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Mass spectrometric strategies for the identification and characterization of Human Serum Albumin covalently adducted by Amoxicillin: ex vivo studies

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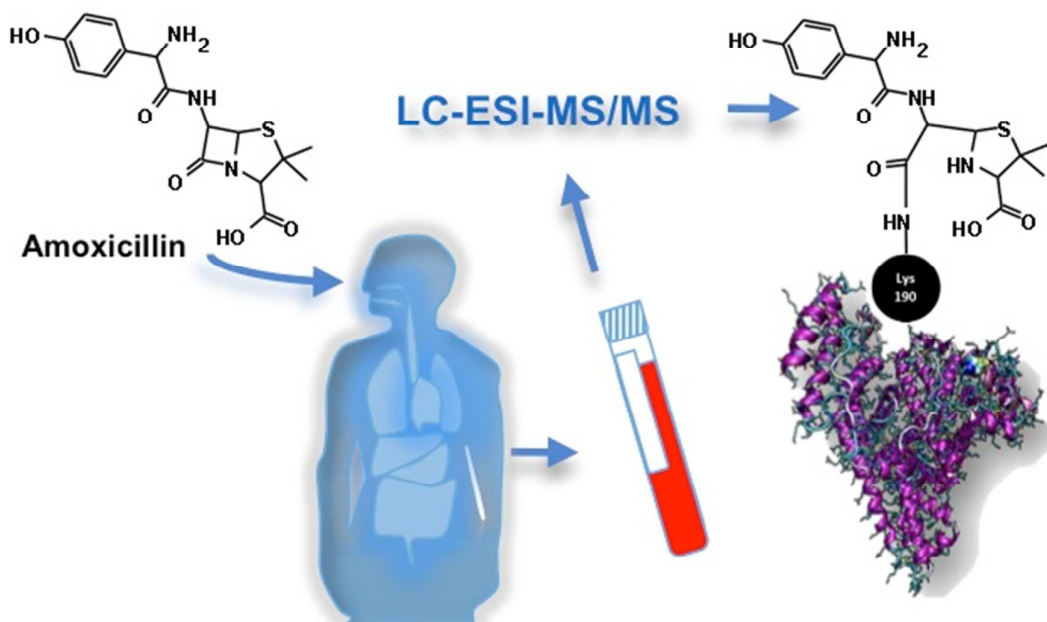
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ABSTRACT

This study addresses the detection and characterization of the modification of human serum albumin (HSA) by amoxicillin (AX) in *ex vivo* samples from healthy subjects under oral amoxicillin administration (acute intake of 1 g every 8 h for 48 h). To reach this goal we have used an analytical strategy based on targeted and untargeted mass spectrometric approaches. Plasma samples withdrawn before AX oral intake represented the negative control samples to test the method selectivity, while HSA incubated in vitro with AX was the positive control. Different MS strategies were developed and in particular: 1) Multiple Reaction Monitoring (MRM) and Precursor Ion Scan (PIS) using a HPLC system coupled to a triple quadrupole MS analyser; 2) a dedicated data-dependent scan and a customized targeted MS/MS analysis carried out using a nano-LC system coupled to a high resolution MS system (LTQ Orbitrap XL).

Lys 190 was identified as the only modification site of HSA in the *ex vivo* samples. The AX adduct was identified and fully characterized by complementary targeted approaches based on triple quadrupole (MRM mode) and orbitrap (SIC mode) as mass analyzers. The SIC mode also permitted to measure the relative amount of AX adducted HSA, ranging from 1 to 2% (6-12 μ M) after 24 and 48 hours from the oral intake. No adduct in any *ex vivo* sample was identified by the untargeted methods (PIS and data dependent scan mode analysis).

The results on one hand indicate that MS and in particular high resolution MS analyser represents a suitable analytical tool for the identification/characterization of covalently modified proteins/peptides; on the other hand they give a deeper insight into the AX-induced protein haptenation, which is required for a better understanding of the mechanisms involved in AX-elicited allergic reactions.

Keywords: Amoxicillin, human serum albumin (HSA), haptenation, Lys 190, protein covalent binding, Multiple Reaction Monitoring, Orbitrap.

Introduction

Allergy to β -lactam antibiotics poses important clinical problems *per se* and reduces the therapeutic possibilities in a time when bacterial infections are again rising as important causes of morbidity and mortality.^{1,2} Covalent binding of drugs to protein structures, in a process known as haptentation, is believed to be essential to give rise to a stable structure capable of eliciting an immune response.^{3,4} Identification and characterization of the protein targets for modification by drugs may help delineate the mechanisms involved in drug sensitization.⁵ Amoxicillin (AX) is a wide spectrum β -lactam antibiotic, the use of which has been increasing steadily during the past decades becoming the most commonly consumed betalactam in many countries.⁶⁻⁹ Incidence of allergy to AX has increased in parallel with its consumption in such a way that nowadays it is the antibiotic eliciting a higher frequency of allergic reactions.^{10,11} A recent data analysis on 333 cases of allergy reaction shows that the 50% of hypersensitivity reactions are due to β -lactam antibiotics and among them AX was incriminated in 65 % of the patients.¹²

Patients who have developed an allergic reaction to AX are evaluated and diagnosed on the basis of either: *i*) the presence of AX-reactive IgE in serum detected by *in vitro* immunoassays, *ii*) the responsiveness of their basophil cells or the specific lymphocyte proliferation in presence of the antibiotic by *in vitro* basophil activation test or lymphocyte transformation test, respectively *iii*) the responsiveness of their mast cells and T cells by skin test and, *iv*) provocation tests by oral administration of AX.¹³ Nevertheless, skin and oral provocation tests imply higher risks than *ex vivo* testing and have to be carried out in a hospital setting.¹⁴ Therefore, diagnosis through *ex vivo* tests is preferable. Strategies for these tests include, besides the use of AX, AX-derivatized dendrimeric structure or extensively modified homopolymers and proteins such as poly-L-lysine and serum albumin, respectively.¹⁵⁻¹⁷ However, the sensitivity of the present diagnostic tools is still limited and oral drug administration is required in a considerable percentage of patients in order to confirm the diagnostic.^{13,18-20} A current interpretation of this phenomenon postulates the contribution of endogenous protein structures to the formation of the antigenic determinant(s) important for antibody recognition. Identification of endogenous targets for haptentation could therefore render a battery of new tools that could be explored to increase diagnostic efficiency.

With a view to fully elucidating the target protein of AX haptentation, we recently described the *in vitro* modification of various serum proteins by AX and characterized the modification of HSA both by immunological and mass spectrometric (MS) approaches. Under the conditions of the previous study, several lysine residues in HSA, in particular Lys 190 and 199, were found to be the most

reactive to AX. In addition, we observed that Lys 190 was modified by AX either using the isolated protein or whole serum.²¹ Figure 1 reports the reaction mechanism between AX and Lys 190 together with the diagnostic fragment ions that were used for the adduct characterization by MS studies.²¹ In the current study we have addressed the detection and characterization of the modification of HSA by AX in *ex vivo* samples from subjects under oral administration of AX. To reach this goal we used both immunological and MS methods. The MS approach we used is based on the most suitable techniques and MS analyzers (triple quadrupole and orbitrap) applied to identify and characterize peptide/protein covalent adducts. Using this approach we have been able to detect the addition of AX to Lys 190 of HSA and to determine the relative content in respect to the native peptide. These findings set the basis for the study of this structure as antigenic determinant and the development of novel diagnostic tools.

MATERIALS AND METHODS

Reagents. AX was from Glaxo SmithKline (Madrid, Spain). Iodoacetamide (IAA), (D)-threo-1,4-dimercapto-2,3-butanediol (DTT), tris (hydroxymethyl) aminomethane, sodium phosphate dibasic and LC-grade and analytical-grade organic solvents were from Sigma-Aldrich. Sequence grade modified trypsin was obtained from Promega (Milan, Italy). The monoclonal antibody anti-amoxicillin (AO3.2) was the generous gift of Dr. Cristobalina Mayorga (Research Laboratory, IBIMA – Hospital Regional Universitario de Málaga - UMA, Málaga, Spain).²¹

AX administration, serum collection and HSA isolation. AX (Glaxo Smithkline, Madrid, Spain) was orally administered (1 g every 8 h for 48 h) to two healthy volunteers, not allergic to this antibiotic and identified as subject A and B within the manuscript. The extraction of peripheral blood using tubes with gel (Vacuette Greiner Bio-One, Kremsmünster, Austria) immediately before (T0) the intake of the first pill and 24 (T24) and 48 h (T48) after the intake of the first pill (with an accumulated dose of 3 and 6 g) was done at the Regional University Hospital of Malaga (Spain). Serum was obtained by centrifugation of the samples at 4000 rpm for 5 min and it was frozen in aliquots at -80°C until their analysis. The study was conducted according to the Declaration of Helsinki principles and was approved by the Malaga Research Ethics Committee (Spain). All subjects included in the study were informed orally about the study and signed the corresponding informed consent form.

HSA isolation. HSA was isolated from the serum by affinity chromatography using Affi-Gel[®] blue gel (Bio-Rad, Segrate, Italy). An aliquot of 50 μ L of serum was diluted with 150 μ L of sodium phosphate dibasic 20 mM, pH 7.1 (buffer A) and kept at room temperature for 15 minutes. In the meanwhile a Sigma Prep Spin column was filled with 500 μ L of BlueGel Affigel and the gel was activated as indicated by the protocol. Aliquots of 200 μ L of the diluted serum were pipetted in the column and washed three times with buffer A. Albumin was eluted by pipetting two aliquots of 250 μ L of NaCl 1.4 M (buffer B) in the column. For each step the columns were centrifuged by means of a mikro 120 Hettich centrifuge for one minute at 20 g. The recovery of HSA and of AX modified HSA ranged from 85 to 92% and did not changes significantly. HSA concentration was determined spectrophotometrically at 280 nm.

In vitro modification of serum proteins by AX. AX was freshly prepared in 50 mM Na₂CO₃/NaHCO₃ pH 10.2. For western blot analysis, HSA was incubated in the presence of 0.5 mg/ml AX for 16 h at 37°C. AX incorporation into serum proteins was assessed by SDS-PAGE followed by western blot and detection with anti-AX monoclonal antibody AO3.2, as previously described.²¹ AX-modified HSA as positive control for MS study was prepared by incubating 150 μ M HSA (10 mg/mL) with 13.5 mM AX (5 mg/mL) in PBS overnight at 37°C (stoichiometric ratio HSA:AX of 1:90)

HSA protein digestion for LC-ESI-MS analysis. Aliquot of 30 μ g of HSA was dissolved in 30 μ L 50 mM Tris-HCl (pH 7.8) and digested with trypsin according to the manufatcurer's procedure. Firstly DTT was added at a final concentration of 2 mM and the sample was incubated for 60 min at 37 °C; then IAA was added to the solution at a final concentration of 4 mM and the sample was incubated for 45 min at 37 °C in the dark. In order to quench the excess of IAA, DTT was added again at a final concentration of 4 mM and the sample was incubated for 60 min at 37 °C degrees. CaCl₂ was spiked at a final concentration of 1 mM and then trypsin was added to the sample at a trypsin to protein ratio of 1:30. After an overnight period at 37 °C the tryptic mixtures were acidified with formic acid up to a final concentration of 10%. The digested HSA was then analyzed by triple quadrupole and orbitrap mass spectrometer, using both targeted and untargeted approaches, as summarized in Figure 2.

Multiple Reaction Monitoring and Precursor Ion Scan analyses. Aliquots of 5 μ g of digested HSA were dissolved in 20 μ L of buffer A (0.1% formic acid in water) and injected onto a reversed-phase Agilent Zorbax SB-C18 column (4 mm i.d., particle size 3.5 μ m, CPS Analitica,

Milan, Italy) protected by an Agilent Zorbax RP guard column. The peptides were eluted with a 55 min gradient from 98% buffer A (0.1% formic acid in water) to 60% buffer B (0.1% formic acid in acetonitrile) at a constant flow rate of 200 $\mu\text{L}/\text{min}$. The triple quadrupole TSQ Quantum Ultra (ThermoQuest, Milan, Italy) was set in positive ion mode and with the following ion source parameters: capillary temperature 275° C; spray voltage 4.5 kV; capillary voltage 35 V; tube lens voltage 120 V. The flow rate of the nebulizer gas (nitrogen) was 50 a.u.

MRM of each of the AX adducted peptides previously identified under in vitro conditions²¹ was carried out by setting as diagnostic fragments the ions at m/z 160.0 and 349.1, corresponding to the thiazolidinic moiety of AX and to AX minus the aminic residue, respectively. The AX modified peptides and the corresponding transitions are summarized in Table1.

The parameters influencing these transitions were optimized as follows: argon gas pressure in the collision Q2, 1.5 mbar; peak full width at half-maximum (FWHM), 0.70 m/z at Q1 and Q3; scan width for all MRM channels, 1 m/z ; scan rate (dwell time), 0.05 s/scan. Data processing was performed by the Xcalibur 2.0 software. Fragmentation was done using CID mode (isolation width, 1 m/z ; normalized collision energy, 28 CID arbitrary units).

Precursor Ion Scan analysis was carried out by setting as diagnostic ions the ions at m/z 160.0 and 349.1 which are characteristic of the AX moiety.²¹ MS parameters were set as follows: collisional voltage at 30 V, mass unit resolution, scan time of 1 s for both Q1 and Q3, m/z 300–1500 as Q1 scan range.

Liquid chromatography electrospray ionization mass spectrometry/mass spectrometry analysis (LC-ESI-MS/MS): LTQ Orbitrap XL mass spectrometer. Peptides from HSA digestion were then separated by reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray tandem mass spectrometry (ESI-MS/MS). Digested peptides arising from 1.5 μg of HSA were dissolved in 10 μL of buffer A (0.1% formic acid in water) and injected into a trapping column in order to purify and concentrate the sample. The trapping column was a Acclaim PepMap 100, nanoviper C18, 100 Å, 100 μm i.d. x 2 cm, Thermo Scientific. The flow rate was of 10 $\mu\text{L}/\text{min}$ for 4 minutes. After 4 minutes the valve switched and the peptide mixture was loaded onto a fused silica reversed-phase column (PicoFrit™ Column, HALO, C18, 2.7 μm , 100 Å, 75 μm i.d. x 10 cm, New Objective). The peptides were eluted with a 55 min gradient from 99% buffer A (0.1% formic acid in water) to 35% buffer B (0.1% formic acid in acetonitrile) at a constant flow rate of 300 nL/min. The nano chromatographic system, an UltiMate 3000 RSLCnano System (Dionex), was connected to an LTQ-Orbitrap XL mass

spectrometer (Thermo Scientific, Milan, Italy) equipped with a nanospray ion source (dynamic nanospray probe, Thermo Scientific, Milan, Italy) set as follows: spray voltage 1.8 Kv; capillary temperature 220 °C, capillary voltage 30 V; tube lens offset 100 V, no sheath or auxiliary gas flow.

The LTQ-Orbitrap XL mass spectrometer was set to perform scan cycles consisting, firstly, of recording a high-resolution (resolving power 60000, FWHM at m/z 400) full scan (200-1500 m/z) in profile mode, then by the acquisition of the MS/MS spectra of the three most intense ions in centroid mode, using both CID and HCD fragmentation (isolation width, 3 m/z ; normalized collision energy, 35 CID arbitrary units, 30 HCD arbitrary units). Protonated phthalates [dibutylphthalate (plasticizer), m/z 279.159086; bis(2-ethylhexyl)phthalate, m/z 391.284286] and polydimethylcyclsiloxane ions $[(\text{Si}(\text{CH}_3)_2\text{O})_6 + \text{H}]^+$; m/z 445.120025] were used for real time internal mass calibration. Dynamic exclusion was enabled (repeat count, 2; repeat duration, 45 s; exclusion list size, 50; exclusion duration, 80 s; relative exclusion mass width, 10 ppm). Charge state screening and monoisotopic precursor selection was enabled, singly and unassigned charged ions were not fragmented.

Identification of the peptides covalently modified by AX as well as the modification sites, was carried out by the MS strategy previously described and based on two different steps.²¹ The first step consisted of identifying the precursor ions of diagnostic fragment ions at m/z 160.04 and 349.08. In more detail, the precursor ions were extracted from the two parent ion maps displayed by setting as product mass the ions at m/z 160.04 (tolerance 0.01) and the ion at m/z 349.08 (tolerance 0.01). The two lists of parent ions were then exported to Excel Microsoft and analyzed using the following Boolean logic: if value1 > ts, value2 > ts, value\$ = value1 + value2 or else value\$ = 0, where value1 and value2 are the relative abundances of the precursor ions setting the product ions at m/z 160.04 (value 1) and 349.08 (value 2) and ts is the threshold relative abundance. By using this approach, each precursor ion was confirmed only when it was present in both the ion maps which were reconstituted by setting the two diagnostic precursor ions, otherwise it was discarded. The second step consisted of matching the precursor ions with a list of theoretical modified ions generated by an in silico digestion of albumin carried out by considering the following parameters: trypsin as enzyme, two missed cleavages and the addition of the amoxicilloyl moiety at the Lys residues.

Targeted MS/MS scan analyses were carried out by setting the LTQ-Orbitrap XL mass spectrometer to perform two scan cycles, one in full scan (200-1500 m/z), the other based on the inclusion list reported in Table 2:

MS/MS experiments were carried out in CID mode (isolation width, 3 m/z ; normalized collision energy, 33 CID arbitrary units). Protonated phthalates [dibutylphthalate (plasticizer), m/z 279.159086; bis(2-ethylhexyl)phthalate, m/z 391.284286] and polydimethylcyclsiloxane ions $[(Si(CH_3)_2O)_6 + H]^+$; m/z 445.120025] were used for real time internal mass calibration.

Bioinformatics. Peptide sequences were identified using the software turboSEQUEST (Bioworks 3.1, ThermoQuest, Milan, Italy), and using a database containing only HSA and assuming trypsin digestion. The protein sequence of HSA was obtained from the SwissProt database (primary accession number P02768). Sequence fragments calculator (Bioworks 3.1.1) was used to obtain the theoretical digested masses setting trypsin as enzyme, all the Cys residues as carbamidomethyl-cysteine and considering the oxidation of methionine residues and the formation of amoxicilloyl-Lys adducts as variable modifications. The predicted y and b series ions were determined using the Peptide Sequence Fragmentation Modeling, Molecular Weight Calculator software program (ver. 6.37), <http://come.to/alchemistmatt>. The chemical structure of amoxicillin diagnostic fragments were predicted by the software Mass Frontier 5.0 (HighChem ltd, Slovakia).

RESULTS

Detection of AX-HSA adducts in human serum by Multiple Reaction Monitoring and Precursor Ion Scan: LC-ESI-MS/MS studies. Multiple Reaction Monitoring: development of allergy towards AX occurs most frequently after oral intake of the antibiotic. We were therefore interested in detecting AX-protein adducts under these conditions. Multiple Reaction Monitoring (MRM) is commonly used for targeted quantitative analyses.²² Concerning β -lactams-induced protein haptentation, it was applied by Jenkins et al.²³ in order to identify the HSA residues modified by flucloxacillin in both in vitro and in vivo conditions.

Firstly, based on its well-established selectivity and sensitivity, the Multiple Reaction Monitoring scan mode was employed in order to identify the adducted peptides arising from the digested HSA isolated from subjects treated with AX. Transitions (fragment ions associated to the precursor ions) were set on the bases of the in vitro studies previously reported²¹ and are summarized in Table 1.

The method was firstly validated by identifying all the previously identified adducted peptides in the positive control sample (HSA incubated with AX)(Figure S1). Each adducted peptide was also identified on the basis of the retention time and the three most intense ions were K199-AX, K541-AX and K190-AX. Adducted peptides are named on the basis of the modified Lys: K190-AX,

K199-AX, K351-AX, K541-AX, K545-AX and K432-AX. All the peptides monitored were not detected in the negative control samples T0A and T0B. The method was then applied to the *ex vivo* samples T1A, T1B, T2A and T2B. Only the peak corresponding to the Lys 190 adducted peptide ion (m/z 628.6 $\rightarrow$ 160.0 + 349.1), and characterized by a retention time of 28 minutes, was detected in all *ex vivo* samples with comparable intensity. Figure 3 (left panel) shows the MRM traces of Lys 190 adducted peptide relative to the negative and positive control and to the set of the *ex vivo* samples. The method was found to be selective since no peak of the adducted peptide ion was present in the negative control samples obtained by HSA isolated from samples collected before AX intake (T0A and T0B).

Although the MRM mode was found to be sensitive enough to identify the Lys 190 adducted peptide, it was unable to characterize the peptide or to identify the site of modification because of the lack of additional information such as accurate mass, isotopic pattern and MS/MS fragmentation pattern. To overcome this limitation, a mixed MRM-data-dependent scan was applied. The approach was effective for the positive control sample while in *ex vivo* samples the fragment ions did not reach suitable intensity values, even when concentrating the sample, so that no MS/MS spectra were recorded for the peptide characterization.

Another limitation of the MRM approach resides in the fact that it cannot perform a semi-quantitative analysis (the relative content of the adducted in respect to the native peptide) due to the fact that the product ions are characteristic only of the adducted peptides since they referred only to the AX moiety.

The PIS method was then used as an untargeted method in order to identify the unknown AX-modified peptides in addition to those already detected by using the MRM mode. However, as reported in Figure S2, this approach was not able to identify any AX modified peptides in the *ex vivo* samples.

By considering that the same set of samples were analysed in both MRM and PIS mode, we can confirm that the PIS mode is less sensitive in respect to the MRM mode and that in this study it was not useful for the untargeted identification due to the low abundance of the analytes.

Detection of AX-HSA adducts by LC-MS/MS Orbitrap. The second step of the work was to apply a high resolution MS analyser coupled to a nano LC system in order to detect and identify the AX-adducted peptides. MS analyses were performed in two different scan modes, a data-dependent scan and a customized targeted MS/MS mode.

Data-dependent scan: The identification of AX-modified peptides was then carried out using a standard data-dependent scan as explained in the methods and in Figure 2. It should be noted that such an approach is *per se* not selective since it does not distinguish native and adducted peptides; hence when it was applied to the HSA digested samples, a large number of MS/MS spectra arising from the fragmentation of both native and adducted peptides was generated. Hence, the identification of AX-modified peptides was by necessity based on a post-run analysis. In particular for this analysis the same MS strategy described in our previous study²¹ was employed in order to identify amoxicilloyl-modified peptides (+365.10454 Da).

Although this approach confirmed all the 6 different adducted peptides in the positive control sample (see supporting information Figure S3), it was not able to identify any modified peptide in the *ex vivo* samples. It should be noted that the lack of adduct identification is not due to a poor sequence coverage, since the protein coverage of both control and AX-treated HSA samples was always higher than 80% (see supplementary Figure S4) and the most reactive Lys residues, as reported in the literature and predicted by molecular modelling studies, were covered.

Customized targeted MS/MS mode: the next step was to search for the adducted peptides using a targeted method, based on reconstituting the single ion chromatograms (SIC) by extracting the current ion relative to the m/z ion of each of the adducted peptides as previously identified, and setting a 5 ppm tolerance.

Figure 3 (right panel) shows the SICs relative to the positive control where only a single and well defined peak is observed for Lys 190 AX modified peptide. The retention time of the adducted peptide ion is clearly quite different from that observed in the MRM analysis, due to the different chromatographic configuration. By contrast, in the blank samples T0A and T0B, several peaks were detected but none at the RT of the analyte. SIC traces show the presence of the adducted peptide ion K190-AX, for all the *ex vivo* samples analysed (T1A, T1B, T2A, T2B). Regarding the peptide K190-AX, the method was found not to be highly selective since several peaks were present in the SIC trace, with a higher intensity compared to the adducted peptide although with a different RT. The adduct was assigned on the basis of the accurate mass and retention time and by considering the isotopic pattern of the multiply charged ion at m/z of 628.63184, overlapping the simulated data (Figure 4). These results also indicate that the failure to identify the K190-AX peptide by using the untargeted methods above reported (Sequest search algorithm and reconstituted precursor ion maps), is not due to the absence of the precursor ion, but to the fact that the target peptide was not fragmented. We then sought the reason for the lack of fragmentation. The first hypothesis was the poor intensity of the target ion. However a manual inspection of the SIC trace revealed that the

intensity of the multiply charged ion relative to the target peptide was higher with respect to the ion threshold set in the data-dependent scan. We then found that several peptide ions eluted at the same retention time as the target peptide and that the dynamic exclusion did not succeed in excluding all the redundant ions.

In order to record the MS/MS spectrum of the target peptide, the threshold intensity was decreased and the dynamic exclusion modified. Although different attempts were made and the ion at m/z 628.63163 ion was always detectable in the full mass spectrum, the adducted peptide was never automatically fragmented (data not shown) and this prompted us to set-up a targeted MS/MS method. In this last approach, the LTQ-Orbitrap XL mass spectrometer continuously performed scan cycles consisting of a high-resolution full scan (200-1500 m/z) followed by the MS/MS analysis of only those ions included in the list of AX adducted peptides as previously detected.²¹

This targeted-target approach was found to be highly informative since each adduct was identified not only by the accurate mass and isotopic pattern but also by the MS/MS fragmentation pattern.

This method identified the K190-AX adduct in all the ex vivo samples which was finally confirmed and fully characterized by the MS/MS spectrum (Figure 5), a result which was not achieved in the other MS approaches as above reported.

The peptide containing Lys190 (¹⁸²LDELRLDEGK#ASSAK¹⁹⁵) contains three potential nucleophilic sites: Arg186, Lys190 and C-terminal Lys 195. The MS/MS fragment ions as detected in CID mode show an unmodified b series from b2 to b8 thus excluding the binding to Arg186. Moreover, in the spectrum there was an unmodified y series from y3 to y5 excluding the adduction on the C-terminal Lys 195. By contrast, the y series from y7 to y13 and the b series from b9 to b10 showed a mass increase of 365.1 Da due to the AX adduction, thus confirming that Lys190 is the modification site. As further confirmation of AX adduction, the spectrum showed the ion at m/z 349 which is a diagnostic fragment of modification by AX.

The areas of the peaks relative to K190-AX and to the corresponding native peptide were determined by reconstituting the selected ion chromatograms (SICs), setting the filter ions at m/z 628.63163 $z=3$ and m/z 537.77492 $z=2$, respectively, and assuming that the ionic responses of the filter ions were similar. The relative content of the modified peptide with respect to the native one was then calculated and the results summarized in Table 3. The relative content of the modified peptides in the ex vivo samples obtained after either 24 or 48 h AX intake ranged from 1 to 2 % corresponding to a concentration range from 6 to 12 μ M given that HSA is 600 μ M.

Comparing MS approaches with immunological methods. We have previously used immunological methods to detect AX-protein adducts formed *in vitro*.²¹ For this reason we also explored the possibility of using immunological approaches for the detection of AX-protein adducts in *ex vivo* samples obtained after AX oral administration. Commercial human serum incubated with AX *in vitro* was used as a positive control. As described earlier, several adducts were detected under these conditions (Figure S5A). The major adducted proteins have been previously identified and can be assigned to transferrin (Trf), HSA, and heavy and light chains of immunoglobulins (Ig HC and Ig LC, respectively). Incubation of serum samples from healthy subjects with AX *in vitro* led to the detection of similar adducts (Figure S5B). However, analysis of serum from the same subjects after oral intake of AX for 24 or 48 h by this method did not allow the detection of signals above background levels (Figure S5C). Nor did the analysis of the same samples by 2D-electrophoresis using 100 µg of total protein allow the detection of specific signals (results not shown). Therefore, detection of AX-adducts formed *in vivo* by use of the available antibody may require enrichment steps. Alternatively, more sensitive methods, like the ones developed herein, need to be employed.

DISCUSSION

As reported elsewhere, when AX is orally administered at a dose of 1 g, the plasma concentration of the antibiotic increases rapidly reaching at 2-3 hours a concentration peak of approximately 10-20 µg/ml. After this period, the AX plasma concentration decreases rapidly, reaching after 8 hours an almost negligible concentration. The kinetics of plasma AX levels upon repeated doses has been modeled and is thought to give rise to a series of peaks, not exceeding 20 µg/mL.²⁵ We have previously shown that immunological methods can detect HSA-AX adducts formed *in vitro* after incubation of HSA in the continuous presence of AX at 25 µg/mL for 16 h at alkaline pH.²¹ However, under these conditions, the extent of modification of HSA is higher than that occurring at neutral pH (not shown). As stated above, oral administration of AX results in the modification of only 1-2% of serum HSA, as detected in *ex vivo* samples by MS. Therefore, immunological methods using the AO3.2 antibody may not be suitable to detect AX-protein adducts in *ex vivo* serum samples withdrawn after AX oral administration, due to either the lower extent of HSA modification or the higher background given by serum samples.

We have previously reported MS strategies for the identification and characterization of the AX modifications.²¹ Therefore, in this paper we turned to MS approaches in order to achieve high

sensitivity. Figure 2 overviews the different targeted and untargeted approaches we used. None of the untargeted approaches considered (Precursor Ions Scan and data-dependent scan mode) were able to identify any AX-adducted peptides generated by the enzymatic digestion of isolated HSA and this is probably due to the lack of sensitivity of the PIS approach and to the limited scan rate of the orbitrap MS analyzer used in the present work. Moreover the limited amounts of the AX adducts present in the *ex vivo* samples with respect to the native peptides should also be considered. To overcome these limitations a sample preparation method should be set-up in order to isolate HSA-AX adducts from unmodified-modified HSA. Moreover the use of a more sensitive MS analyzer for PIC analysis and of a HRMS characterized by a faster scan rate would facilitate the untargeted search.

The targeted approaches set-up on the basis of the known AX adducts previously identified in *in vitro* conditions, confirmed that AX only modifies Lys190 *in vivo*. The MRM approach was found to be highly selective and sensitive but limited with regard to the analyte structure confirmation. The SIC approach carried out in the HRMS orbitrap, although less selective, gave additional information concerning the accurate mass, the isotopic pattern and the MS/MS fragmentation pathway, leading to the structure confirmation of the AX adduct. The SIC approach also permitted the establishment of the relative content of the adduct with respect to the native peptide. Although the approach is limited since it assumes that the ionization efficiency of the native and adducted peptides overlaps, it represents the first attempt of measuring the formation of the adducted peptide in a semi-quantitative way. A MRM method based either on isotope labelled or unlabelled standards is needed to measure the absolute amount of native and modified peptides.

In conclusion, Lys 190 was identified as the modification site of AX orally given to human volunteers. The AX adduct was identified and fully characterized by complementary targeted approaches based on triple quadrupole (MRM mode) and orbitrap (SIC mode) as mass analyzers. The SIC mode also permitted the semi-quantitative measurement of AX-HSA amount, ranging from 1 to 2% (6-12 μM) of the total amount of HSA, 24 and 48 hours after the beginning of the oral intake.

It should be noted that several analytical methods have been developed towards the identification of the adducts formed between proteins and β -lactams. Because HSA is the most abundant protein in serum, most works have attempted the characterization of HSA adducts. The first pioneering studies employed the HPLC separation of tryptic peptides and Edman degradation sequencing coupled to

the Enzyme Immuno Assay analysis (EIA).²⁶ Such strategies were able to identify 6 different Lys sites modified by penicillin. More recent works have studied the interaction between HSA and flucloxacillin, piperacillin and benzylpenicillin^{27,28} by using different mass spectrometric strategies which permitted the characterization of the adducted peptides and the identification of the modification sites. As an example of MS strategy, the Accurate Inclusion Mass Screening²⁹ was developed to identify and characterize the penicilloyl-HSA adducts. It is interesting to note that, in contrast to our results, several adducted sites were detected in the previous works. Several possibilities could account for these differences. In some of the studies the routes of administration were different (i.e. intravenous or intramuscular). In addition, it is possible that if the modified proteins are stable, adduct accumulation at other sites of the protein or on other proteins could occur after prolonged administration of the antibiotic. Moreover, the presence of concomitant treatments or the pathophysiological conditions could affect plasma protein concentrations.

Accumulating evidence indicates that HSA adduction by AX occurs upon administration of the antibiotic in healthy subjects, not developing AX allergy. Therefore, other factors should intervene for the allergic reaction to occur. Apart from genetic predisposition, repeated administration, concurrent infections, inflammatory settings or other circumstances associated, known generically as “danger signals”, could potentiate the sensitization towards the antibiotic.⁵ In addition, whether the nature or the amount of adducts formed are important factors for allergy development requires further study.

The methods so far reported together with the approaches herein described represent a starting point to stimulate further studies aimed at setting-up advanced MS strategies for untargeted identification and for the accurate quantitative analysis of penicillin protein adducts. The application of such methods would permit a better understanding of the hapteneation pathways and the development of more specific allergy diagnostic tools.

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Notes

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SUPPORTING INFORMATION

The MRM traces of all the target peptides; Chromatograms recorded in Precursor Ion Scan analysis mode (PIS); the SICs of all the target peptides; Sequence coverage of HSA after trypsin digestion and LC-MS/MS analyses; Immunological detection of adducts of AX with serum proteins. Samples analyzed were: (A) commercial human serum or (B) serum from two different healthy subjects incubated *in vitro* with AX, and (C) serum samples from healthy subjects before and after the intake of AX. This material is available free of charge via the Internet at <http://pubs.acs.org/>

ABBREVIATIONS

AX, amoxicillin; CID collision induced dissociation; HCD high energy collision induced dissociation HSA, human serum albumin; MRM, Multiple Reaction Monitoring; MS, mass spectrometry; PIS, Precursor Ion Scan; SIC, single ion chromatogram; TQ, triple quadrupole, TSQ, triple stage quadrupole.

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TABLES

Table 1: AX modified peptides searched by MRM analysis. For each peptide the sequence and the *m/z* of the corresponding precursor ion and product ions used for the transition are reported. The symbol # indicates the modification site and \$ the carbamidomethylated Cys

Multiple reaction monitoring: transition list			
Modified residue	Sequence	Precursor ion (<i>m/z</i>)	Product ions (<i>m/z</i>)
K 190	¹⁸² LDELRDEGK#ASSAK ¹⁹⁵	628.6	160.0 +349.1
K 199	¹⁹⁸ LK#C\$ASLQK ²⁰⁵	656.8	160.0 +349.1
K 351	³⁴⁹ LAK#TYETTLEK ³⁵⁹	554.6	160.0 +349.1
K 432	⁴²⁹ NLGK#VGSK ⁴³⁶	584.3	160.0 +349.1
K 541	⁵³⁹ ATK#EQLK ⁵⁴⁵	591.8	160.0 +349.1
K 545	⁵⁴² EQLK#AVMDDFAAFVEK ⁵⁵⁷	736.0	160.0 +349.1

Table 2: Inclusion list used for targeted orbitrap MS/MS analysis. For each targeted peptide the sequence, the exact mass and the charge are reported. Mass tolerance was set at 10 ppm. The symbol # indicates the modification site and \$ the carbamidomethylated Cys.

Targeted Orbitrap MS/MS analysis: inclusion list			
Modified residue	Sequence	Exact mass (m/z)	Charge (z)
K 190	¹⁸² LDEL RDEGK#ASSAK ¹⁹⁵	628.63163	+3
K 199	¹⁹⁸ LK#C\$ASLQK ²⁰⁵	656.82303	+2
K 351	³⁴⁹ LAK#TYETTLEK ³⁵⁹	554.60787	+3
K 432	⁴²⁹ NLGK#VGSK ⁴³⁶	584.29496	+2
K 541	⁵³⁹ ATK#EQLK ⁵⁴⁵	591.79479	+2
K 545	⁵⁴² EQLK#AVMDDFAAFVEK ⁵⁵⁷	736.01134	+3

Table 3: Relative content of K190-AX in the in vitro and ex vivo samples. The relative content of K190-AX with respect to the native peptide (K190-native) was calculated by integrating the area of the peaks identified in the SICs traces as shown in Figure 3. The standard deviation of each value is calculated on three replicates for each sample. The following equation was used to calculate the % of adduction: $[K190-AX / (K190-AX + K190-native)] * 100$ where K190-AX and K190-native refer to the peak areas.

Sample	K190-AX Area (10^6)	K190-native Area (10^6)	Modification (%)
HSA-AX	146.0 ± 5.7	4.5 ± 0.6	97.0 ± 0.5
T0 A	0.0	11.1 ± 0.6	0.0
T0 B	0.0	11.0 ± 1.4	0.0
T1 A	0.5 ± 0.1	47.3 ± 1.8	1.1 ± 0.1
T1 B	0.13 ± 0.0	11.7 ± 0.6	1.5 ± 0.1
T2 A	0.3 ± 0.0	16.5 ± 0.6	1.8 ± 0.3
T2 B	0.9 ± 0.1	44.1 ± 2.2	1.9 ± 0.0

FIGURE LEGENDS

Fig.1 - Reaction mechanism between Lys 190 and AX and chemical structure of AX diagnostic fragment ions. The amoxicilloyl modification induces a mass increase of +365.1 Da. HCD or CID fragmentation of the Lys 190 AX modified peptide generates two diagnostic fragment ions : AX1 referred to the thiazolidinic ring and AX2, attributed to the cleavage of AX from Lys 190 and by the concomitant loss of the amine group. The two fragment ions structures were predicted by Mass Frontier software.

Fig. 2 – Overview of the MS targeted and untargeted methods used to identify AX covalent adducts to HSA.

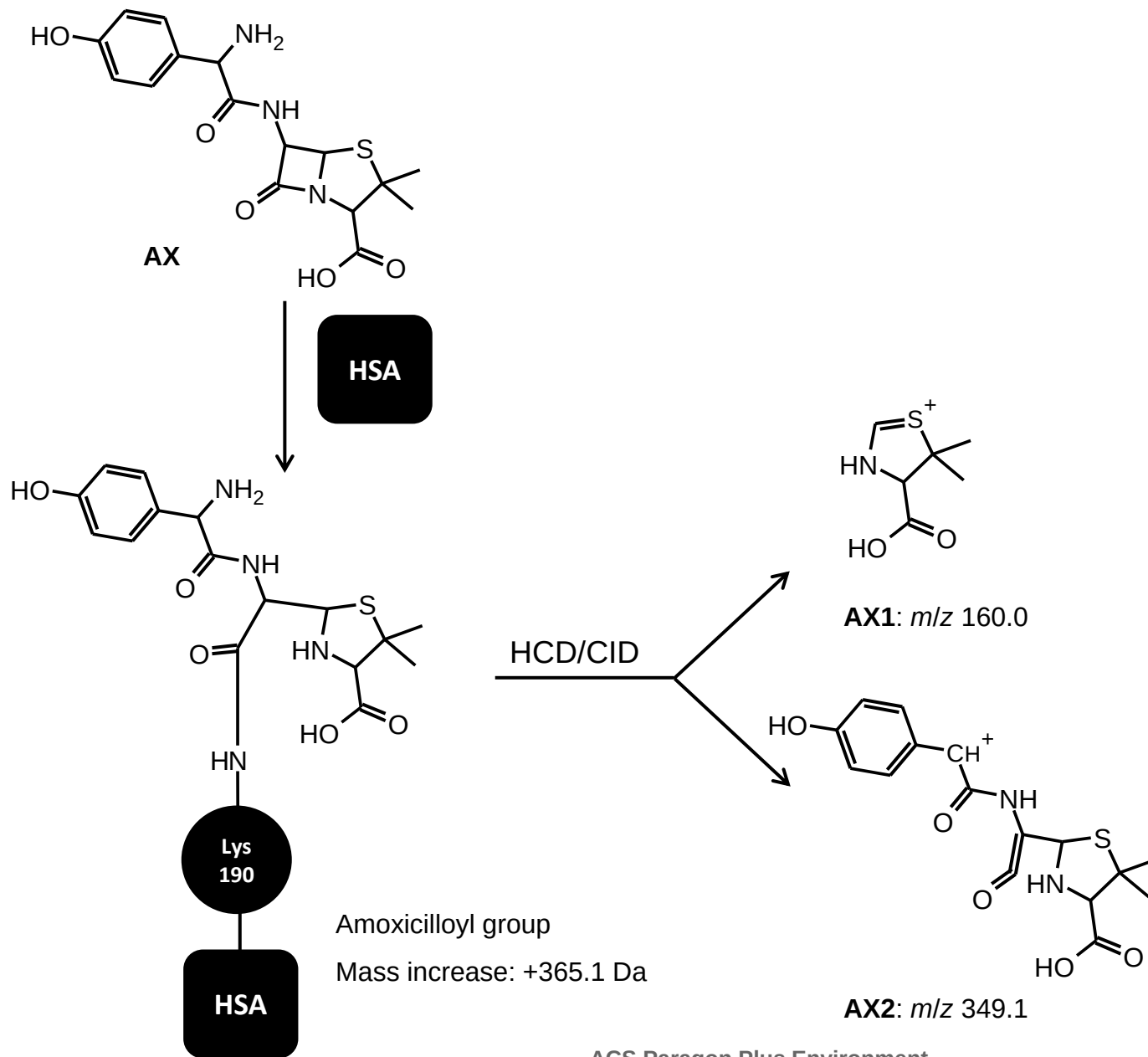
Fig. 3 – *Left panel*: Multiple Reaction Monitoring. The Multiple Reaction Monitoring trace of the Lys 190 AX-modified peptide was reconstituted by setting the corresponding transition reported in table 1. The method was found to be selective (no peak before AX administration; T0A and T0B) and able to identify the Lys 190 AX modified peptide formed under in vitro conditions (HSA-AX). In samples collected after AX administration (24 h for T1A, T1B and 48 h for T2A, T2B) the peak at retention time 28 min was attributed to K190-AX (m/z 628.6 $\rightarrow$ 160.0+349.1).

Right panel: Single Ion Chromatogram (SIC). Single Ion Chromatogram (SIC) of Lys 190 AX modified peptide was reconstituted by setting the $[M+3H]^{3+}$ of the modified peptide as filter ion. Orbitrap was used as MS analyser. The peak eluting at 11.2 min was attributed to K190-AX and it was identified in all the samples collected after AX administration (T1A, T1B, T2A, T2B) but not in samples T0A and T0B.

Fig. 4 - Experimental and calculated isotopic pattern of K190-AX. The upper panel shows the experimental isotopic pattern of the ion at m/z 628.63184 ($z=3$) extracted from the peak eluting at 11.2 min for T1A and referred to as K190-AX. The lower panel shows the theoretical isotopic pattern of K190-AX calculated by considering the chemical formula $C_{78}H_{126}N_{22}O_{30}S$ and $z=3$.

Fig. 5 - MS/MS analysis of K190-AX. Tandem MS spectrum of the adducted peptide $^{182}LDEL RDEGK\#ASSAK^{195}$ (K190-AX) was recorded in CID mode and set a mass range 180-1020 m/z . The spectrum is characterized by the diagnostic y and b fragment ion series confirming the modification of Lys 190. AX adduction is further confirmed by the diagnostic ion fragment at m/z 349.

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Chemical Research in Toxicology
HSA from ex vivo samples

Enzymatic
digestion

Peptide Mixture

Targeted MS approaches

Untargeted MS approaches

Multiple Reaction Monitoring

- Q1: Filters the selected ions relative to the AX adducted peptides as reported in the paper by Ariza et al.²¹
- Q2: Collision Induced dissociation of the selected ions
- Q3: Monitors the two diagnostic AX fragment ions at m/z 160.0 and 349.1

Precursor Ion Scan

- full mass scan (mass range m/z 300-1500)
- Collision Induced dissociation of all the ions trasmitted by Q1
- Select two diagnostic AX fragment ions, at m/z 160.0 and m/z 349.1.

← Triple Quadrupole →
Conventional LC

On Target Analysis

- high resolution full mass scan (mass range m/z 200-1500)
- MS/MS analysis of the selected ions relative to the adducted peptides²¹
- SICs reconstituted by setting as filter ions those referred to the adducted peptides

Data Dependent Analysis

- high resolution full mass scan (mass range m/z 200-1500)
- MS/MS analyses (Dynamic Exclusion Activated, Threshold $\geq 5 \times 10^4$ counts, z : 2, 3)
- precursor ion maps generated by setting the two diagnostic AX fragment ions at m/z 160.0 and 349.1 as product ions.

← Orbitrap →
Nano-LC

MRM
K190-AX

T0A

T0B

28 T1A

28 T1B

T2A

28 T2B

28 HSA-AX

Intensity (cps)

Time (min)

SIC
K190-AX

T0A

T0B

11.2 T1A

11.2 T1B

T2A

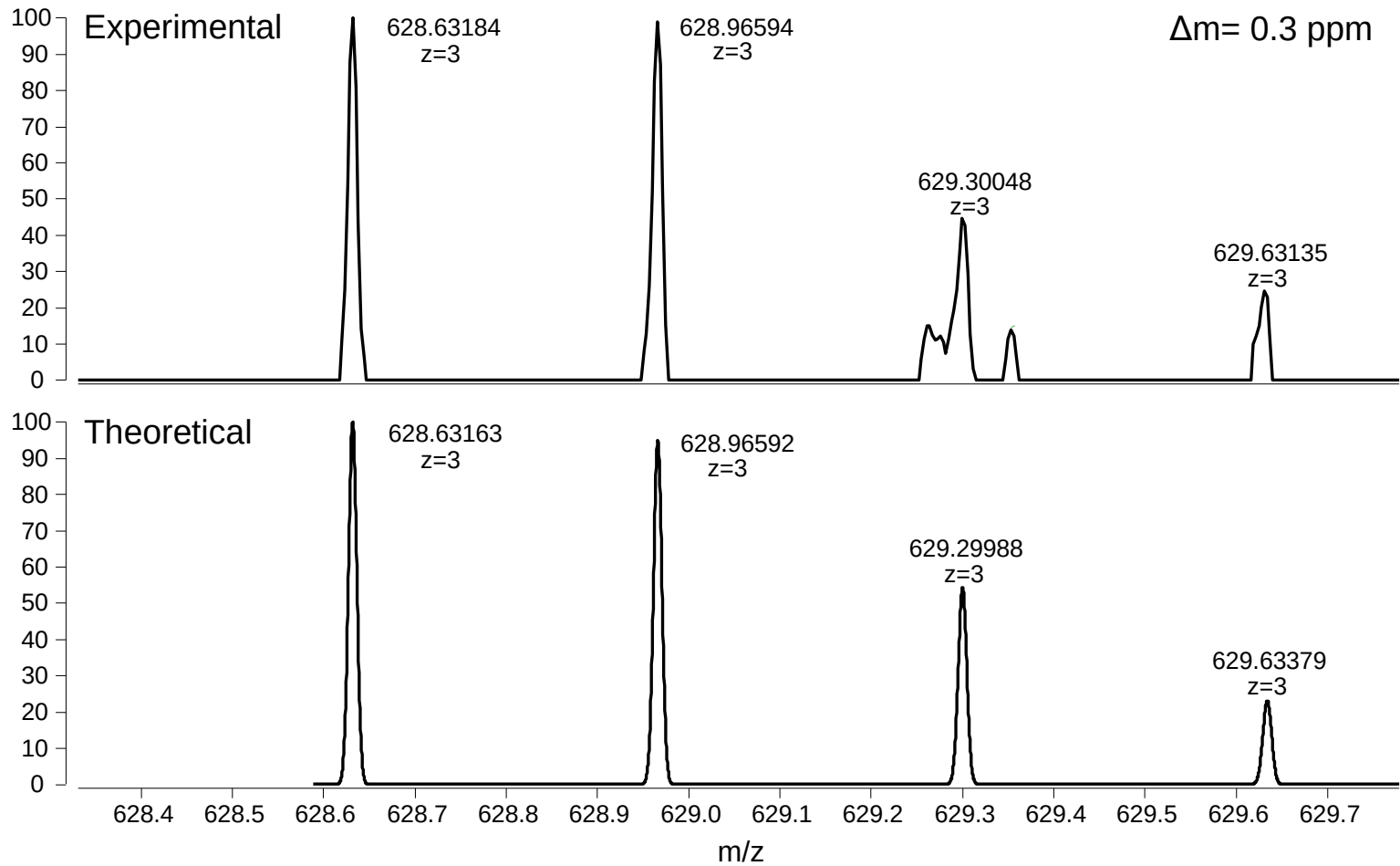
11.2 T2B

11.2 HSA-AX

Intensity (cps)

Time (min)

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m/z 628.63, $z=3$
MS2 spectrum

