points of lead structures and allow for specific modifications at unstable sites.

2. Experimental

2.1. Test compounds and chemicals

The parent compound NVP-AAV490, 1-(2-chlorophenyl)1.H-[1,2,4]triazole-3-carboxylic acid {(*S*)-1-[2 - (4 - methoxy - phenylamino) - ethylcarbamoyl] - 3-methyl-butyl}-amide was prepared in Novartis Pharma AG, Pharmaceutical Research, Basel, as reported recently [7]. For intravenous administration, the compound was dissolved in DMSO at 10 mg/ml, to obtain a final injection volume of 1.0 ml/kg total body weight (=10 mg/kg).

2.2. Administration to animal and sampling

All animal experiments described below were carried out under licence no. 1630, issued by the Cantonal Veterinary office Basel-Stadt, Switzerland. Female Sprague–Dawley rats (OFA), weight 280–350 g were housed under standard conditions with free access to food and water throughout the experiment. On the day of the experiment, animals were anaesthetised immediately before intravenous (i.v.) dosing with a bolus dose of 100 mg/kg ketamine (Ketaminol® 10, Veterinaria, Zürich, Switzerland) and 10 mg/kg xylazine (Narcoxyl®, Veterinaria), both administered i.p. During the experiment, the anaesthesia was maintained, if necessary, by additional doses of ketamine (~15 mg/kg/h). The animals were immediately transferred to a heating pad, which allowed accurate control of body temperature via a flexible rectal temperature probe connected to an electronic control unit. For rapid blood sampling, one of the carotid arteries, for i.v. bolus administration, and one of the jugular veins was cannulated using polyethylene tubing (0.5 mm I.D., 1 mm O.D., portex, GB). The urinary bladder was also cannulated for the later collection of the urine samples. Liver samples were taken 1 h post administration after sacrificing the animals. The samples were prepared immediately for analysis by HPLC–MS.

2.3. Study design and sample preparation

Immediately before and 60 min after administration of NVP-AAV490, blood samples with a total volume not exceeding 3 ml were collected in EDTA-coated Eppendorf-tubes.

Plasma was separated from blood cells by centrifugation (the samples were kept on ice not longer than 1 h).

Urine was obtained by puncture and emptying of the urinary bladder before administration of the compound. After cannulation, the urine was collected for 1 h post administration. Aliquots of 200 μl urine were treated as plasma and liver samples (see Section 2.4).

After sacrificing the animals, the liver was immediately removed from the abdominal cavity and transferred to a beaker with isotonic saline solution at 4 °C. Within the next 30 min, the liver was finely minced with scissors on a cooled petri dish and homogenized with a Polytron PT1200 tissue homogenizer (2 min at 2000 rev./min, then 2 min at 5000 rev./min). Four tissue aliquots of 1 g each were transferred to 3-ml Eppendorf tubes and 1 ml of ice cold saline solution added. This mixture was vortexed and centrifuged for 2 min at 20,000 *g*. Two hundred μl of the clear, red supernatant were removed and extracted as described below (Section 2.4).

2.4. Protein precipitation

Proteins of rat plasma and liver were precipitated by addition of a triple volume of acetonitrile and cooling at 4 °C in three steps. After 45 min, the protein precipitate was removed by centrifugation (13,000 rev./min). The supernatant was evaporated under vacuum. The dry residue was taken up in 150 μl methanol–water (1:9). An aliquot of 10 μl was used for capillary HPLC–MS analyses.

2.5. Instrumental

2.5.1. Capillary high-pressure liquid chromatography

In order to protect the analytical column and to enrich the target compounds, the samples were injected via a pre-column in back-flush mode. The system consisted of an automated injection device

with cycle-composer (HTS PAL, CTC Analytics, Zwingen, Switzerland) and a trapping column, YMC H80, 5 mm×1 mm, particle size 4 μm (YMC Europe, Schermbeck, Germany) [8,9].

The liquid chromatographic separation was performed using an Eldex-MicroPro Capillary-HPLC system (Stagroma, Reinach, Switzerland) and a laboratory-made glass capillary column, 150 mm× 0.4 mm, filled with Xterra MS C_{18}, particle size 2.5 μm (Waters, Milford, MA, USA). Gradient mobile phase programming was used with a flow-rate of 6 μl min^{-1}. Eluent A was water–acetonitrile (9:1)+ 0.2% formic acid. Eluent B was acetonitrile–water (9:1)+0.2% formic acid. The mobile phase was held 3 min isocratic at 5% B, followed by a linear gradient from 5% B to 95% B over 30 min and a 5-min hold at 95% B.

For the hydrogen–deuterium (H/D) exchange, the experimental conditions remained the same but water was replaced by deuterium oxide.

2.5.2. Mass spectrometry

The column effluent was introduced directly either into the electrospray ion source of an ion trap mass spectrometer (LCQ-Deca, Finnigan MAT, San Jose, CA, USA) or into a two-channel Z-spray (LockSpray™) of a time-of-flight mass spectrometer (LCT, Micromass, Manchester, UK). The mass reference standard (0.001% reserpine diluted in water–methanol (1:1, v/v)+0.2% formic acid) for exact mass measurement was delivered via the second spray channel at a flow-rate of 10 μl/min from a HT syringe pump (Infors, Bottmingen, Switzerland). The TOF-MS was operated at a resolution of 6000 $m/\Delta m_{FWHM}$. Spectra were acquired from 150 to 1000 amu at an acquisition rate of 20 spectra/s. The ionization technique employed was positive electro-spray (ES). The sprayer voltage was kept at 3400 V for both the sample and the reference. The cone voltage of the ion source was kept at a potential of 30 V. The H/D exchange experiment was performed under the same conditions, but with D_2O–acetonitrile as mobile phase.

The ion trap was tuned by fuel injection analysis (10 μl/min) of NVP-AAV490 (10 ng/μl) dissolved in water–methanol (80:20) containing 0.2% formic acid. The following tune parameters were used: capillary temperature, 240°; sheath gas flow, 45 (arbitrary units); source voltage, 3.5 kV; source current, 80 μA; capillary voltage, 12 V; tube lens offset, −30 V; octapole RF amplifier, 780 V_{p-p}; octapole 1 offset, −6.75 V; octapole 2 offset, −23.5 V; interoctapole lens voltage, −16 V; entrance lens, −62 V; trap DC offset voltage, −10 V.

The IT-MS was operated in MS as well as in MS^n scan mode with automatic gain control on three microscans and 200 ms maximum ion injection time. The isolation width during MS^n experiments was 2.0 u and the collision energy was set to 30% (relative units).

3. Results and discussion

3.1. The role of mass spectrometry in early drug metabolism

The characterization of compounds whose structures are totally unknown by MS can often seem daunting, when identical standard compounds are not available. However, structure elucidation of metabolites exclusively by mass spectrometry, in particular in combination with high-performance liquid chromatography, has a much better prognosis. Metabolites are derivatives of known parent compounds, so that MS-techniques provide an excellent means for revealing almost any kind of modification and, occasionally, its site.

In studies with small animal models, where only small sample volumes are available, the detection sensitivity of the analytical systems is of fundamental importance. In this context, beside the general performance of the mass spectrometer, the advances in instrument design have led to an impressive availability of various mass analyzers such as TOF-, triple-stage quad-, Q-TOF-, IT-MS etc. Each of these instruments is warmly recommended for metabolite identification by its supplier, but as mentioned above, each has its particular merits and limitations. Direct sensitive and comprehensive structure elucidation of in vivo metabolites by mass spectrometry, requires the full potential of the available mass spectrometric techniques such as product ion-, precursor ion- and neutral loss scans, accurate mass measurements, determination of the exchangeable hydrogen atoms etc. Our daily practice has shown that direct on-line

in vivo metabolite characterization performed with only one type of mass analyzer, very often leads to non coherent results and that the risk to miss metabolites depends strongly on the intuition of the specialist and on his know-how in bio-transformation reactions. The situation can be significantly improved by the operation of various complementary mass analyzers, whereby the appropriate MS technique has to be selected on a case-by-case basis.

3.2. The role of capillary HPLC in early drug metabolism

As mentioned above, early metabolism studies mean very often structure elucidation of trace amounts in the sub-nanogram range, in particular when performing studies with small animal models, the chromatographic method can play a crucial role for the achievement of the ultimate detection sensitivity. When using a mass spectrometer in conjunction with HPLC, the electrospray (ES) ion source behaves like a concentration-sensitive detector, where the ion-current is directly proportional to the analyte concentration in the chromatographic peak which in turn is dependent on the internal diameter of the analytical column. Consequently, capillary columns are the separation systems of choice. The column effluent should be introduced directly into the ion source of the mass spectrometer, without any splitting device [1].

A significant increase of sensitivity can only be achieved when the injection volumes, commonly used for wider columns, can be transferred to packed capillary columns. This can be achieved by on-line solid-phase extraction (SPE) of the biological samples, using column switching, to focus the analytes in the sample onto a trapping column, followed by backflushing of the target compounds on the packed analytical capillary column [1,8,9]. Moreover, this approach helps to protect the analytical column against polar parts of the endogenous matrix and salts.

3.3. Metabolite characterization by single analyzing MS

Direct metabolite characterization in biological fluids is, first of all, a problem of orientation within rather complex mixtures and the recognition of trace amounts of potential metabolites. In most of the cases the target compounds were covered by endogenous matrix compounds and/or vehicles used in experimental formulations. For a first overview, the instrument of choice is the time-of-flight mass spectrometer. Modern single analyzing time-of-flight mass spectrometers with orthogonal extraction optics are demonstrating routine resolution of about 6000 ($m/\Delta m_{\mathrm{FWHM}}$). Higher resolution is enabling more selective ion chromatograms within the range of some millidaltons which can lead to improved metabolite recognition.

The selected ion chromatograms (SIC) of rat plasma, urine and liver homogenate, over the protonated molecular ions of NVP-AAV490 and its predicted main metabolites, recorded with a mass window of 0.1 Da, 1 h after intravenous administration of 10 mg/kg parent compound, are shown in Figs. 2–4. The results (see Section 3.9) are in accordance with the pharmacokinetic findings.

The bulk amount of parent compound and related metabolites were detected in urine. The parent compound and only little portions of metabolite m3 and m8 were found in plasma whereas only a small quantity of parent could be detected in liver homogenate (see also Section 3.9).

The recognition and characterization of potential metabolites by single analyzing MS is speculative, although, in the actual case, the search for potential metabolites was simplified by the fact that the parent compound and, presumably all biotransformation products, are bearing one chlorine atom. The search was based on the assumption that the drug would either undergo, in phase I, sp^2 hydroxylation at one of the aromatic moieties, O-dealkylation at the methoxy group, oxidative deamination or enzymatic hydrolysis at various points of the molecule in phase I, followed by conjugation reactions at the newly formed phenolic functions in phase II.

3.4. Confirmation of the elemental composition of the proposed metabolites by exact mass measurement

Exact mass measurement within a few ppm, even in complex sample matrices under which in vivo metabolite structure characterization is performed,

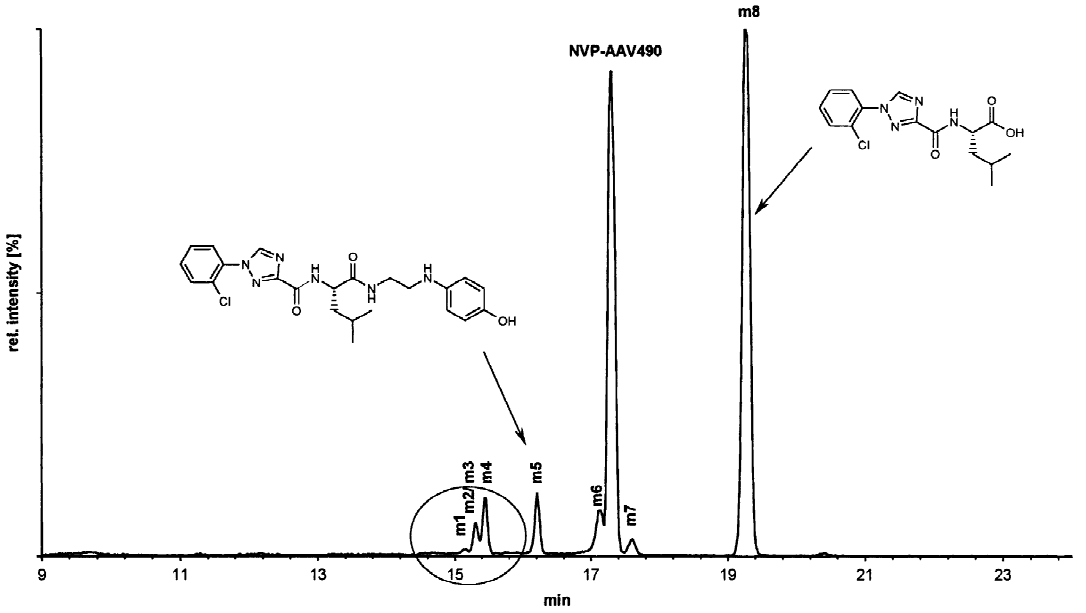


Fig. 2. Urine chromatogram. Urine: SIC over MH^+ ions of NVP-AAV490 (m/z 485) and its metabolites m1–m8 (m/z 677, m/z 647, m/z 379, m/z 501, m/z 471, m/z 380, m/z 394 and m/z 337).

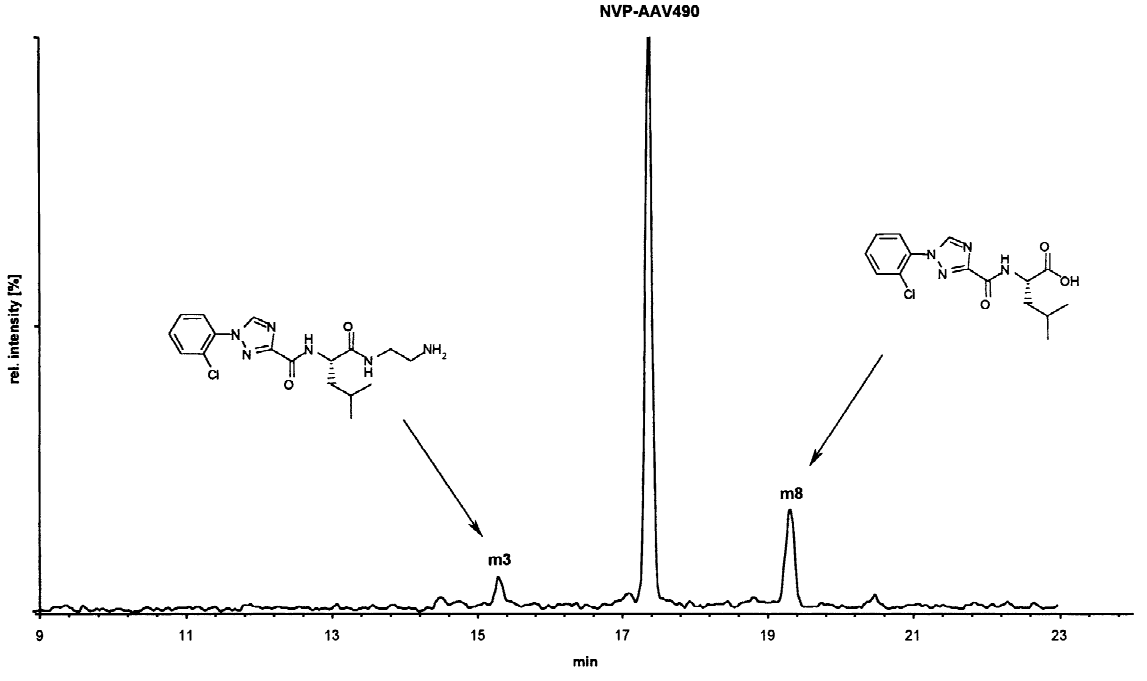


Fig. 3. Plasma chromatogram. Plasma: SIC over MH^+ ions of NVP-AAV490 (m/z 485) and its metabolites m1–m8 (m/z 677, m/z 647, m/z 379, m/z 501, m/z 471, m/z 380, m/z 394 and m/z 337).

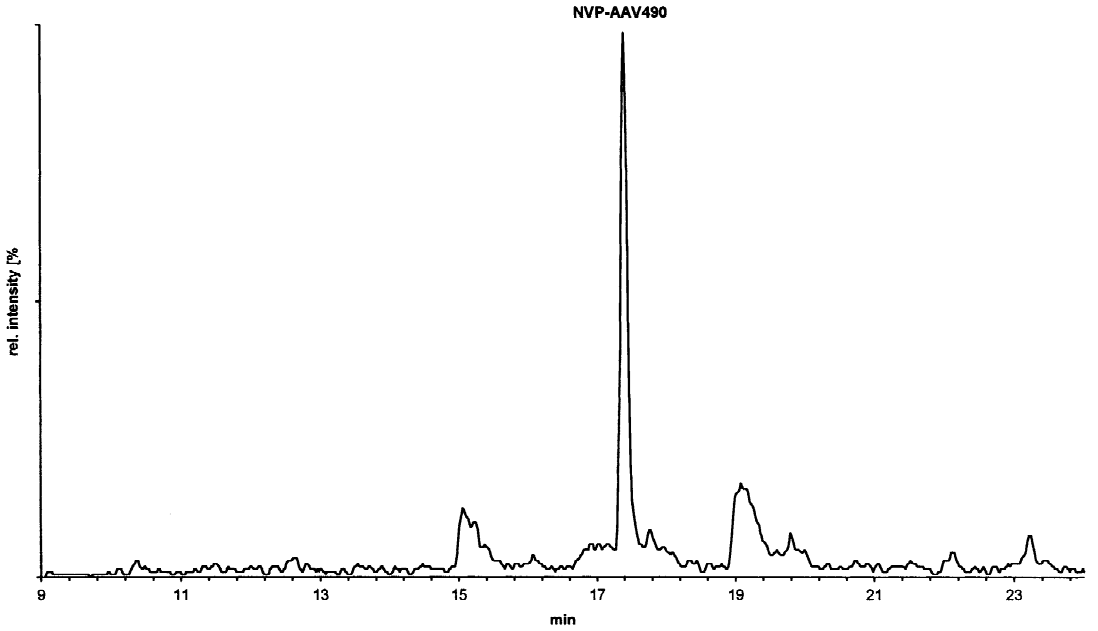


Fig. 4. Liver chromatogram. Liver: SIC over MH^+ ions of NVP-AAV490 (m/z 485) and its metabolites m1–m8 (m/z 677, m/z 647, m/z 379, m/z 501, m/z 471, m/z 380, m/z 394 and m/z 337).

can be made on time-of-flight mass analyzers with orthogonal extraction optics and only intermediate resolution capability, provided the instrument is calibrated with a reference compound over the mass range at which an analysis is performed [1,10].

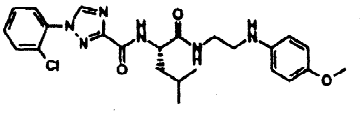
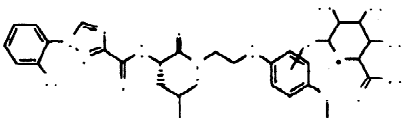
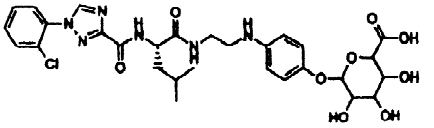
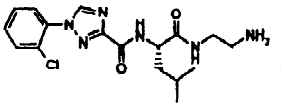
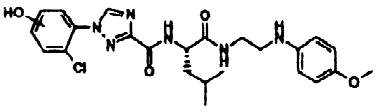
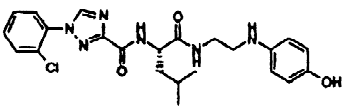
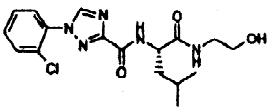
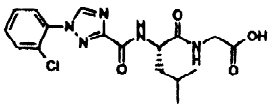
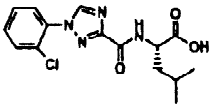
In practice, any effect of the reference can be excluded, and the accuracy of the mass measurement can be significantly improved, by the use of a two-channel electrospray interface (LockSpray™) adding an internal reference compound by a second electrospray inlet, monitored independently, and closely in time to the sample, calibrating the target sample data automatically scan by scan, without interfering with the HPLC–MS measurement [1,10].

The protonated molecular ion masses of NVP-AAV490 and its metabolites m1–m8 as well as their variation from calculated values are summarized in Table 2. The results of this measurement corroborated the proposed metabolite structures derived from the TOF data. The achieved mass accuracy was about 5 ppm which is acceptable for small molecules (molecular mass <1000 Da) [11] (Table 1).

Table 1
Mass accuracy measurements of the MH^+ ions of the metabolites m2 and m3

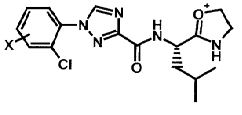
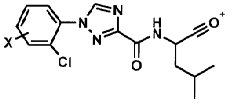
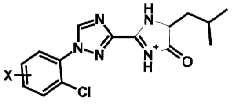
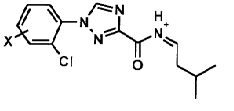
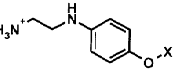
Compound	Formula	Exact mass	Measured mass	Mass error (ppm)
p	$C_{24}H_{30}ClN_6O_3$	485.2068	485.2037	6.4
m1	$C_{30}H_{37}ClN_6O_{10}$	677.2338	nm	nm
m2	$C_{29}H_{36}ClN_6O_9$	647.2232	647.2261	−4.5
m3	$C_{17}H_{24}ClN_6O_2$	379.1649	379.1657	−2.1
m4	$C_{24}H_{30}ClN_6O_4$	501.2017	501.2022	−1.0
m5	$C_{23}H_{28}ClN_6O_3$	471.1911	471.1918	−1.5
m6	$C_{17}H_{23}ClN_5O_3$	380.1489	380.1466	6.1
m7	$C_{17}H_{21}ClN_5O_4$	394.1282	394.1316	−8.6
m8	$C_{15}H_{18}ClN_4O_3$	337.1067	337.1082	7.4

Table 2
Chemical structures and protonated/deuterated molecular ions of metabolites m1–m8

Compound	Chemical structure	MH^+ (m/z)	$[MD\text{-}d_x]^+$ (m/z)
NVP-AAV490		485/487	489/491 $d_x = 3$
Metabolite m1		677/679	685/687 $d_x = 7$
Metabolite m2		647/649	655/657 $d_x = 7$
Metabolite m3		379/381	384/386 $d_x = 4$
Metabolite m4		501/503	506/508 $d_x = 4$
Metabolite m5		471/473	476/478 $d_x = 4$
Metabolite m6		380/382	384/386 $d_x = 3$
Metabolite m7		394/396	398/400 $d_x = 3$
Metabolite m8		337/339	340/342 $d_x = 2$

d_x, number of exchangeable hydrogen atoms.

Table 3
Proposed principle parent and product ions of NVP-AAV490 and its metabolites m1–m8

Compound	MH^+	$[MH-18]^+$	$[MH-176]^+$	Fragment ion A	Fragment ion B	Fragment ion C	Fragment ion D	Fragment ion E	Other fragment ions
Parent	485/487	467 (100)		362 (18)	319 (5)	318 (16)	291 (8)	167 (12)	
m1	677/679	659 (5)	501 (100)	362 (14)	319 (3)		291 (4)		579(6), 483(13), 438(8)
m2	647/649	629 (9)	471 (100)		319 (3)	318 (2)	291 (8)	329 (3)	611(3), 549(3), 453(12)
m3	379/381	361 (100)			319 (3)		291 (1)		
m4	501/503	483 (100)		362 (2)	335 (15)	334 (3)		167 (24)	465(3), 289(2), 150(7)
m5	471/473	453 (100)		362 (20)	319 (3)	318 (12)	291 (9)	153 (6)	340(2)
m6	380/382	362 (100)			319 (58)		291 (4)		
m7	394/396	376 (1)			319 (100)		291 (11)		
m8	337/339								
Fragment ion A		Fragment ion B		Fragment ion C		Fragment ion D		Fragment ion E	
									
m1, m5, parent: X=H m4: X=OH		m2, m5, parent: X=H m4: X=OH		m2, m5, parent: X=H m4: X=OH		m2, m5, parent: X=H m4: X=OH		m2, m5, parent: X=H m4: X=OH	

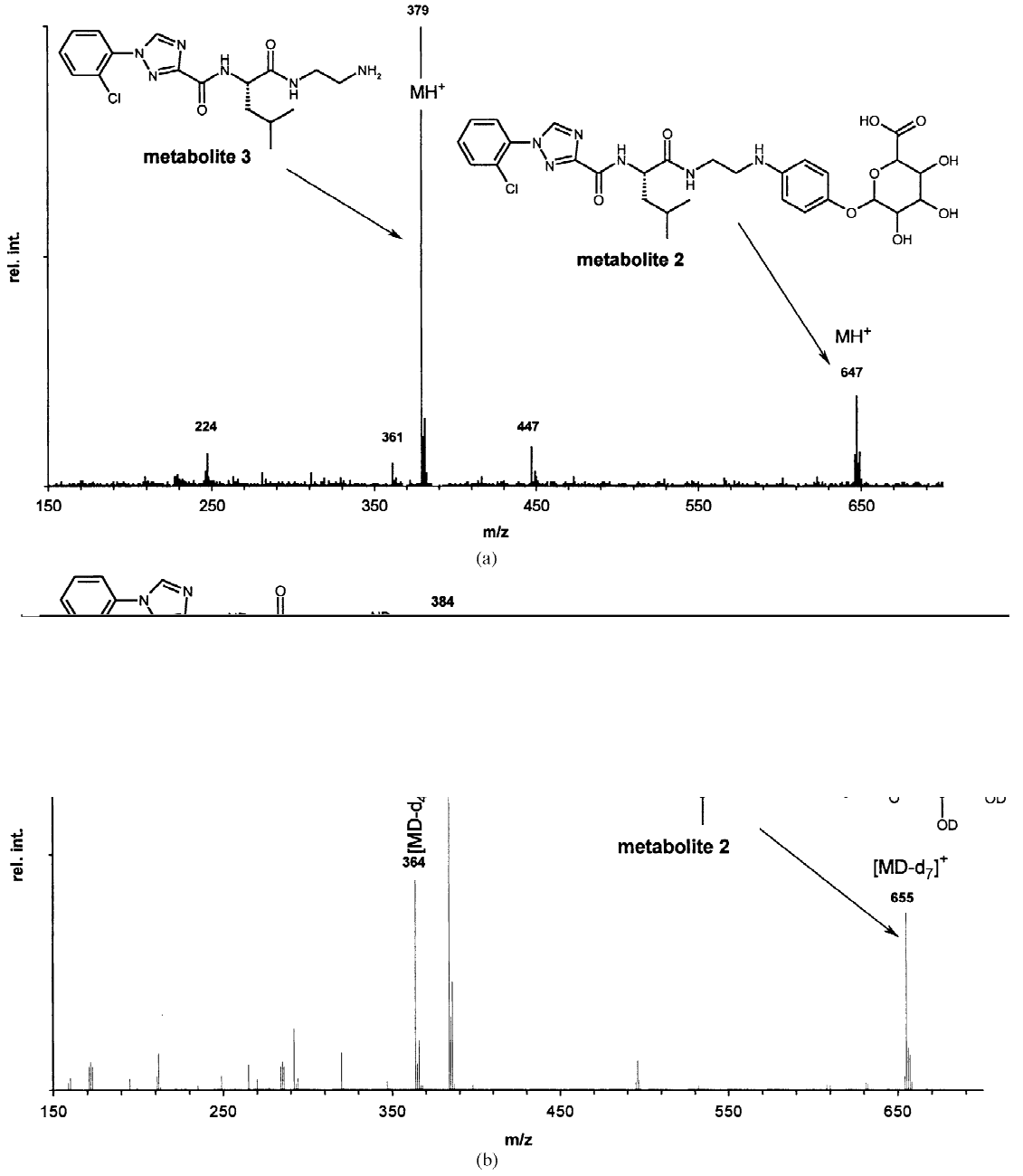


Fig. 5. (a) ES mass spectrum of co-eluting metabolites m2 and m3. (b) ES mass spectrum of co-eluting metabolites m2 and m3 after H/D exchange. (c) Product ion spectrum of m/z 647 (MH^+ metabolite m2). (d) Product ion spectrum of m/z 379 (MH^+ metabolite m3).

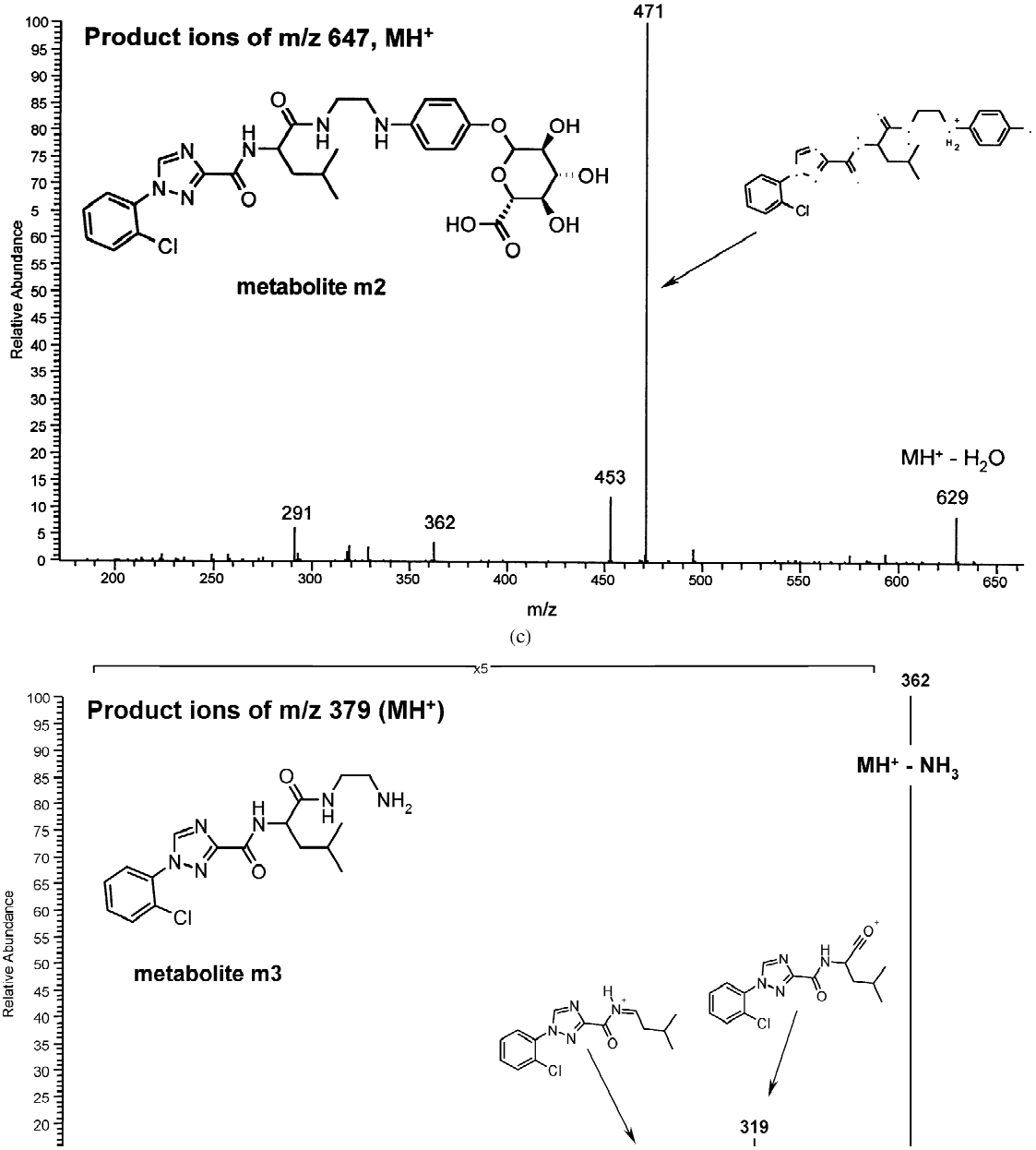


Fig. 5. (*continued*)

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