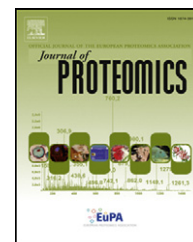


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Protein haptentation by amoxicillin: High resolution mass spectrometry analysis and identification of target proteins in serum

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ARTICLE INFO

Article history:

Received 11 June 2012

Accepted 24 September 2012

Available online 4 October 2012

Keywords:

Drug allergy

β -Lactam antibiotics

Protein haptentation

Amoxicillin binding

High resolution mass spectrometry

Amoxicillin targets

ABSTRACT

Allergy towards wide spectrum antibiotics such as amoxicillin (AX) is a major health problem. Protein haptentation by covalent conjugation of AX is considered a key process for the allergic response. However, the nature of the proteins involved has not been completely elucidated. Human serum albumin (HSA) is the most abundant protein in plasma and is considered a major target for haptentation by drugs, including β -lactam antibiotics. Here we report a procedure for immunological detection of AX–protein adducts with antibodies recognizing the lateral chain of the AX molecule. With this approach we detected human serum proteins modified by AX in vitro and identified HSA, transferrin and immunoglobulins heavy and light chains as prominent AX-modified proteins. Since HSA was the major AX target, we characterized AX–HSA interaction using high resolution LTQ orbitrap MS. At 0.5 mg/mL AX, we detected one main AX–HSA adduct involving residues Lys 190, 199 or 541, whereas higher AX concentrations elicited a more extensive modification. In molecular modeling studies Lys190 and Lys 199 were found the most reactive residues towards AX, with surrounding residues favoring adduct formation. These findings provide novel tools and insight for the study of protein haptentation and the mechanisms involved in AX-elicited allergic reactions.

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1. Introduction

The widely prescribed β -lactam antibiotics are among the drugs most frequently eliciting allergic reactions, thus posing an important clinical problem. In the most severe cases allergic reactions may be life-threatening and reduce the therapeutic options against infections. Protein haptentation

plays a key role in immunological reactions to β -lactams. This process occurs through the nucleophilic opening of the β -lactam ring, generally by the attack of free amino groups in proteins, and gives rise to a penicilloyl–protein adduct which is able to elicit an immune response (reviewed in [1]). See Fig. 1A for a schematic representation of the formation of an amoxicilloyl–protein adduct.

Abbreviations: AX, amoxicillin; HSA, human serum albumin; ECL, enhanced chemiluminescence.

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<http://dx.doi.org/10.1016/j.jprot.2012.09.030>

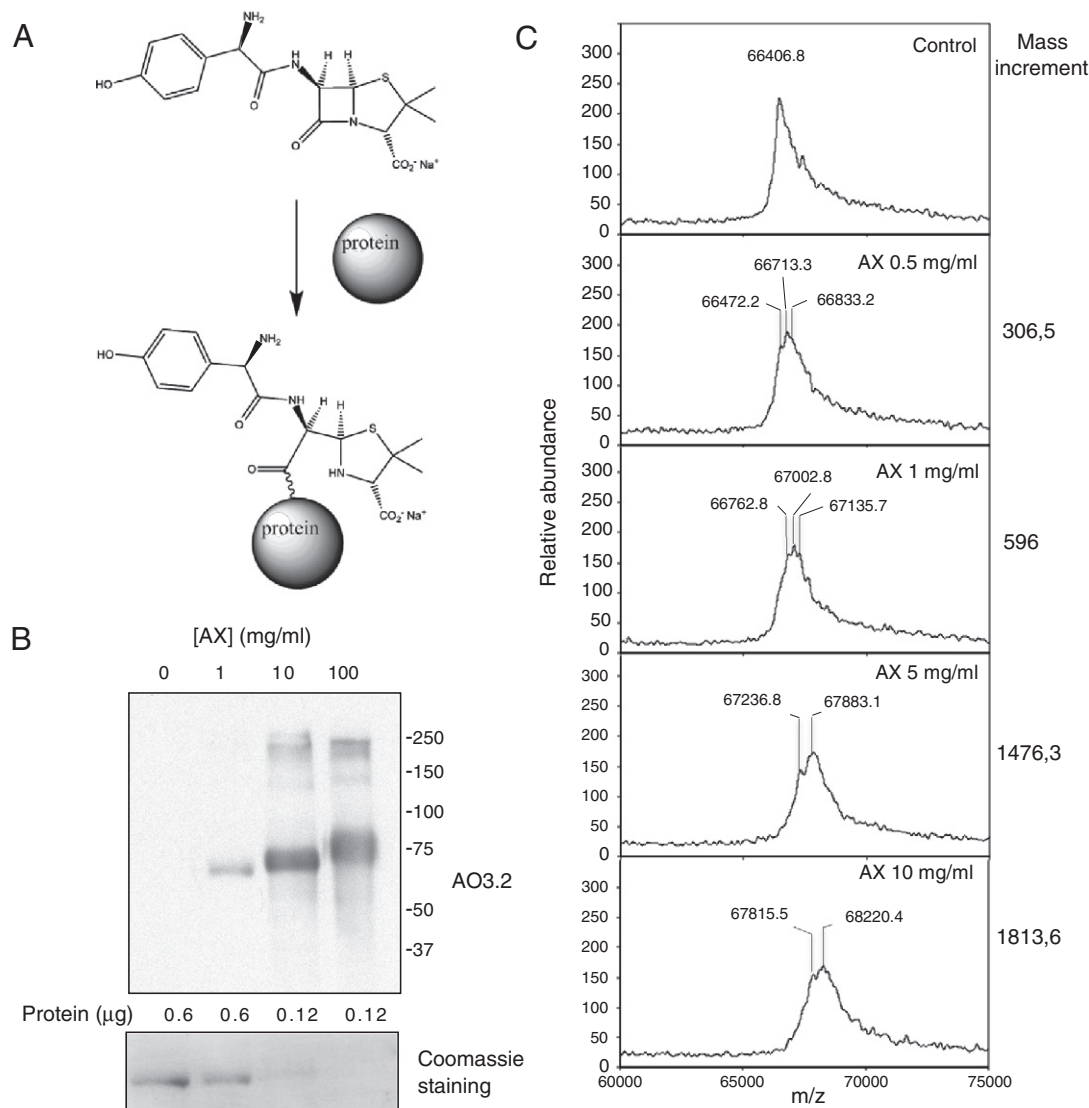


Fig. 1 – Interaction of AX with HSA as detected by immunological and MALDI-TOF MS approaches. (A) Structure of AX and the adduct formed with a protein (amoxicilloyl–protein adduct) through a non-specified residue. The main nucleophilic sites from the protein potentially able to react with the β -lactam ring of AX include lysine, histidine, cysteine and the amino terminal group. (B) Immunological detection of AX-modified HSA by Western blot with the AO3.2 monoclonal antibody. HSA was incubated in the presence of increasing concentrations of AX under the conditions used for antibody generation (see the [Materials and methods](#) section) and analyzed by SDS-PAGE followed by Western blot and ECL detection. Aliquots of the incubations containing decreasing amounts of protein (indicated at the bottom) were loaded in each lane to avoid saturation of the signal. The Coomassie staining of the blot is shown in the lower panel. The protein amount of the two lanes on the right was close to the detection level. (C) MALDI-TOF MS analysis of HSA incubated with the indicated concentrations of AX for 16 h at 37 °C. The mass increment observed in every condition is indicated on the right. Results are representative of at least 4 assays with similar results.

Important efforts have been devoted towards the understanding of the pathogenic role of protein haptenation by β -lactams, the identification of the adducts formed and their ability to activate the immune system. Because human serum albumin (HSA) is the most abundant protein in serum, most works have attempted the characterization of penicilloyl–HSA adducts. The first pioneering studies on these aspects, employing HPLC separation of tryptic peptides and EDMAN degradation sequencing, were published by Yvon et al. [2,3] and reported the sites of adduct formation in albumin obtained

from a penicillin-treated patient or prepared by *in vitro* conjugation. More recently, mass spectrometry (MS) has been applied to the study of adducts of HSA with various β -lactam antibiotics, including benzylpenicillin [4], flucloxacillin [5] and piperacillin [6], formed either *in vitro* or in the serum of patients. Noteworthy, there seems to exist a high degree of variability among the adducts detected and the factors that influence this process are not completely understood.

Unlike the other investigated penicillins, amoxicillin (AX) is a zwitterionic molecule which possesses a primary amino

group and belongs to the class of aminopenicillins, characterized by a greater activity against gram-negative bacteria and a better pharmacokinetic profile [7]. This unique feature of AX can heavily influence its reactivity towards nucleophilic centers.

The detection and characterization of the amoxicilloyl-HSA (AX-HSA) adducts formed could be very helpful for the understanding of the pathogenic role of protein haptentation in the allergic reaction, as well as for the design of more relevant antigens to be used in diagnostic tests. HSA modified in the presence of high concentrations of β -lactam antibiotics, including benzylpenicillin, ampicillin or AX has been widely used in the development of diagnostic assays to detect penicillin-reactive IgE antibodies in the serum of patients, to evaluate cross-reactivity and as immunogens for the development of anti- β -lactam antisera [8–11]. However there is little information on the structural features of HSA modified by AX concentrations close to those occurring during treatment with this antibiotic. Pharmacokinetic studies of AX in humans and in animal models reveal that the plasma concentrations after oral or intravenous administration may be in the order of 20 or 250 $\mu\text{g/mL}$, respectively [12,13], whereas HSA concentration in plasma is approximately 30 mg/mL. Therefore, the molar ratio of antibiotic to protein *in vivo* is not expected to exceed 2:1.

Here we have undertaken the study of the formation of covalent adducts of low AX concentrations with HSA and potentially with other serum proteins, as a first step to understand the processes that govern protein haptentation, resulting in the identification of a novel set of serum proteins as potential targets for modification by AX. Moreover, in the present study we explored the potentialities of a novel MS approach based on a high resolution MS analyzer (orbitrap) coupled to nanoscale capillary liquid chromatography for the full elucidation of the covalent binding of AX to HSA and in particular to obtain the following information: stoichiometry of reaction, chemical nature of the covalent adduct and the sites of modification. LTQ Orbitrap mass spectrometry is emerging as a powerful tool for protein identification and characterization due to its capabilities of high resolution (up to 100,000 for small molecules), and excellent mass accuracy (<5 ppm with external calibration) in a robust manner even within HPLC time scales [14]. Orbitrap has been successfully applied to investigate post-translational modifications as well as to fully elucidate protein modifications induced by xenobiotics [15]. However, to our knowledge, no applications of orbitrap and in general of high resolution MS analyzers have been reported for the study of penicillin binding to proteins and for this reason a top-down and an innovative bottom-up MS approach are here reported to fully elucidate the covalent binding of AX towards HSA.

2. Materials and methods

2.1. Reagents

Amoxicillin was from GlaxoSmithKline. Human serum was obtained from Sigma-Aldrich or prepared from blood samples

(male healthy donors) collected by venipuncture and allowed to clot at room temperature for no longer than 30 min and centrifuged for 10 min at 1500 g. Serum aliquots were stored in liquid nitrogen until their use. Iodoacetamide, (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. Anti-AX monoclonal antibodies, raised against HSA modified in the presence of high concentrations of AX prepared in 50 mM $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ pH 10.2, have been previously described [10]. Anti-penicillin antibody was from AbD Serotec. Reagents for enhanced chemiluminescence (ECL) detection were from GE Healthcare. Human serum albumin was obtained from Sigma or purified from serum from young healthy donors, as previously described [16]. Custom-synthesized LQQCPF, HPYFYAPELLFFAK, LKASLQK, and LVNEVTEF peptides, with 90% purity were supplied by Sigma-Aldrich (Milan Italy). Sequence grade modified trypsin was obtained from Promega (Milan, Italy) and chymotrypsin from Roche Diagnostics S.p.A. (Monza, Italy).

2.2. *In vitro* modification of synthetic HSA peptides by AX

Synthetic HSA peptides containing nucleophilic sites such as LQQCPF (Cys34), HPYFYAPELLFFAK (His146), LKASLQK (Lys199), and LVNEVTEF (control) were dissolved in 10 mM PBS (pH 7.4) and diluted to reach a final concentration of 150 μM . Aliquots of an AX solution in PBS pH 7.4 were then mixed with each peptide to reach a final concentration of 14 mM. In another set of experiments AX (14 mM final concentration) was incubated in the presence of the mixed peptides (150 μM each peptide). After an overnight incubation at 37 °C, an aliquot of each sample was analyzed by ESI-MS as described below. Stability analyses were carried out by incubating the AX-peptide mixtures in the presence of trypsin and/or by freezing at –20 °C and thawing.

2.3. *In vitro* modification of HSA or serum proteins by AX

AX was freshly prepared in 50 mM $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ pH 10.2 or 10 mM PBS pH 7.4, as indicated. For modification by AX, HSA at 10 mg/mL in PBS was incubated with AX at the indicated concentrations, for most assays the concentration of AX used was 0.5 mg/mL. Mixtures were incubated overnight at 37 °C. For modification of serum proteins, human serum was incubated with the indicated concentrations of AX for 16 h at 37 °C.

2.4. SDS-PAGE electrophoresis and Western blot

Samples from incubations containing 1–4 μg of protein were separated in 12.5% polyacrylamide gels. Proteins were transferred to Immobilon-P membranes using a three buffer system according to the manufacturer instructions on a semi-dry Western blot system (Bio-Rad). Proteins of interest were detected using various primary antibodies typically at 1:500 dilution followed by incubation with HRP-conjugated secondary antibodies (Dako) at 1:2000 dilution and ECL detection. Coomassie staining of blots was used as a control for protein loading and transfer.

2.5. Two-dimensional electrophoresis and protein identification

For two-dimensional electrophoresis, aliquots of control and AX-treated human serum containing 100 µg of protein were precipitated with 10% TCA, and the pellet was washed once with 80% ethanol and twice with acetone. The dried pellet was resuspended in 140 µL of IEF sample buffer (4% CHAPS, 2 M thiourea, 7 M urea, 100 mM DTT, and 0.4% Bio-lyte ampholytes) and loaded on ReadyStrip IPG Strips (pH 3–10, Bio-Rad) for isoelectric focusing on a Protean IEF cell (Bio-Rad), following the instructions of the manufacturer. Before the second dimension strips were equilibrated in 6 M urea, 2% SDS, 0.375 M Tris pH 8.8, 20% glycerol, containing 130 mM DTT for the first equilibration step and 135 mM iodoacetamide for the second step. Strips were then placed on top of 12.5% polyacrylamide SDS gels. Gels were run in duplicate. One of the gels was subsequently transferred to Immobilon P membrane (Millipore) and used for localization of AX-positive spots by Western blot. The duplicate gel was stained for total protein with Simplyblue stain (Invitrogen) or SYPRO Ruby (Bio-Rad) and it was used for spot excising and identification. The EXQuest Spot Cutter (Bio-Rad) was used for gel imaging and picking the selected spots. Excised spots were deposited in 96-well plates and digested automatically using a DigestPro MS (Intavis AG). The digestion protocol used was based on Schevchenko et al., [17] with minor variations: gel pieces were washed first with 50 mM ammonium bicarbonate (Sigma-Aldrich) and secondly with acetonitrile (Scharlau). Trypsin (Promega), at a final concentration of 12.5 ng/µL in 50 mM ammonium bicarbonate solution, was added to the gel pieces for 8 h at 37 °C. Finally, 70% acetonitrile containing 0.5% TFA (Sigma-Aldrich) was added for peptide extraction. Tryptic eluted peptides were dried by speed-vacuum centrifugation and resuspended in 4 µL of 30% acetonitrile–0.1% TFA. One µL of each peptide mixture was deposited onto an 800 µm AnchorChip (Bruker-Daltonics) and dried at RT. One µL of matrix solution (3 mg/mL α -cyano-4-hydroxycinnamic acid) in 33% acetonitrile–0.1% TFA was then deposited onto the digest and allowed to dry at RT. Samples were analyzed with an Autoflex III TOF/TOF mass spectrometer (Bruker-Daltonics). Typically, 1000 scans for peptide mass fingerprinting (PMF) and 2000 scans for MS/MS were collected. Automated analysis of mass data was performed using FlexAnalysis software (Bruker-Daltonics). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z 842.510 and m/z 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the m/z 800–3200 region. The typical error observed in mass accuracy for calibration was usually below 20 ppm. MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker-Daltonics) to interrogate the NCBI non-redundant protein database (NCBI: 20100306) using MASCOT software 2.3 (Matrix Science). Relevant search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); oxidation (M); 1 missed cleavage allowed; peptide tolerance, 50 ppm; MS/MS tolerance, 0.5 Da. Protein scores greater than 75 were considered significant ($p < 0.05$).

2.6. Mass spectrometry analysis of AX-modified HSA by MALDI-TOF

For MALDI-TOF MS analysis, HSA incubated in the presence of various AX concentrations was purified by ZipTip (C4) from Millipore as indicated by the manufacturer. The 2,5-dihydroxyacetophenone (2,5-DHAP) matrix solution was prepared by dissolving 7.6 mg (50 µmol) in 375 µL ethanol followed by the addition of 125 µL of 80 mM diammonium hydrogen citrate aqueous solution. For sample preparation, 2.0 µL of the sample (reconstituted in water) was diluted with 2.0 µL of 2% trifluoroacetic acid aqueous solution and 2.0 µL of matrix solution. A volume of 1.0 µL of this mixture was spotted onto the 800 mm AnchorChip MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. The MALDI experiments were performed on an Autoflex III MALDI-TOF-TOF instrument (Bruker Daltonics, Bremen, Germany) with a smartbeam laser. The spectra were acquired using a laser power just above the ionization threshold. Samples were analyzed in the positive ion detection and delayed extraction linear mode. Typically, 1000 laser shots were summed into a single mass spectrum. External calibration was performed, using bovine albumin from Sigma, covering the range from 20,000 to 70,000 Da.

2.7. Direct infusion electrospray mass spectral analysis (ESI-MS): LTQ XL Orbitrap mass spectrometer

2.7.1. Peptide analysis

Peptides incubated in the absence or presence of AX as described above were analyzed by direct infusion on a LTQ XL Orbitrap mass spectrometer (Thermo Scientific, Milan, Italy) equipped with an Electrospray Finnigan Ion Max source. An aliquot of each sample was diluted 300 fold with CH₃CN/H₂O/HCOOH (30:70:0.1 v/v/v) for the MS analysis and infused into the mass spectrometer at a flow rate of 5 µL/min. Analyses were carried out under the following instrumental conditions: full scan mode, mass range m/z 200–2000, positive-ion mode, AGC target 5×10^5 , 500 ms maximum injection time, 1 microscan, scan time 1.9 s, resolving power 100,000 (FWHM at 400 m/z), capillary temperature 275 °C, spray voltage applied to the needle 3.5 kV; capillary voltage 40 V; tube lens voltage 100 V; nebulizer gas (nitrogen) flow rate set 2 a.u.; and acquisition time 0.5 min. A list of 20 common inquinants of ESI background was used for internal mass calibration, i.e. 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). MS/MS experiments were performed in collision induced dissociation (CID) and high energy collision dissociation (HCD) modes (isolation width, 3 m/z ; normalized collision energy, 30 to 50 CID arbitrary units, 25 to 35 HCD arbitrary units).

2.7.2. HSA infusion

Prior to infusion HSA incubated in the presence or absence of AX was filtered and desalted by Amicon filter devices, 30 kDa MWCO (Millipore S.p.A., Milan, Italy). Three different aliquots were withdrawn from each sample and washed using three different procedures: the first aliquot was washed four times with 0.3 mL of H₂O, the second one twice with 0.3 mL H₂O and twice with 0.3 mL of a 1:1 (v:v) H₂O:EtOH, the third one four

times with 1:1 (v:v) H₂O:EtOH. Then the retained aliquots were dried using speedvac and kept frozen at –20 °C until analysis. To detect changes in protein mass and to determine the stoichiometry of the reaction, undigested native, and AX-treated HSA were analyzed by direct infusion on a linear ion trap LTQ Orbitrap™ XL mass spectrometer (Thermo Scientific, Milan, Italy) equipped with an Electrospray Finnigan Ion Max source. An aliquot of the dried protein (96 µg) prepared as described above was dissolved with 10 µL of H₂O, diluted with 200 µL of H₂O and 200 µL of CH₃CN/H₂O/HCOOH (60:40:0.4 v/v/v) and infused into the mass spectrometer at a flow rate of 5 µL/min. The linear ion trap was set under the following instrumental conditions: mass range m/z 800–2000, positive-ion mode, capillary temperature 300 °C, spray voltage applied to the needle 3.5 kV; capillary voltage 48 V; tube lens voltage 190 V; nebulizer gas (nitrogen) flow rate set 8 a.u.; and acquisition time 5 min.

2.8. Liquid chromatography electrospray ionization mass spectrometry/mass spectrometry analysis (LC–ESI-MS/MS): LTQ Orbitrap XL mass spectrometer

All digested peptide mixtures were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray tandem mass spectrometry (ESI-MS/MS). For sample preparation, the lyophilized protein (30 µg) was dissolved in 30 µL 50 mM Tris–HCl (pH 7.8) and digested with trypsin and trypsin/chymotrypsin according to the manufacturer's procedure. The tryptic mixtures were acidified with formic acid up to a final concentration of 10%. One microliter of tryptic digest was injected into a nanochromatographic system, UltiMate 3000 RSLCnano System (Dionex). The peptide mixtures were loaded on a fused silica reversed-phase column (PicoFrit™ Column, HALO, C18, 2.7 µm, 100 Å, 75 µm i.d. × 10 cm, New Objective). The peptides were eluted with a 30 min gradient from 4% buffer A (0.1% formic acid in water) to 60% buffer B (0.1% formic acid in acetonitrile) at a constant flow rate of 300 nL/min. The liquid chromatography system was connected to an LTQ XL-Orbitrap mass spectrometer (Thermo Scientific, Milan, Italy) equipped with a nanospray ion source (dynamic nanospray probe, Thermo Scientific, Milan, Italy) set as follows: spray voltage 1.7 kV; capillary temperature 220 °C, capillary voltage 30 V; tube lens offset 100 V, no sheath or auxiliary gas flow. During analysis, the mass spectrometer continuously performed scan cycles in which first a high-resolution (resolving power 60,000, FWHM at m/z 400) full scan (200–1500 m/z) in profile mode was made by the Orbitrap, after which MS2 spectra were recorded in centroid mode for the 3 most intense ions, using both CID and HCD modes (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 $[(Si(CH_3)_2O)_6 + H]^+$, m/z 445.120025) were used for real time internal mass calibration. Dynamic exclusion was enabled (repeat count, 3; repeat duration, 10 s; exclusion list size, 50; exclusion duration, 120 s; relative exclusion mass width, 5 ppm). Charge state screening and monoisotopic precursor selection were enabled, singly and unassigned charged ions were not fragmented.

Identification of the adduct-bearing peptides and the sites of modification was carried out by a novel MS strategy based on two different steps: the first step consists of the analyses of the MS/MS spectra recorded in HCD mode, in order to identify the precursor ions of those found to be diagnostic in this work, namely, ions at m/z 160.04 and 349.08 (see Results). In more detail, a list of precursor ions is extracted from the parent ion map displayed by setting as product mass the ions at m/z 160.04 (tolerance 0.01). A second ion map is then generated by setting as product ion that at m/z 349.08 (tolerance 0.01). The two lists of parent ions are then exported to Excel 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 ($value1$) and 349 ($value2$) and ts is the threshold relative abundance (Fig. S1). Thus, by using this approach, each precursor ion is confirmed only when it is present in both the ion maps reconstituted by setting the two diagnostic precursor ions, otherwise it is discarded. The second step consists in 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/chymotrypsin as enzymes, two missed cleavages and the addition of the amoxicilloyl moiety.

2.9. Bioinformatics

Direct infusion ESI-MS spectra were deconvoluted using the software packages Bioworks 3.1.1 (Thermo-Quest, Milan, Italy) and the software MagTran 1.02 [18]. Peptide sequences were identified using the software turboSEQUENT (Bioworks 3.1, ThermoQuest, Milan, Italy), and using a database containing only the protein of interest and assuming trypsin and trypsin/chymotrypsin digestion. The protein sequence of HSA was obtained from the Swiss-Prot database (primary accession number P02768). Sequence fragment calculator (Bioworks 3.1.1) was used to obtain the theoretical digested masses setting trypsin and trypsin/chymotrypsin as enzymes, 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>.

2.10. Molecular modeling

The starting structure of human serum albumin was retrieved by PDB Database (PDB ID: 1A06) [19]. The experimental structure was completed by adding the hydrogen atoms using VEGA software [20] and underwent preliminary minimizations keeping fixed the backbone atoms to preserve the experimental folding. The optimized structure was then used to predict the ionization constants for lysine residues using PropKa [21] and in the following docking simulations. AX was considered in its zwitterionic form and its conformational profile was explored by a clustered MonteCarlo simulation to generate 1000 minimized conformers. The so obtained lowest

energy structure was used in the docking analyses. For each identified site of addition, a docking search was performed using the AutoDock 4.0 software and considering a 12 Å sphere around the adducted lysine, which was considered in its neutral form. AX was docked into this grid with the Lamarckian algorithm and the ligand flexible bonds were left free to rotate. The genetic-based algorithm generated 100 poses with 2,000,000 energy evaluations and a maximum number of generations of 27,000. The crossover rate was increased to 0.8, and the number of individuals in each population to 150. All other parameters were left at the AutoDock default settings [22]. The obtained complexes were ranked considering both the docking scores and the distance between the lysine's amino group and the AX β -lactam carbonyl group. The chosen complexes were finally minimized keeping fixed the atoms outside a 15 Å radius sphere around the bound substrate and then used to recalculate docking scores.

3. Results

3.1. Detection of AX–HSA adducts by Western blot

Anti-AX antibodies have been previously used for a variety of applications. However, the detection of AX–protein adducts by Western blot has not been previously documented. To explore this possibility, we incubated HSA with AX concentrations close to those used to generate AX–HSA for immunological studies or for the generation of antibodies (1 to 100 mg/mL, Fig. 1B) [8–11]. Interestingly, analysis of HSA incubated with high concentrations of AX by Western blot with an anti-AX antibody, previously used for ELISA (AO3.2), showed a clear immunoreactive signal. Moreover, an increase in the apparent molecular weight of the HSA band was observed, as expected from the increment of mass produced by the incorporation of several AX molecules. In addition, higher molecular weight species appeared suggestive of the formation of HSA aggregates during incubation with the antibiotic. As a control for this assay, AX-modified HSA samples were analyzed by MALDI-TOF MS. Incubation of HSA in the presence of increasing concentrations of AX induced a concentration-dependent increase in the mass of the protein as detected by MALDI-TOF MS (Fig. 1C). A displacement of the centroid of the HSA peak was evident even with the lowest concentration of AX tested (0.5 mg/mL). In the spectrum of HSA treated with 5 mg/mL of AX, two poorly defined peaks could be observed showing m/z increments of 830 and 1476 with respect to the native protein, compatible with the incorporation of 2 and 4 molecules of AX, respectively. Finally, the spectrum of HSA treated with 10 mg/mL AX, conditions which are close to those routinely used for obtaining the AX–HSA complex for immunological applications, showed a major peak of m/z 68,220.4, representing a m/z increment of approximately 1813.6 with respect to control HSA. This increment could be due to the incorporation of up to five AX molecules and/or to the occurrence of other modifications in the HSA molecule during incubation in the presence of the β -lactam, as suggested by the Western blot assay (Fig. 1B).

3.2. Immunological detection of AX-modified HSA with various anti- β -lactam antibodies

In previous works several monoclonal antibodies were obtained using HSA treated with high concentrations of AX as immunogen [10]. Competition studies with various moieties of the AX molecule in ELISA indicated that the antibodies obtained were directed towards different parts of the molecule. Therefore, we assessed the ability of several of these monoclonal antibodies to detect AX-modified HSA by Western blot. As it can be observed in Fig. 2, two monoclonal antibodies, AO3.2 and AO19.1, effectively detected the AX–HSA adduct. The AO3.2 antibody, which recognizes the lateral chain plus a part of the nuclear region of AX, allowed the detection of an immunoreactive signal above the background even after incubation of HSA in the presence of 0.025 mg/mL AX, which is in the range of the concentrations expected to occur in vivo [12,13] and indicates that this procedure shows high sensitivity. The AO19.1 antibody, which recognizes the

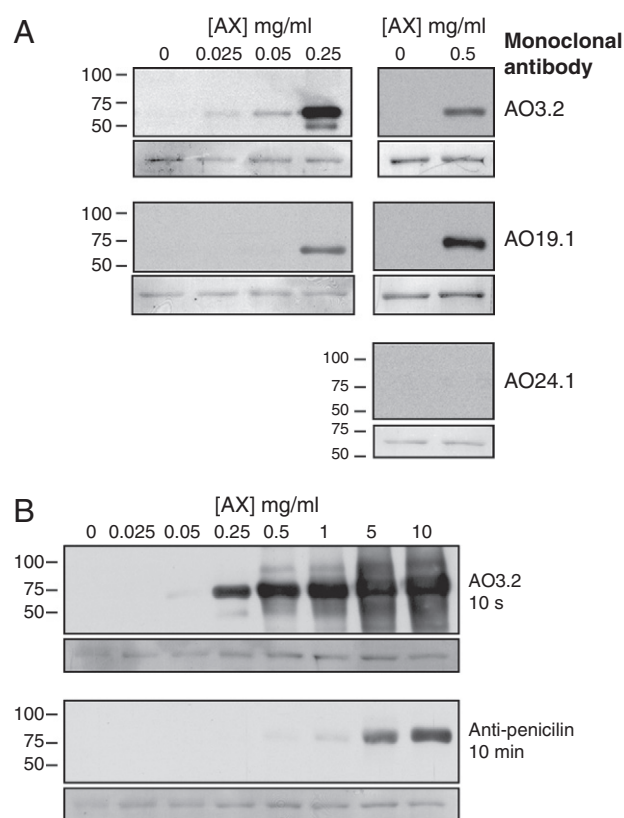


Fig. 2 – Immunological detection of AX-modified HSA by Western blot with various antibodies. HSA was incubated in the presence of increasing concentrations of AX. Aliquots of the incubations containing 2 μ g of protein were analyzed by SDS-PAGE followed by Western blot with the indicated antibodies and ECL detection. In panel B, the times of exposure of films for signal detection is indicated on the right. Results are representative of four assays. Panels underneath each blot show the corresponding Coomassie staining.

lateral chain plus the open β -lactam ring, also gave a positive signal although it showed less sensitivity. In contrast, several monoclonal antibodies reportedly recognizing the lateral chain of AX (with a β -lactam ring absent or open), including AO24.1 (Fig. 2), AO14.1 and AO18.2 (results not shown), gave no signal in this assay, although they previously showed immunoreactivity in direct and inhibition ELISA [10] using exhaustively modified protein as antigen. Finally, we used a commercial anti-penicillin antibody for comparison. This antibody showed much lower sensitivity towards AX-modified HSA than the AO3.2 antibody and, used at the same concentration, only detected HSA modified by high concentrations of AX after long exposures.

3.3. Detection of AX candidate target proteins in human serum

HSA is the most abundant protein in human plasma and therefore it is considered the main target for protein haptentation. However, as binding of haptens to proteins depends on their structure, other plasma proteins may result covalently modified by AX to a significant proportion and therefore, may also be good candidates for mediating the immunological reactions towards this antibiotic. To assess this possibility we incubated human serum with AX and applied immunological detection. This allowed the observation

of multiple positive bands, even with the lowest concentration of amoxicillin used (Fig. 3A). In order to get insight into the nature of these proteins we carried out two-dimensional electrophoresis followed by peptide fingerprint analysis by tryptic digestion and MALDI-TOF MS (Fig. 3B, Table 1). Protein identification was confirmed by MALDI-TOF-TOF MS/MS analysis of selected peptides from every protein. Identified proteins included HSA, several forms of transferrin, and heavy and light immunoglobulin chains. In contrast, other abundant proteins, like apolipoprotein or haptoglobin were negative in this assay. It should be noted that according to the signal given by the antibody, the relative modification of transferrin under these conditions was more extensive than that of HSA, thus suggesting that this and other serum protein(s) may play an important role in AX-induced reactions. Of particular interest is the modification of immunoglobulins, which could have functional consequences related to the immune response.

3.4. AX reaction with His, Lys and Cys containing peptides from HSA

As reported above, HSA is the main plasma protein target of AX, and for this reason, we undertook a detailed characterization of the AX-HSA interaction, including the elucidation of the reactivity and the reaction mechanism, and identifying

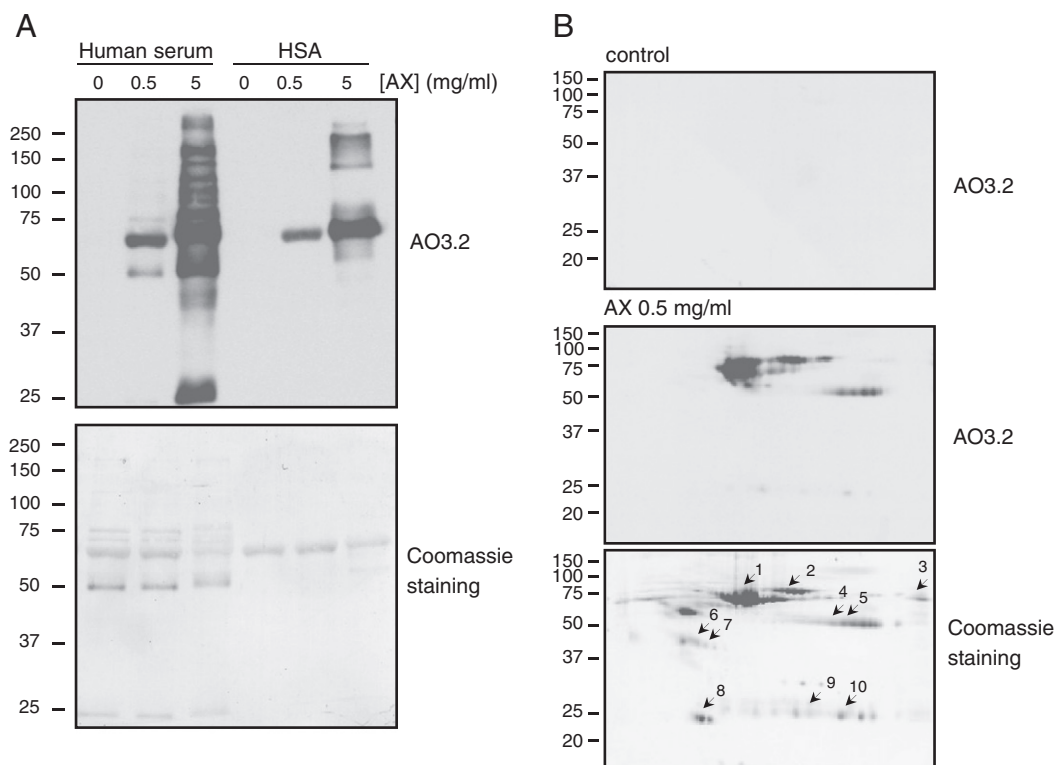


Fig. 3 – Immunological detection of adducts of AX and serum proteins and identification of targets. (A) Human serum or HSA was incubated with the indicated concentrations of AX for 16 h at 37 °C and analyzed by Western blot. AX-modified proteins were detected with the AO3.2 antibody. Lower panel shows the Coomassie staining of the blot. (B) Samples of serum incubated in the absence or presence of 0.5 mg/mL AX were subjected to 2D-electrophoresis on duplicate gels, after which, one of the gels was used for detection of modified proteins by Western blot and the other one was used for protein staining with Coomassie. Matched spots were excised from the gel and used for tryptic digestion and peptide fingerprint analysis. Results are representative of multiple assays.

Table 1 – Protein identification by mass spectrometry.

Spot number ^a	Protein name	Accession code ^b	Total score ^c	Ion score ^d	MW (Da) ^e	pI ^f	Matched peptides ^g	Cover (%) ^h	Identif. TOF/TOF/LTQ ⁱ	AO3.2 signal ^j
1	Human serum albumin	gi 168988718	570	41, 19, 91, 84, 81	67,690	5.63	21	37	Yes	(+)
2	Apo-human serum transferrin (chain A)	gi 110590599	524	55, 93, 84	76,988	6.85	22	42	Yes	(+)
3	Human serum albumin	gi 168988718	363	36, 79	67,690	5.63	19	39	Yes	(+)
4	Immunoglobulin G heavy chain	gi 185362	115	52	52,687	8.6	4	13	Yes	(+)
5	Immunoglobulin G heavy chain	gi 185363	150	52, 19	52,687	8.6	5	16	Yes	(+)
6	Haptoglobin 2	gi 47124562	280	18, 28, 58, 61	31,647	8.48	7	26	Yes	(–)
7	Haptoglobin 2	gi 47124562	270	18, 28, 58, 61	31,647	8.48	7	26	Yes	(–)
8	Apolipoprotein A1	gi 90108664	121	24, 42	28,061	5.27	4	16	Yes	(–)
9	Immunoglobulin kappa4 light chain	gi 170684480	187	94	24,507	6.1	5	35	Yes	(+)
10	Immunoglobulin kappa4 light chain	gi 170684480	313	94, 80, 60	24,507	6.0	5	48	Yes	(+)

Data under superscripts c through h are from MASCOT.

^a Spot numbering as shown in 2-DE Coomassie gel in Fig. 3.

^b Protein accession code from NCBI database.

^c Mascot total score.

^d Mascot ion score.

^e Theoretical molecular weight (Da).

^f Theoretical pI.

^g Number of matched peptides.

^h Protein sequence coverage for the most probable candidate as provided by Mascot.

ⁱ Proteins identified by MALDI-TOF/TOF (TOF–TOF) and proteins identified by nano-LC coupled to LTQ.

^j Results of Western blot analysis with AO3.2 antibody indicated.

and characterizing the reaction products of AX towards the main nucleophilic sites of HSA potentially able to covalently react with the β -lactam ring, namely Lys, Cys, and His residues and the amino terminal group. As a first approach to achieve a detailed characterization of AX–HSA adducts, peptides containing the most reactive HSA nucleophilic residues, as determined by previous studies [16], were used for the in vitro reactions and in particular: Lys199 (¹⁹⁸LKASLQK²⁰⁵), His146 (¹⁴⁶HPYFYAPELLFFAK¹⁵⁹), and Cys34 (³¹LQQCPF³⁶). The reactivity of the amino terminal group was tested by considering ⁴²LVNEVTEF⁴⁹ as model peptide, since it does not contain any nucleophilic amino acid residue. The MS spectra of each peptide incubated in the absence of AX showed the corresponding [M+H]⁺ and [M+2 H]²⁺ ions (data not shown). When AX was added at a 1:90 molar ratio and incubated for 18 h, all the adduct-bearing peptides were characterized by an increase of 365.10454 Da, compatible with the addition of the amoxicilloyl moiety (empirical formula C₁₆H₁₉N₃O₅S), with respect to the native forms and they were easily determined in the case of peptides containing Lys199, His146 and to a lesser extent (<0.5% relative abundance), but still detectable, for the peptide LVNEVTEF and that containing Cys34 (Fig. 4). If we consider that the amoxicilloyl modification impacts the response factors of the adducts to a similar extent, the relative abundance of the adducts can be considered as a good index of the chemical reactivity of the peptide towards AX. The relative abundances of the adduct-bearing with respect to the native peptides were

found to be different: the Lys199-containing peptide was the most reactive (12%) followed by the His146-containing peptide (3%), while LVNEVTEF and the Cys34-containing peptide were modified to a negligible extent (<0.5%). The modification site of each peptide was then identified by MS/MS experiments. The peptide ¹⁹⁸LKASLQK²⁰⁵ contains three potential nucleophilic sites: Lys199, the C-terminal Lys205 and the amino terminal group. MS/MS fragmentation pathway of the native peptide was carried out by selecting the [M+2 H]²⁺ as precursor ion at *m/z* 445.76 Da. The peptide sequence agrees well with the y and b ion series as determined by MS/MS experiments carried out in CID and HCD modes (data not shown). The fragmentation of the amoxicilloyl-peptide adduct was then analyzed by selecting the [M+2 H]²⁺ ion at *m/z* 628.31 (365.1 Da increase) as precursor ion (Fig. 5). The MS/MS fragment ions as detected in CID mode show an unmodified y ion series from y₂ to y₆, excluding the formation of an amoxicilloyl–Lys205 adduct and this is well explained by considering that the amino group forms a salt bridge with the carboxyl group; by contrast the b-ion series (from b₂ to b₇) increased by 365.1 Da although their relative abundance is very low, excluding the amino terminal involvement and confirming Lys199 as a main site for adduct formation. As a further confirmation, the y ion series was found to be modified from y₇ to y₈ (see the abundant ion at *m/z* 619.8 relative to y₈⁺⁺–NH₃). By comparing the MS/MS spectrum of the adducted with respect to the native peptide, several other abundant fragment ions were observed and in particular the ion

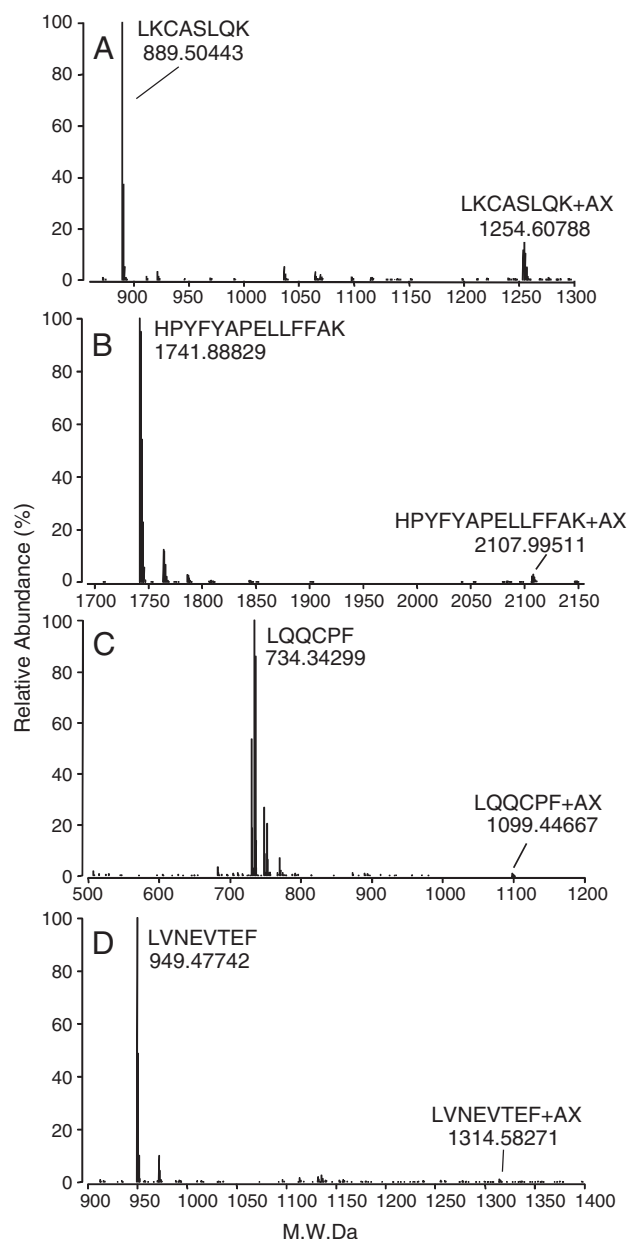


Fig. 4 – In vitro modification of HSA peptides by AX. Deconvoluted MS spectra obtained by ESI-MS direct infusion experiments of HSA peptides incubated overnight at 37 °C with AX. See material and methods for experimental details. Native and AX-modified peptides are labeled in each spectrum.

at m/z 553.78 (base peak) which can be attributed to the y_8^{*2+} ion adducted by the amoxicilloyl moiety with the loss of the 4-hydroxybenzylamine group (M_1 ion series). Two other

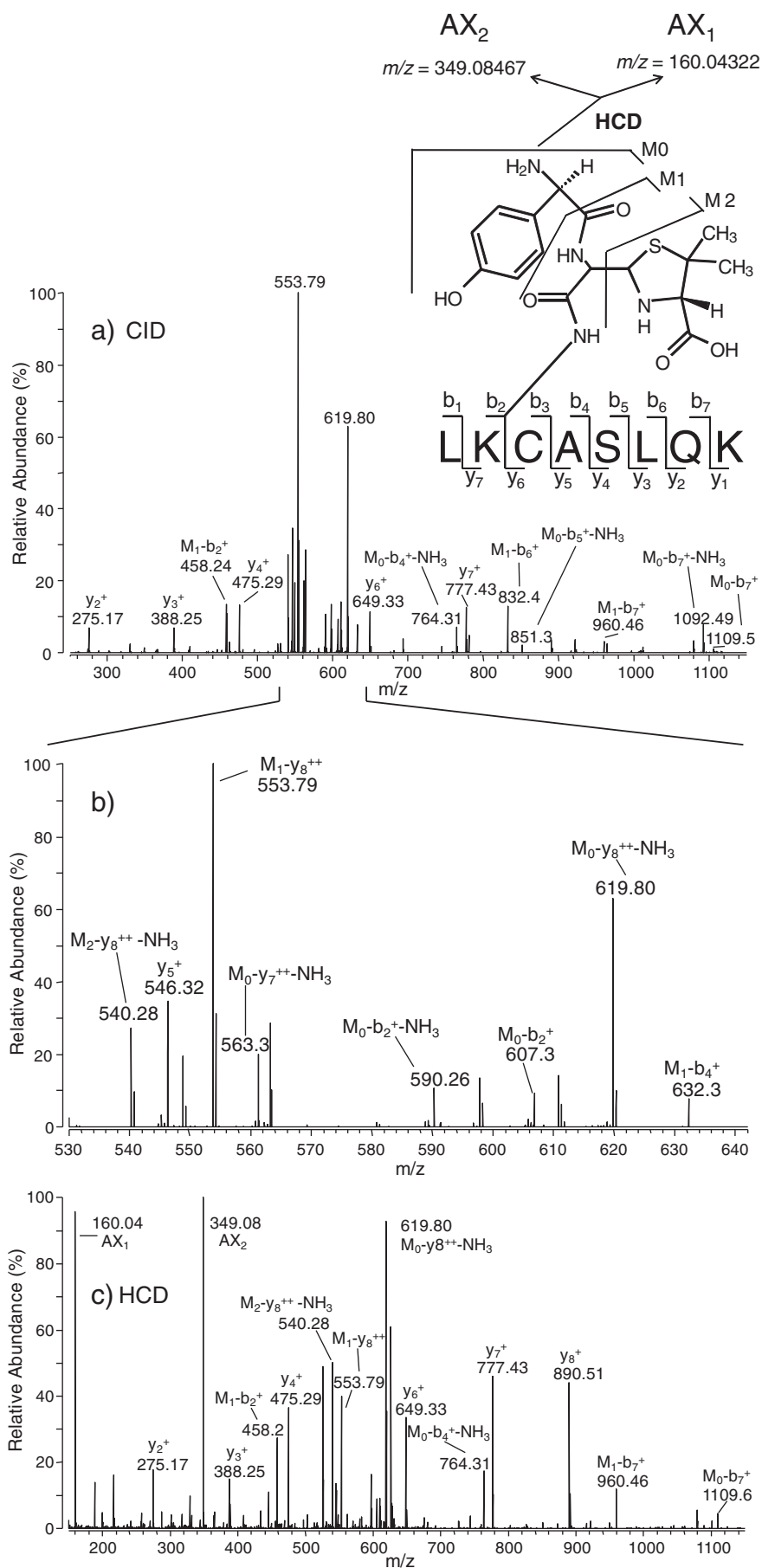
diagnostic abundant ions were then observed at m/z 548.79 and 540.28 which were assigned to the y_8^{*2+} and $y_8^{*2+}-NH_3$ of the amoxicilloyl group with the loss of the thiazolidinic group (M_2 ion series).

As shown in Fig. 5, the main difference between the MS/MS spectra of the adducted peptide recorded in CID and HCD is that the spectrum recorded in the latter mode is characterized by the presence of the ions at m/z 160.04222 (AX_1) and 349.08467 (AX_2), corresponding to the thiazolidinic moiety of AX and to AX minus the aminic residue, respectively.

The $^{146}HPYFYAPELLFFAK^{159}$ peptide contains three potential nucleophilic residues, the amino terminal group, His146 and the C-terminal Lys159, although the latter should be excluded since it forms a salt bridge with the terminal carboxyl group as reported above. The MS/MS spectrum recorded in CID mode shows the unmodified y ion series from y_4 to y_{13} as well as the b ion series from b_5 to b_{13} to indicate that the modification is unstable and is lost during the collision. No diagnostic y and b fragment ions of the AX-adducted peptide were found (M_0 series). The comparison of the MS spectra of the native and adducted peptides indicates that the latter is characterized by intense ions at m/z 980.47 and 975.48 relative to the y_{14}^{2+} of the M_1 and M_2 series, accompanied by the corresponding $-NH_3$ ions. HCD spectrum shows a similar fragmentation pattern and in contrast to that observed for Lys199 no ions relative to the thiazolidinic ring of AX or to AX minus the aminic residue of the R group were detected (data not shown).

Taken together, these data indicate that among the nucleophilic sites present in HSA and potentially able to covalently react with the β -lactam ring, lysine residues are the most reactive sites towards AX and that the amoxicilloyl adduct represents the main reaction product. It should be noted that the order of reactivity as determined using HSA peptides takes into account only the intrinsic reactivity of the nucleophilic amino acid as well as the effect of the vicinal amino acids, while it does not consider other parameters related to the arrangement of the amino acid in the three-dimensional protein structure which can increase or reduce the reactivity, for example surface exposure or the presence of surrounding residues that can affect the nucleophilicity of the residues. The determined reactivity appears to be roughly proportional to the basicity of the nucleophilic site. This result is in line with other reported studies and confirms the key role of general acid catalysis exerted by the protonated amine to promote the breakdown of the tetrahedral intermediate by donating a proton to the β -lactam nitrogen atom [23]. The unexpected lack of reactivity of the cysteine residue, which is in contrast with several studies reporting the thiolysis of penicillins [24], could be justified by considering the effect of the protonated amine which attracts the reactive thiolate function hampering its proper attachment to the β -lactam carbonyl group.

Fig. 5 – ESI-MS/MS spectra and fragmentation pathway of the AX-modified peptide LK#CASLQK. Panels (a) and (b) show the ESI-MS/MS spectrum recorded in CID mode and with a mass range of 250–1100 m/z and 530–640, respectively. Panel (c) shows the ESI-MS/MS spectrum recorded in HCD mode. In the upper part is shown the fragmentation pathway of the adducted peptide LKCASLQK leading to the M_0 , M_1 and M_2 ion series as well as the two diagnostic fragment ions at m/z 160 and 349 generated in HCD mode.



From an analytical point of view we found that the fragmentation patterns of the adduct-bearing peptides differ on the basis of the modified amino acid. In the case of Lys, both CD and HCD are characterized by diagnostic y and b fragment ions bearing fragmented AX and in particular the AX moiety arising by the loss of the 4-hydroxybenzylamine group (M_1 ion series) and the thiazolidine ring (M_2 ion series). Moreover, HCD but not CID spectra are characterized by the cleaved thiazolidine ring at m/z 160 and by the fragment at m/z 349.08 arising from the cleavage of the AX moiety and concomitant loss of the amino group.

We then evaluated the effect of trypsin incubation as well as sample freezing–thawing (both procedures used for the bottom-up analysis of the HSA sites modified by AX, vide infra) on the stability of the AX-peptide adducts. Trypsin digestion, freezing–thawing, as well as both the procedures did not affect the stability of adducts since the relative abundances were unchanged with respect to control samples before and after sample treatment.

3.5. AX reaction with HSA: top-down experiments

A summary of the MS strategy used to characterize the AX–HSA adduct, including top-down and bottom-up approaches is shown in Fig. S1. The ability of AX to covalently react with HSA and the stoichiometry of the reaction were firstly evaluated by top-down experiments, consisting of directly infusing the AX–HSA reaction mixture. Fig. 6, panel (A) shows the deconvoluted MS spectrum of HSA incubated in absence of AX, which is characterized by the main peak at 66,439 Da relative to the mercaptoalbumin and by two minor peaks at 66,559 Da (+120 Da) and 66,603 Da (+164 Da), which are attributed to the cysteinylated and glycated forms, respectively, as previously reported [16]. Fig. 6 panels (B) and (C) show the deconvoluted MS spectra of HSA incubated at pH 7.4 for 18 h in the presence of AX at a molar ratio HSA:AX 1:9 (AX 0.5 mg/mL) and 1:90 (AX 5 mg/mL), respectively. When HSA was incubated with the lowest concentration of AX, an easily detectable single adduct at 66,804 Da was observed, shifted by 365 Da with respect to mercaptoalbumin and referred to as the amoxicilloyl derivative. This adduct represented 20% of the native protein. By increasing AX concentration to 5 mg/mL, the relative abundance of the three native isoforms of albumin was drastically reduced and three main HSA adducts appeared at 66,804, 67,169 and 67,534 Da, shifted by 365, 730 and 1095 Da, respectively, with regard to the native forms, which can be attributed to AX–HSA adducts with the following stoichiometric ratios: 1:1, 1:2; 1:3 (Fig. 6 panel C). The cysteinylated and glycated HSA forms were also found to be modified to a similar extent than mercaptoalbumin, thus indicating that Cys34 (the site of cysteinylation) as well as Lys525 (the main glycation site) are not involved in the reaction with AX. It should be underlined that all samples were washed several times with an EtOH:H₂O mixture before MS analysis, until the relative abundances of the adducts were constant. This procedure was adopted in order to remove non-covalent protein–AX adducts that could be maintained in the gas-phase, although the sample is sprayed under denaturing conditions. In fact, when HSA was incubated with AX at a

molar ratio of 1:9 and injected after a washing step carried out only with water, apart from mono-adduct, adducts with stoichiometric ratios 1:2 and 1:3 were observed due to the presence of non-covalent interactions which disappeared upon extensive washing with the EtOH:H₂O mixture (data not shown).

3.6. Identification of the HSA sites modified by amoxicillin: bottom-up experiment

Identification of the HSA sites modified by the amoxicilloyl residue was carried out by a bottom-up approach using trypsin and trypsin plus chymotrypsin for protein digestion (Fig. S1). Double digestion with trypsin plus chymotrypsin was used since the modified Lys residues are not recognized by trypsin and this would increase the MW and the length of the adduct-bearing peptides to values not suitable for the LC–ESI-MS analysis. The protein coverages of both native and AX-modified HSA were always higher than 90% and 85% for trypsin and trypsin/chymotrypsin digestions, respectively (see Fig. S2). It should be noted that the most reactive Lys residues as reported in the literature and predicted by molecular modeling studies were covered.

The first attempt to identify the AX-modified peptides and the corresponding sites of modification was based on the Sequest algorithm search by setting the amoxicilloyl modification (+365 Da) as a variable modification on Lys, His, Cys and Ser. Such an automatic approach is of great value for the identification of the amino acids covalently modified by different xenobiotics as well as by post-translational modifications. However, this approach was not suitable to identify the AX-modified peptides and this can be clearly explained by considering the typical MS/MS pathways of the AX-peptide adducts, which are characterized, as reported above, by the cleavage of the amoxicilloyl moieties, leading to the formation of unpredictable y and b ion series.

The MS approach was then optimized on the basis of the fragmentation pathway of the peptides adducted on the Lys residue as described above and summarized in Fig. S1. As shown above, using model peptides we found that the MS/MS spectra of the AX-Lys adducts recorded in HCD mode are characterized, besides by the y and b fragment ions, by the cleaved thiazolidine ring at m/z 160 and by the fragment at m/z 349.08, which arises from the cleavage of the amoxicilloyl moiety and the concomitant loss of the amino group. Therefore, such specific and diagnostic fragments were then set as product ions to generate the maps of precursor ions. The ion map relative to the sample of HSA incubated in the presence of AX at the higher molar ratio (1:90) reported 11 precursor ions and 8 of them were attributed to 6 adducted peptides which were reduced to 3 peptides (five precursor ions) at the lowest molar ratio, as reported in Table 2. The method was found highly specific since no precursor-ions were detected in the control sample. To further confirm that the precursor ions are referred to the adducted peptides, SIC traces were then reconstituted for each of them by setting a 5 ppm tolerance. A well detectable peak was identified for each of the precursor ions selected and whose area dose dependently increased by increasing AX concentration in the reaction mixture, while it was absent in the control.

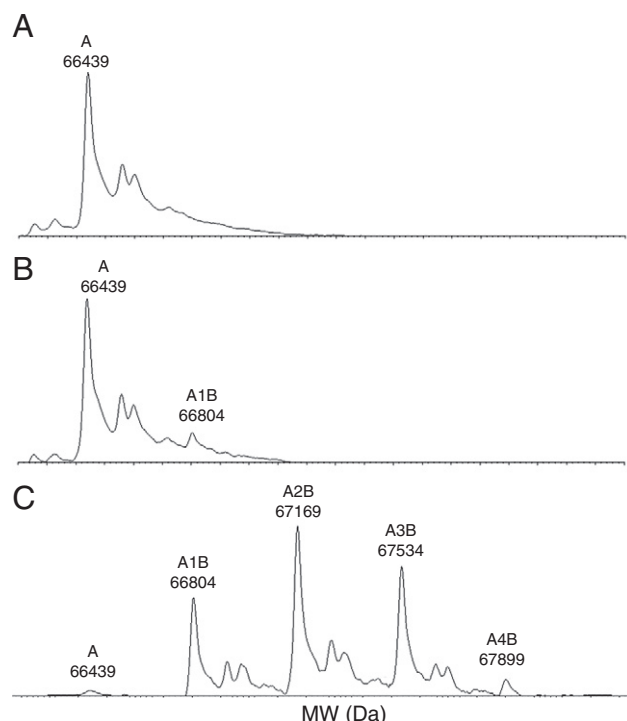


Fig. 6 – Top-down approach for characterization of HSA modification by AX. Deconvoluted MS spectra of HSA incubated in the presence of AX. Deconvoluted MS spectra recorded by direct infusion experiments of HSA incubated in the absence (A) and presence of 0.5 mg/mL (B) and 5 mg/mL (C) of AX. Mercaptoalbumin isoform is at 66,439 (peak A). The peaks A1B, A2B, A3B and A4B are referred to the HSA:AX adducts at the following stoichiometric ratio: 1:1, 1:2, 1:3, and 1:4.

3.6.1. Identification of the sites modified by AX in purified HSA

By matching the precursor ions with a list of theoretical adducted ions, 8 precursor ions, relative to 6 adducted peptides, were identified and the sequence as well as the sites of adduct formation were then determined by analyzing the MS/MS spectrum and attributing the y and b ions. In particular, Lys190, 199, 351, 432, 541 and 545 were found adducted when purified HSA was incubated with AX at 5 mg/mL and Lys 190, 199 and 541 when incubated in the presence of AX at 0.5 mg/mL (Table 2).

As an example of peptide matching and identification of the modified site, here below is described the approach we used to identify Lys 541 as adduction site. The ion map and Boolean logic identified two multi-charged precursor ions at m/z 591.79474 and m/z 394.86562 referred to the $z=2$ and $z=3$ of the adducted peptide. The SIC ion trace relative to the sample incubated with 0.5 mg of AX was characterized by a well detectable peak at RT of 8 min and whose area increased by almost ten-fold in the presence of 5 mg of AX and was absent in the control sample (Fig. 7). Matching such precursor ions with a list of theoretical AX-modified precursor ions, permitted to identify the peptide with the sequence $^{539}\text{ATK}\#\text{EQLK}^{545}$ and whose identity and the site of modification were determined by analyzing the MS/MS spectrum as reported in Fig. 7.

3.6.2. Identification of the sites modified by AX in HSA in complete human serum

We then analyzed the AX–HSA adducts formed when complete human serum was incubated in the presence of the antibiotic. For this, after incubation, HSA was purified as described in the Materials and methods section and subjected both to immunological detection of the modification (Fig. S3) and to proteomic analysis following the same approach described above. In general, adducts identified when using isolated HSA were confirmed in these experiments. In particular, when serum was incubated with AX at 5 mg/mL, the following HSA Lys residues were found to be modified by AX: Lys 190, 199, 351, 432, 541 and 545. At 0.5 mg/mL AX, a slight difference in the modification sites was observed with respect to the experiments carried out with purified HSA, since Lys 190 and Lys 432 but not Lys199 or Lys541 were found to be modified.

3.7. Peptide consumption

The relative consumption of the peptides undergoing AX modification was determined as here below reported and compared to that calculated for peptides not covalently modified. The ion responses of the selected peptides were determined by measuring the peak areas in the selected ion chromatograms (SICs) reconstituted by using the $[\text{M}+\text{nH}]^{\text{n}+}$ as filter ions. The peptide responses were normalized with respect to peptide LVAASQAALGL, chosen as reference peptide because it does not contain nucleophilic residues and hence represents an off-target of AX. Relative consumption of the adducted peptides in AX-treated HSA with respect to

Table 2 – Matched precursor ions.

Peptide adduct I.D.	Precursor ion (m/z)	Charge (z)	Predicted adducted ions	Accuracy (ppm)	Sequence	Adducted site	0.5 mg/mL amoxicillin	5 mg/mL amoxicillin
P1	628.63147	3	628.63163	0.25	$^{182}\text{LDELRLDEGK}\#\text{ASSAK}_{195}$	Lys 190	X	X
	471.72574	4	471.72554	0.4	$^{182}\text{LDELRLDEGK}\#\text{ASSAK}_{195}$	Lys 190	X	X
P2	528.74624	2	528.74626	0.04	$^{198}\text{LK}\#\text{C}^*\text{ASL}_{203}$	Lys 199	X	X
P3	480.72837	2	480.72839	0.04	$^{349}\text{LAK}\#\text{TY}_{353}$	Lys 351	O	X
P4	584.29468	2	584.29496	0.48	$^{429}\text{NLGK}\#\text{VGSK}_{436}$	Lys432	O	X
P5	591.79474	2	591.79481	0.12	$^{539}\text{ATK}\#\text{EQLK}_{545}$	Lys 541	X	X
	394.86562	3	394.86563	0.02	$^{539}\text{ATK}\#\text{EQLK}_{545}$	Lys 541	X	X
P6	780.83907	2	780.83891	–0.20	$^{542}\text{EQLK}\#\text{AVMDDF}_{551}$	Lys 545	O	X

#Amoxicilloyl adduct, *Carbamidomethyl-cysteine.

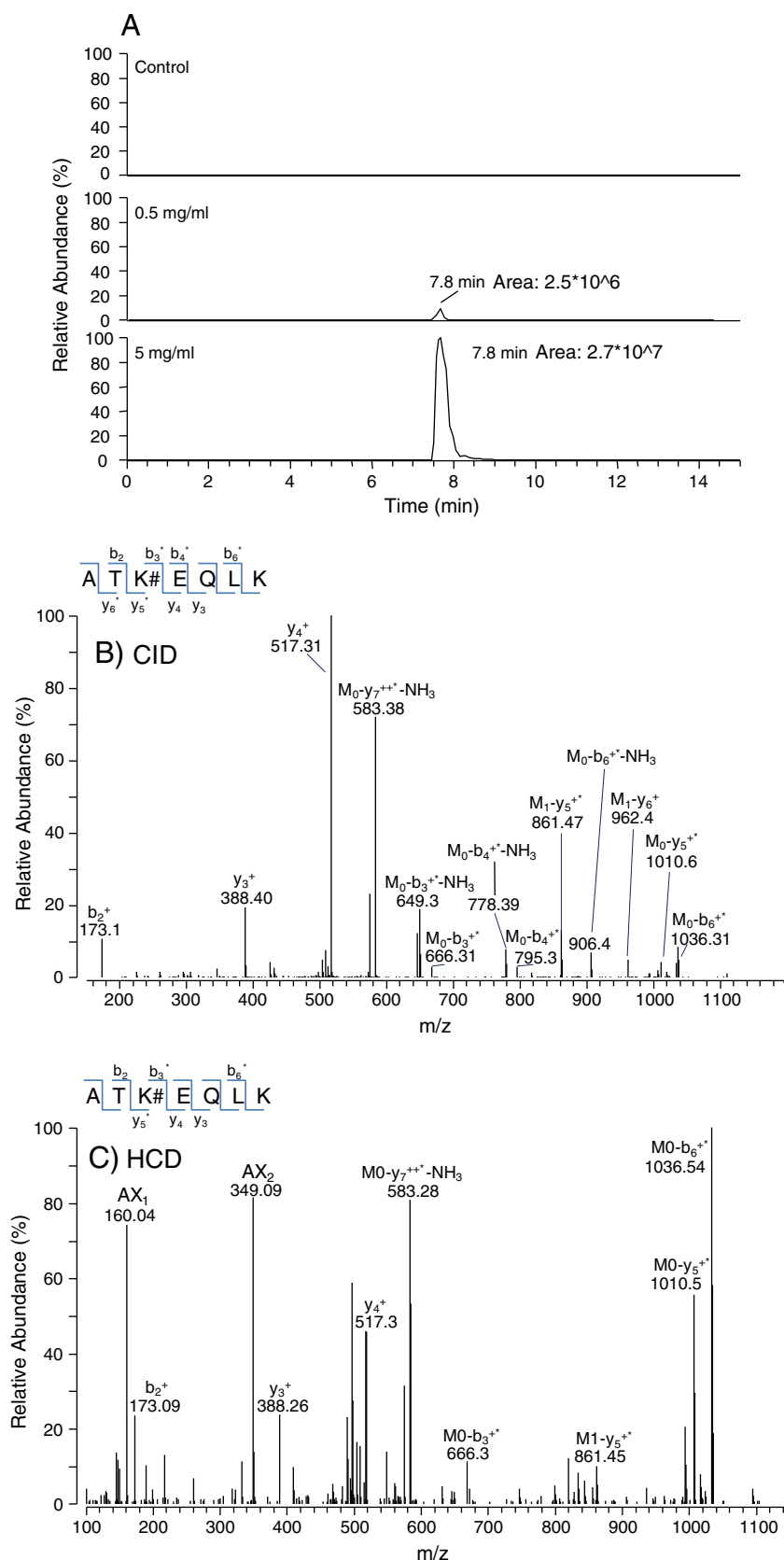


Fig. 7 – Bottom-up approach for the study of HSA modification by AX showing the identification of the adduct at Lys541. Panel A: SIC ion traces reconstituted by setting the ion at m/z 591.79474 relative to the $z=2$ of the adducted peptide $^{539}\text{ATK}\#\text{EQLK}^{545}$ as filter ion. SICs are referred to HSA incubated in the absence, and in the presence of AX 0.5 mg/mL and 5 mg/mL. Panels B and C show the MS/MS spectra of the adducted peptide $^{539}\text{ATK}\#\text{EQLK}^{545}$ in CID and HCD modes, respectively.

native HSA was determined by using the following equation: peptide consumption (%) = $100 - [(A - AX) / (A - \text{native}) * 100]$ where $A - AX$ and $A - \text{native}$ are the normalized peptide ion response of the target peptide from HSA incubated in the presence and absence of AX, respectively.

As shown in Table 3 the peptides which undergo missed cleavages due to Lys modification by AX, and in particular P18–19 (Lys190), P21–22 (Lys 199), P39 (Lys 351), P60 (Lys 541) and P61 (Lys 545), were found to be concentration-dependently consumed. In particular, when AX was added at 0.5 mg/mL the peptide P18–19 underwent the highest consumption (85%), followed by P21–22 (54%). By considering the peptide consumption as an index of reactivity the following rank can be drawn: Lys 190 > Lys 199 > Lys 541 ≈ Lys 351 > Lys 545. The consumption of the native peptide containing Lys 432 (⁴²⁹NLGK⁴³²) was not determined because it was not covered due to its small length. The method was then validated by calculating the peptide consumption of four peptides not undergoing AX adduction as determined by MS studies (negative controls). As summarized in Table 3, the consumption of the peptides considered as negative controls at either of the two AX concentrations was well below 20%, a value selected as the threshold level. At the highest AX concentration (5 mg/mL), the consumption of other peptides, namely, those containing Lys12, Lys73, Lys402 and Lys525, was higher than the threshold level. However, as the corresponding AX adducts were not found by either the precursor ion scanning approach or the manual search, their modification cannot be confirmed.

3.8. Molecular modeling

With a view to rationalizing in-depth the reactivity of the adducted lysine residues, molecular modeling studies were performed investigating the intrinsic reactivity of albumin Lys residues as well as the specific recognition between AX and the regions surrounding the modified residues. Analysis of some relevant physicochemical properties of all HSA Lys residues indicated that the reactivity of the modified Lys residues can be rationalized in terms of exposure and abundance of neutral form (Table S2). In particular, the comparison of all the 58 Lys residues of HSA emphasizes that the adducted Lys residues are characterized either by a very low basicity which render them extremely reactive regardless of their accessibility (as in the case of Lys190 and Lys 199), or by a significant accessibility which markedly facilitates their approach by AX (as seen for Lys351, Lys541 and Lys545). Yet again, Table S2 evidences that the adducted residues are surrounded by other positively charged residues whose abundance appears in line with the reported reactivity. This finding has two explanations. First, positively charged residues contribute to low basicity of the adducted lysine; second, they can act as general acid catalysts breaking down the tetrahedral intermediates.

Furthermore, docking simulations were utilized to investigate whether the region surrounding the adducted Lys residues can stabilize convenient complexes with AX so further promoting adduct formation. Firstly, it should be noted that the docking scores for the minimized complexes are in agreement with the reported reactivity thus suggesting that the capacity to suitably harbor AX is another key factor

Table 3 – Peptide consumption for the peptides undergoing modification by AX and for four selected peptides not modified by AX and considered as negative controls.

Residue	Peptide	Peptide consumption	
		AX 0.5 mg/mL	AX 5 mg/mL
Peptides undergoing AX adduction			
P18-P19	¹⁸² LDELRLDEGK ¹⁹⁰	85	97
P21-P22	²⁰⁰ C [*] ASLQKFGGER ²⁰⁹	54	92
P39	³⁵³ TYETTLK ³⁶⁰	29	63
P60	⁵⁴² EQLK ⁵⁴⁵	31	66
P61	⁵⁴⁶ AVMDDFAAFVEK ⁵⁵⁷	19	54
Peptides not modified by AX (negative controls)			
P5	⁴² LVNEVTEFAK ⁵¹	10	12
P24	²¹³ AWAVAR ²¹⁸	8	7
P37	³³⁸ HPDYSVVLRL ³⁴⁸	6	9
P42	³⁹⁰ QNC [*] ELFEQLGEYK ⁴⁰²	12	11
* Carbamidomethyl-cysteine.			

* Carbamidomethyl-cysteine.

which influences the reactivity of Lys towards AX for adduct formation. As an example, Fig. 8 illustrates the minimized complexes showing the interactions stabilizing AX in the proximity of Lys190 (Fig. 8A) and Lys199 (Fig. 8B). It is worth noting that in both complexes the adducted lysine is clearly close to the β -lactam carbonyl carbon atom with an arrangement favorable for adduct formation. In both cases, the β -lactam carbonyl oxygen atom elicits a H-bond (with Arg186 for Lys190 and with His242 for Lys199) which polarizes the carbonyl group favoring the aminolysis and mimicking the effect of the oxyanion hole of hydrolases. Moreover, Fig. 8A and B shows that the AX ionizable groups are always involved in salt bridges (with Arg186 and Glu425 for Lys190 and with Arg257 and Glu292 for Lys199) which play a pivotal stabilizing role and should minimize electrostatic interferences with adduct formation. Additional polar interactions involving the AX phenol ring characterize both complexes, which are also stabilized by apolar contacts between the dimethyl thiazolidinic ring and surrounding aliphatic residues. Besides the conducive distance between the adducted lysine and the β -lactam ring, all AX complexes obtained for the other reactive sites share similar salt bridges involving at least the AX carboxylate group, thus suggesting a third role for the basic residues surrounding the adducted lysine, namely attracting the AX negative center to reduce its interference with adduct formation.

4. Discussion

Drug allergy constitutes an important clinical problem, the molecular basis of which is not fully understood. AX is among the drugs most frequently eliciting allergic reactions. Untangling the mechanisms underlying allergy to β -lactam antibiotics will require multidisciplinary approaches. Here we have set up an in vitro model that may shed light into the

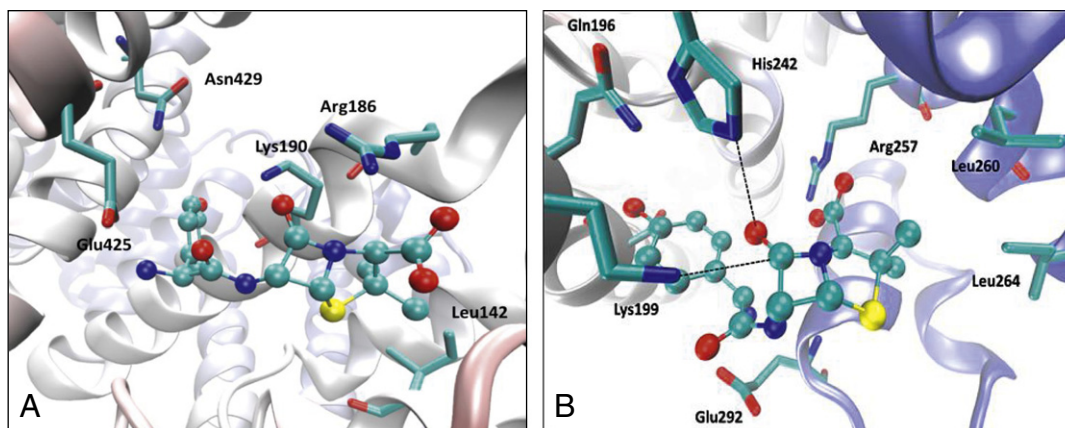


Fig. 8 – Main interactions stabilizing the AX molecule in the proximity of the target residues. The residues potentially establishing interactions with AX in the region surrounding Lys190 (A) and Lys199 (B) are shown.

process of protein haptentation. Our results show that HSA can be modified *in vitro* by concentrations of AX in the range of those expected to occur *in vivo*. Moreover, we have detected this modification by high resolution MS, proteomic and immunological methods with high sensitivity. Using these approaches we have identified other potential candidates for protein haptentation in human serum. Furthermore, we have highlighted the structural features of the protein and of the antibiotic that would be important for the interaction. These findings may help to design and interpret *in vivo* studies addressing the mechanisms of drug allergy.

Immunological methods have been previously used to detect adducts of proteins and β -lactam antibiotics [25], and HSA and transferrin were identified as target proteins for binding of ampicillin [26]. Our results show that, in addition to these major targets, other serum proteins are modified by AX. Therefore, the potential involvement of these newly identified adducts in the immune response to AX deserves further study. In particular, modification of immunoglobulins by AX will surely need to be taken into account in the context of the immune response. In addition, covalent AX binding to serum proteins including immunoglobulins will need to be considered for the interpretation of the results of immunoassays aimed at detecting anti-AX antibodies in patients.

Importantly, it is clear that the extent of AX addition is protein-specific. Transferrin appears to be modified by AX in a high proportion, which, taking into account the plasma concentration of this protein, could represent a modification 10-fold higher than that of HSA. Therefore, although HSA is the most abundant protein in serum, adducts of AX with other proteins may play an important role as providers of haptens. Interestingly, other relatively abundant serum proteins do not form detectable adducts under our experimental conditions. Therefore, factors other than protein abundance may influence AX binding.

In the early works by Yvon and co-workers, six major penicilloyl binding sites were identified in HSA, namely Lys 190, 195, 199, 432, 541 and 545 [2,3]. Recently, using proteomic approaches, the formation of adducts between HSA and

flucloxacillin, piperacillin and benzypenicillin has been characterized [4–6]. In this case, the MS approach applied consisted of generating multiple reaction monitoring (MRM) transitions, specific for drug-modified peptides by selecting the m/z values calculated for all the possible peptides with a missed cleavage at a Lys residue plus specific fragments such as that at m/z 160 attributed to the cleaved thiazolidine ring, combined with MS/MS sequencing of the identified peptides. Interestingly, the nature of the residues modified appears to vary widely between subjects, and adducts with residues Lys190, 195, 432 and 541 have been detected in the plasma of patients treated with piperacillin, whereas multiple adduct-bearing peptides have been detected in patients treated with benzylpenicillin or flucloxacillin. Under our experimental conditions, both covalent and non-covalent modes of interaction of AX with HSA were detected, the latter being evidenced by its reversal by extensive washing of AX-incubated HSA. Our data indicate that the covalent coupling takes place by amide bond formation between primary amines from Lys residues and the carboxylic group from the β -lactam ring, to afford the amoxicilloyl moiety. This is in agreement with what has been described for other penicillins [27] and it is also supported by data from the NMR characterization of an AX-butylamine conjugate (Montañez, M.I., personal communication). The major sites for addition of AX at low concentrations in isolated HSA were Lys 190, 199 and 541. Modeling studies were able to rationalize the marked reactivity of the targeted Lys residues confirming that their reactivity can be vastly influenced by the surrounding microenvironment and that residue accessibility, albeit relevant, cannot be seen as the sole factor governing the reactivity. The microenvironment can enhance the intrinsic reactivity of the Lys residues and it can contribute to the binding of AX through a sort of recognition process which can stably constrain AX in a pose conducive to adduct formation thus explaining the fine selectivity of the modification sites. Nevertheless, at high AX concentrations other sites of modification were found. Moreover, the modification of other residues *in vivo* cannot be excluded. Indeed, we

noted that the main HSA sites modified by low AX concentrations when incubation was carried out in complete serum were Lys190 and Lys 432. The differences found in the residues modified by AX depending on the incubation conditions, either purified HSA or complete serum, suggest that the protein context may influence the nature of the adducts formed. It should be noted that β -lactam-peptide adducts will be formed both in non-allergic subjects and in subjects developing allergic reactions, the factors determining the development of allergy not being currently understood. It would be interesting to assess whether changes in serum composition occurring in pathophysiological situations may influence protein haptation.

The so-called danger signals are thought to play an important role in allergic reactions. It has been shown that sulfamethoxazole forms adducts with proteins in antigen presenting cells and that the amount of adducts formed increases when cells are exposed to bacterial endotoxin, cytokines or a variety of inflammatory or stress-inducing stimuli [28]. This underlines the importance of concomitant factors for protein haptation. It is known that HSA is the target of various modifications by reactive lipids generated under situations of inflammation or oxidative stress, including 4-hydroxy-2-nonenal and cyclopentenone prostaglandins [16,29,30]. Moreover, HSA is sensitive to various oxidative modifications [31]. It has been reported that HSA modification may drastically alter its binding properties and even its immunogenic and proinflammatory potential [32]. In preliminary experiments we have observed that pretreatment of HSA in vitro in the presence of certain oxidative agents alters its modification by amoxicillin, as evidenced by immunological detection (Ruiz-Abánades et al., unpublished observations). Further studies are needed to assess the biochemical basis of these effects.

In summary, this study sets the basis for the highly sensitive detection and identification of AX–protein adducts. Future studies will allow the identification of AX binding sites in other proteins as well as in vivo thus broadening the information on protein haptation by AX and hopefully contributing to the development of diagnostic tools and to the characterization of the pathogenic mechanisms of allergic reactions towards AX.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2012.09.030>.

Acknowledgments

We are indebted to MJ Carrasco for valuable technical help and to Drs. A.B. Blázquez, A. Aranda and C. Mayorga for help with preliminary experiments. The insightful contributions of Drs. M. Blanca, MI Montañez and FJ Cañada are gratefully appreciated. This work was supported by grants SAF2009-11642 from Ministerio de Economía y Competitividad (financed in part by “Plan E”) and RETIC RD07/0064/0007 from ISCIII to DPS, by grant Project PRIN 2009 Z8YTYC to GA and by grants from ISCIII PI-0545-2010, RETIC RD07/0064/0000, and Junta de Andalucía CTS06603 and PS09/01768 to MJT. The collaboration and mobility between DPS and GA laboratories have been supported by EU COST Action CM1001.

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