

RESEARCH ARTICLE

Analysis of electrochemical and liver microsomal transformation products of lasalocid by LC/HRMS

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Rationale: Lasalocid (LAS), an ionophore, is used in cattle and poultry farming as feed additive for its antibiotic and growth-promoting properties. Literature on transformation products (TP) resulting from LAS degradation is limited. So far, only hydroxylation is found to occur as the metabolic reaction during the LAS degradation. To investigate potential TPs of LAS, we used electrochemistry (EC) and liver microsome (LM) assays to synthesize TPs, which were identified using liquid chromatography high-resolution mass spectrometry (LC/HRMS).

Methods: Electrochemically produced TPs were analyzed online by direct coupling of the electrochemical cell to the electrospray ionization (ESI) source of a Sciex Triple-TOF high resolution mass spectrometer. Then, EC-treated LAS solution was collected and analyzed offline using LC/HRMS to confirm stable TPs and improve their annotation with a chemical structure due to informative MS/MS spectra. In a complementary approach, TPs formed by rat and human microsomal incubation were investigated using LC/HRMS. The resulting data were used to investigate LAS modification reactions and elucidate the chemical structure of obtained TPs.

Results: The online measurements identified a broad variety of TPs, resulting from modification reactions like (de-)hydrogenation, hydration, methylation, oxidation as well as adduct formation with methanol. We consistently observed different ion complexations of LAS and LAS-TPs (Na^+ ; 2Na^+ K^+ ; NaNH_4^+ ; KNH_4^+). Two stable methylated EC-TPs were found, structurally annotated, and assigned to a likely modification reaction. Using LM incubation, seven TPs were formed, mostly by oxidation/hydroxylation. After the identification of LM-TPs as Na^+ -complexes, we identified LM-TPs as K^+ -complexes.

Conclusion: We identified and characterized TPs of LAS using EC- and LM-based methods. Moreover, we found different ion complexes of LAS-based TPs. This knowledge, especially the different ion complexes, may help elucidate the metabolic and environmental degradation pathways of LAS.

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1 | INTRODUCTION

The drug lasalocid (LAS) belongs to the class of ionophore antibiotics and is used to control coccidiosis. Furthermore, LAS acts against gram-positive mastitis pathogens¹ and protects cells against multiple bacterial toxins.² In accordance with other ionophore antibiotics, an exhibited potent anticancer activity for LAS was described.^{3–5} Overall, LAS is commercially used as coccidiostat (feed additive) and growth promoter in cattle and poultry farming. The biotransformation process of LAS is well investigated; LAS is mainly metabolized in the liver.^{6–8} The quantity and structure of the resulting metabolites depend on the investigated animal. For example, in chicken liver, LAS remained unchanged and also mono- and multi-hydroxylated metabolites were excreted.⁷ Hydroxylation was determined as the main biotransformation pathway of LAS in animals, while additional information on structural details of the LAS-derived metabolites is not known. In addition to animal metabolism, environmental degradation of secreted compounds is of high significance for veterinary drugs, and several studies investigate the degradation of LAS under different conditions.^{9–11} However, degradation products were not elucidated.

The structure of LAS is presented in Figure 1. Polyether ionophore antibiotics are known to form alkali metal complexes. LAS, specifically, forms complexes with monovalent (Li^+ ; K^+ ; Na^+) and divalent cations (Ca^{2+} ; Mg^{2+}) and also with ammonium salts (NH_4^+).¹² LAS-potassium complexes are more stable than LAS-sodium complexes.^{13,14} In general, the formation and stability of different LAS-ion complexes is dependent on solvent polarity and ion concentration.^{14,15}

Transformation product (TP) is a generic term that comprises metabolites (formed by biotransformation processes in living organisms) and degradation products (formed by various environmental processes).^{16,17} A simple method (in relation to the complexity) to simulate metabolic reactions is the cell-free incubation with human liver microsomes (HLM) or rat liver microsomes (RLM).¹⁸ After incubation, metabolites can be analyzed using appropriate detection methods like liquid chromatography high-resolution mass spectrometry (LC/HRMS). HRMS allows an accurate mass determination of TPs resulting in molecular formulas, containing information to infer the causal modification reactions.¹⁹ The combination of electrochemistry (EC) with mass spectrometry (MS) offers an approach to simulate redox processes.^{20–22} Depending on the chosen potential polarity oxidation or reduction reactions are induced.²³ Two analytical strategies to analyze EC-derived TPs exist,

online and offline mode. The direct (online) coupling of EC outlet and the electrospray ionization (ESI) source of an MS enables the detection of stable and reactive (short-living) TPs because of the short time difference between the generation of the TPs and detection. In offline mode, the effluent after the EC treatment is collected and analyzed later using LC/HRMS. This allows one to include a separation step but enables predominantly the detection of stable TPs. Several studies showed that EC is capable of mimicking selective redox processes of the cytochrome-P450 (CYP450) enzyme family. These include primarily functionalization of the phase I metabolism like oxidation, reduction, hydrolysis, and isomerization.²² Jurva et al.²⁴ investigated the potential of EC to mimic oxidation performed by CYP450 and described which oxidation reactions are simulated. The application of EC is constantly increasing, its usage for pharmaceutical research is well established, and as an example Szultka-Mlynska and co-workers presented that EC-generated TPs of six chemotherapeutics are also found by metabolism tests with HLM or RLM. In addition, some TPs are found in urine samples (in vivo tests).²⁵ After metabolic reactions, a good overlap between EC-generated TPs and photochemical TPs was presented for the veterinary drug sulfadiazine by Hoffmann et al.²⁶ Further application areas are the reduction of disulfide bonds of proteins or peptides using EC, described by Zhang and co-workers.²⁷ These selected examples showed the broad range of possible approaches using EC, and the pure instrumental setup of EC/MS shows some benefits especially, but not exclusive in relation to, metabolism tests with liver microsomes. The EC investigation is time and cost saving (no incubation time, no costly enzymes, or no microsomes are needed). The absence of the matrix of the microsomes leads to a better identification of the TPs, and the production of TPs can be easily scaled up to reach mg quantities.

The aim of the study was to simulate TPs of LAS by electrochemistry and liver microsome incubation using HRMS for identification. The focus was placed on the suspected target analysis of the HRMS data of measured TPs to obtain information on compound formula, transformation processes (modification reaction), and compound structure.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Lasalocid sodium (purity 98%) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). KH_2PO_4 was obtained from Chemsolute (Renningen, Germany), and K_2HPO_4 from Carl Roth (Karlsruhe, Germany). NADPH tetrasodium salt was purchased from Carl Roth (Karlsruhe, Germany). Potassium biphthalate was obtained from Fluka Chemika (Buchs, Switzerland). Acetonitrile and methanol were purchased from Chemsolute (Renningen, Germany). Ammonium formate was obtained from Fluka Chemika (Buchs, Switzerland) and formic acid from Merck (Darmstadt, Germany). Ultrapure water was produced by a Purelab Flex 2 system, ELGA Veolia Water

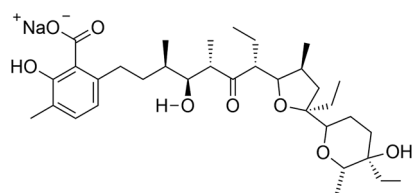


FIGURE 1 Chemical structure of lasalocid (LAS)

Technologies (Celle, Germany). The purity of all standard chemicals was PA grade. Acetonitrile and methanol were of LC–MS grade.

2.2 | Electrochemical measurements

An electrochemical reactor (ECR) was used to investigate the electrochemical conversion of LAS. The system consisted of a ROXY™ potentiostat (Antec Scientific, Zoeterwoude, The Netherlands) and an electrochemical flow-through cell equipped with a glassy carbon (GC) working electrode (12 × 30 mm), a HyREF™-reference electrode (Pd/H₂), and a titanium auxiliary electrode. The applied potential was ramped between 0 and 2.5 V with a scan rate of 10 mV/s controlled by the potentiostat. The instrument was controlled via Dialogue Elite software, version 2.0.0.81 (Antec Leyden). A manufacturer-provided pulse cleaning program was used before each measurement to activate the GC-working electrode. A Legato 110 dual rate system syringe pump (KD Scientific, Holliston, MA, USA) was used to pump the LAS-solvent mixture consisting of 20 μM LAS in methanol:acetonitrile:water (3:1:1, v/v/v) with 1 mM ammonium formate through the cell with a flow rate of 40 μL/min. The ECR was combined with the ESI source of a TripleTOF 6600 quadrupole time-of-flight (QTOF) mass analyzer (Sciex, Darmstadt, Germany). The online coupling enabled the recording of mass voltammograms, and the measurements were performed in triplicate. Control measurements were performed by using the solvent without the analyte. After the online measurements, aliquots were collected in a HPLC vial from the EC cell and were used for further offline LC/HRMS measurements.

2.3 | Microsomal assay

RLMs were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). The RLMs were prepared from Sorague Dawley male rats with a protein concentration of 20 mg/mL and a CYP450 content of 319 pmol/mg protein. The total protein content and CYP450 concentrations were provided by the manufacturer. HLMs were purchased from Thermo Fisher Scientific. The HLMs were prepared from 20 human female donors with a concentration of 20 mg/mL and a CYP450 specific content of 309 nmol/mg protein (concentration provided by the manufacturer). Incubations with liver microsomes (LM) were carried out in a volume of 200 μL. Microsomes (1.0 mg/mL microsomal protein) were mixed with 0.1 M potassium phosphate buffer (pH 7.4) and 0.01 mM MgCl₂. First, a pre-incubation was carried out for 5 min at 37°C, then LAS (6.25 μmol/L) was dissolved in acetonitrile (total amount < 3%) and 0.6 mM NADPH was added to the mixture to start the enzymatic reaction (incubation time: 90 min, 37°C, centrifuged at 800 rpm). To stop the reaction, 50 μL of acetonitrile (–20°C) was added, and the sample was mixed thoroughly for 30 s (final LAS concentration: 5 μM). Afterward, the incubation mixture was centrifuged at 12 000 rpm. The supernatant was analyzed using LC/HRMS (Agilent Technologies, Waldbronn,

Germany/Sciex, Darmstadt, Germany). Control incubations, where the amount of NADPH was replaced by the potassium buffer, were performed in duplicate. The reaction was performed in triplicate.

2.4 | Liquid chromatography

The HPLC was performed on an Agilent 1290 Infinity II (Agilent Technologies), consisting of a 1290 Infinity II multisampler, a 1290 Infinity II flexible pump, a 1260 Infinity II diode array detector HS, and a 1290 Infinity II multicolumn thermostat. The liquid chromatograph was connected to the ESI source of the TripleTOF 6600 quadrupole time-of-flight (QTOF) mass analyzer (Sciex). The system was controlled by Analyst TF1.8.0 (AB Sciex) software. The chromatographic determination was performed on an analytical column (ZorbaxEclipse Plus C18, particle size 1.8 μm, pore size 95 Å, 50 × 2.1 mm [Agilent Technologies]). The column oven was set to 25°C. A mobile phase of (A) H₂O + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid was used for separation of the different samples. The injection volume was 2 μL, and the flow rate was 0.8 mL/min. Different gradient programs were used for the separation. The gradient program for the separation of the EC-generated reaction mixture started with 50% of B, within 2 min it was raised to 90%. After 5 min it was decreased to 50%, and the column was recalibrated for 3 min. The gradient for the microsomal assay samples started with 50% of B, within 0.5 min it was raised to 90%. After 3.5 min it was decreased to 50%, and the column was recalibrated for 3 min.

2.5 | Mass spectrometric conditions and data analysis

A TripleTOF 6600 quadrupole time-of-flight (TTOF) mass analyzer (Sciex) was used. The qTOF system was controlled by Analyst TF1.8.0, including data processing. The ECR/HRMS and LC/HRMS runs were performed in positive ionization mode with following parameters: gas temperature, 400°C; ion source gas, 1 50 L/min; ion source gas 2, 55 L/min; curtain gas, 45 L/min; and ion spray voltage, +5500 V. For the EC/HRMS coupling, a collision energy of 30 V, a declustering potential of 80 V, and a mass range from 100 to 800 Da (full scan) were used. For the LC/HRMS measurements, a collision energy of 10 V and a declustering potential of 80 V were used. For the MS/MS experiments, the collision energy was 85 V for LM samples and 70 V for EC samples. The collision energy spread was 20 V, and the declustering potential was 80 V. The mass range was from 100 to 900 Da. For MS/MS experiments an information-dependent acquisition (IDA) was included. The mass spectrometer was tuned according to the manufacturer instructions before any measurement to obtain mass accuracy of <2 ppm. Data were processed by SciexOS and using in-house scripts of the statistical working environment R.²⁸ Sum formulas were calculated for the resulting measured ion mass (nominated to *m/z* of LAS), allowing

only C, H, O, Na, K, and N as potential elements and using ± 5 ppm deviation as a threshold. If more than one sum formula proposal remained, a fit of the isotopomer distribution (sigma value) was used to rank candidates. In addition, the modifications in relation to LAS were calculated and divided into gain and loss of atoms. Then, the best proposal was selected with a focus on reasonable gain and loss groups. MS/MS spectra, which were acquired in IDA mode, allowed a further inference of structural confirmation of the precursor molecule. The software Origin 2019 (OriginLab, Northampton, MA, USA) was used for the graphical representation of the mass spectra.

3 | RESULTS AND DISCUSSION

3.1 | Online EC

We initially optimized conditions of the ECR for reproducibility. Inside the EC cell an electric potential was applied, and TPs of LAS were generated. The electric potential was ramped from 0 to 2.5 V (oxidation reaction). Different experimental conditions were tested, and detailed information is presented in electronic supplementary material (Table S1 [supporting information]). The chosen parameters (best reproducibility) were achieved using a GC working electrode and a solvent mixture consisting of methanol:acetonitrile:water (3:1:1, v/v/v) with 1 mM ammonium formate.

The advantage of using a direct coupling of ECR outlet with ESI/HRMS is the possibility to record the accurate masses of TPs and determine potential molecular formulas. In MS detection (positive ESI mode), the dominant molecular ion of LAS was the protonated sodium adduct (m/z 613.371). In addition, the sodiated-sodium salt was observed (m/z 635.356). Both m/z traces are displayed in Figure 2, where one full-scan cycle from 0 up to 2.5 V and back to 0 V is shown. With increasing potential, the m/z trace of LAS-sodium $[(M-H + Na) + H]^+$ (black) and LAS-disodium $[(M-H + Na) + Na]^+$

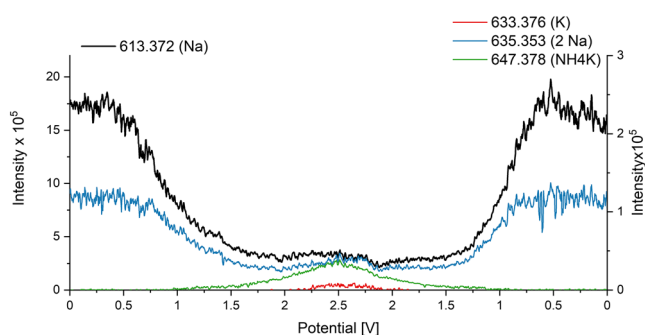


FIGURE 2 Spectrum of electrochemical oxidation of lasalocid. Mass traces (m/z) of different ion complexes of LAS: $[(M-H + Na) + H]^+$ (black), $[(M-H + Na) + Na]^+$ (blue), salt $[(M-H + NH_4) + K]^+$ (green), and $[(M-H + K) + H]^+$ (red). All mass traces are shown in dependence to the applied oxidation potential ramped from 0 to 2.5 to 0 V (one cycle) [Color figure can be viewed at wileyonlinelibrary.com]

(blue) decreased. The evaluation of m/z traces with maxima at 2.5 V confirmed additional ion complexes of LAS. At around 1 V a potassiated-ammonia salt $[(M-H + NH_4) + K]^+$ of LAS with m/z of 647.378 (green) was observed. At even higher potential above 2 V the protonated potassium salt $[(M-H + K) + H]^+$ with m/z of 633.376 (red) was observed. The detected protonated potassium salt contained four additional hydrogen atoms. In general, with increasing potential many additional m/z -traces could be detected. Given that LAS forms several ion complexes (sodium, potassium, and ammonium) we could expect TP-generating modifications to lead to redundant or parallel products.

A comprehensive overview of the TPs detected in online experiments is given in Table 1 and Table S2 (supporting information). The online EC-TPs were ordered by their modification in relation to the origin LAS molecule and divided by their complexed cation(s). In addition, the occurrence of TPs at different potential ranges was given, 1.0 V (a), 1.5 V (b), 2.0 V (c), and 2.5 V (d). Some online EC-TPs were observed over the whole potential range, whereas others were observed only at a specific potential. The highest number of TPs was observed at the highest potential (2.5 V). Based on the accurate mass of the online EC-TPs, sum formulas were calculated, resulting in information about the ion complex and modification reactions. The identified online EC-TPs of 2.5 V are displayed in Figure 3 (additional plots for 1.0, 1.5, 2.0 V are given in Figures S1–S3 [supporting information]). The plot contains the m/z values as x-axis, and the y-axis is ordered by the number of carbon and oxygen atoms of the identified sum formulas. In addition to aforementioned detected ion complexes of LAS (NaH, 2 Na, KH, and NH_4K) the sodiated-ammonia complex is found. This complex shows the loss of three hydrogen atoms in relation to the protonated sodium complex. The different complexes were color coded. Most m/z values were identified as sodium complexes (blue). Seven predominant modification reactions were identified, and related m/z values were connected by color coded lines, respectively. Typical EC modification reactions like (de-)hydrogenation ($-/+H$), oxidation ($+O$) as hydroxylation ($+OH$), (de-)hydration ($-/+H_2O$), and (de-)methylation ($-/+CH_3$) were identified. The solvent mixture contained 60% methanol. It is known from the literature that adduct formation with methanol occurs at a double bond or aromatic system within the EC cell.^{29–31} The single methanol accumulation was identified nine times. The double methanol adduct formation occurred thrice. Also, the combination of methylation and methanol adduct formation was found thrice. As discussed earlier, the presence of different LAS-ion complexes transformed by the same modification reaction led to an increased number of m/z traces. Consider the four ion complexes in Figure 2 (m/z 613.371, 633.379, 635.356, 647.381), with two of them showing a double methanol adduct formation (613.371 to 673.393 and 635.356 to 695.374) and the other two showing the methylation in combination with methanol adduct formation (633.379 to 677.401 and 647.381 to 691.410). Most of the identified m/z values could be attributed to the ion-LAS complex. The sodiated-ammonia-LAS complex did not exist, only the oxidated one ($+O$, m/z 643.371).

TABLE 1 Comparison of TPs found in online EC-MS experiments. The modification is given in relation to the parent LAS molecule

| Modification/ adduct | $[(M-H + Na) + H]^+$ | $[(M-H + Na) + Na]^+$ | $[(M-H + NH_4) + Na]^+ (-3H)$ | $[(M-H + NH_4) + K]^+ (+H)$ | $[(M-H + K) + H]^+ (+4H)$ | | | |
|-------------------------------------|--|--|---|---|---|------|--|------|
| M-CH ₄ O ₂ | | | | C ₃₃ H ₅₄ O ₆ NK | _bc_ | | | |
| M-CH ₂ O | C ₃₃ H ₅₂ O ₇ Na | ab_d | C ₃₃ H ₅₂ O ₇ NNa | ___d | C ₃₃ H ₅₆ O ₇ NK | _b_d | | |
| M-CO | C ₃₃ H ₅₄ O ₇ Na | abc_ | C ₃₃ H ₅₄ O ₇ NNa | ___d | | | | |
| M-H ₂ O | C ₃₄ H ₅₂ O ₇ Na | a___ | | | | | | |
| M-CH ₂ | C ₃₃ H ₅₂ O ₈ Na | a___ | | | | | | |
| M-C | C ₃₃ H ₅₄ O ₈ Na | _b_d | C ₃₃ H ₅₄ O ₈ NNa | abcd | | | | |
| M-2H | C ₃₄ H ₅₂ O ₈ Na | abcd | | | | | | |
| LAS | C ₃₄ H ₅₄ O ₈ Na | abcd | C ₃₄ H ₅₃ O ₈ Na ₂ | Abcd | C ₃₄ H ₅₈ O ₈ NK | _bcd | C ₃₄ H ₅₈ O ₈ K | ___d |
| M + CH ₂ | | C ₃₅ H ₅₅ O ₈ Na ₂ | ___d | | | | | |
| M-H + O | C ₃₄ H ₅₃ O ₉ Na | abcd | | | | | | |
| M + O | C ₃₄ H ₅₄ O ₉ Na | a___ | C ₃₄ H ₅₃ O ₉ Na ₂ | ___d | C ₃₄ H ₅₄ O ₉ NNa | Abcd | | |
| M + H ₂ O | C ₃₄ H ₅₆ O ₉ Na | _bcd | | | C ₃₄ H ₅₆ O ₉ NNa | __cd | | |
| M + H ₂ O ₂ | | | | | C ₃₄ H ₅₈ O ₁₀ NNa | ___d | | |
| M + C-O | | | | | | | C ₃₅ H ₅₈ O ₇ K | _bcd |
| M + CO | C ₃₅ H ₅₄ O ₉ Na | _bcd | | | | | | |
| M + CH ₂ O | | C ₃₅ H ₅₅ O ₉ Na ₂ | __cd | | | | | |
| M + CH ₄ O | C ₃₅ H ₅₈ O ₁₀ Na | ___d | | | C ₃₅ H ₅₈ O ₁₀ NNa | _bcd | | |
| M + C ₂ H ₂ | | | | | | | C ₃₆ H ₆₀ O ₈ K | ___d |
| M + C ₂ H ₄ O | C ₃₆ H ₅₈ O ₁₀ Na | abcd | C ₃₆ H ₅₇ O ₁₀ Na ₂ | __cd | C ₃₆ H ₆₂ O ₉ NK | ___d | C ₃₆ H ₆₂ O ₉ K | ___d |

Notes: The different columns show the different cation complexes. M represents the LAS molecule. The abcd code represents the occurrence at different potential ranges: 1.0 V (a), 1.5 V (b), 2.0 V (c), 2.5 V (d).

Abbreviations: EC-MS, electrochemistry-mass spectrometry; LAS, lasalocid; TP, transformation products.

3.2 | Offline EC

After the online measurements were made, the EC reaction mixture was collected and analyzed using LC/HRMS to obtain information about stable EC-TPs and to use the MS/MS-data to predict structures. LC/HRMS analyses led to two offline EC-TPs (see Table 2; Table S2 [supporting information]) with the same m/z of 624.447. The resulting sum formula of C₃₅H₆₂O₈N corresponds to a combination of methylation, di-hydrogenation, and an ion exchange between sodium and ammonium. In accordance with the online results, different ion complexes could be identified within the MS spectra. In particular, the potassium complex and the sodiated-ammonia complex were clearly visible. In addition, the spectra of the offline EC-TP-2 shows the sodium complex. MS/MS data were evaluated to obtain more information about the structure. The MS/MS spectra of both offline EC-TPs were identical, and a representative spectrum, together with associated fragments of LAS, is displayed in Figure 4. The ESI fragmentation pathway of LAS was previously described by Lopes et al.³² Here, the evaluation of the MS/MS fragments indicates that the modification (methylation and hydrogenation) occurred at the terminal end of LAS (aromatic ring, marked in Figure 4).

3.3 | Comparison of online and offline EC

For LAS, two offline EC-TPs and numerous online EC-TPs were obtained. Different modification reactions (+/-H, +H₂O, +CH₂, +O, +CH₂O) were observed in the EC method. Alkali metal-ion complexes of Na⁺; 2Na⁺ K⁺; NaNH₄⁺; KNH₄⁺ were identified, especially for the online EC-TPs. The identification of online EC-TPs using ESI-MS detection is challenging, because in-source fragmentation can occur during ionization of the ESI source and similar modification reactions can occur. Possible ionization reactions are, for example, oxidation, reduction, or (de-)hydration after ion-adduct formation. The formed ion complexes by ESI ionization of LAS were well investigated, and a wide variety were described, starting from the labile [M + H]⁺-complex to protonated alkali metal-ion complexes [(M-H + X) + H]⁺ (X: e.g., Na, K, Li) to two alkali metal-ion complexes [(M-H + X) + X]⁺. Cluster ions with ammonia or acetonitrile were also formed, and the ammonia complex and protonated complex were found to be labile.³³ We noticed a systematic loss of hydrogen atoms in the ammonia-ion complexes and gain of hydrogen atoms in potassium complexes. Potentially, complexes were stabilized by (de-)hydrogenation. For differentiation, if the modification occurs inside the ECR or by ionization of the ESI source, the potential-dependent intensity course

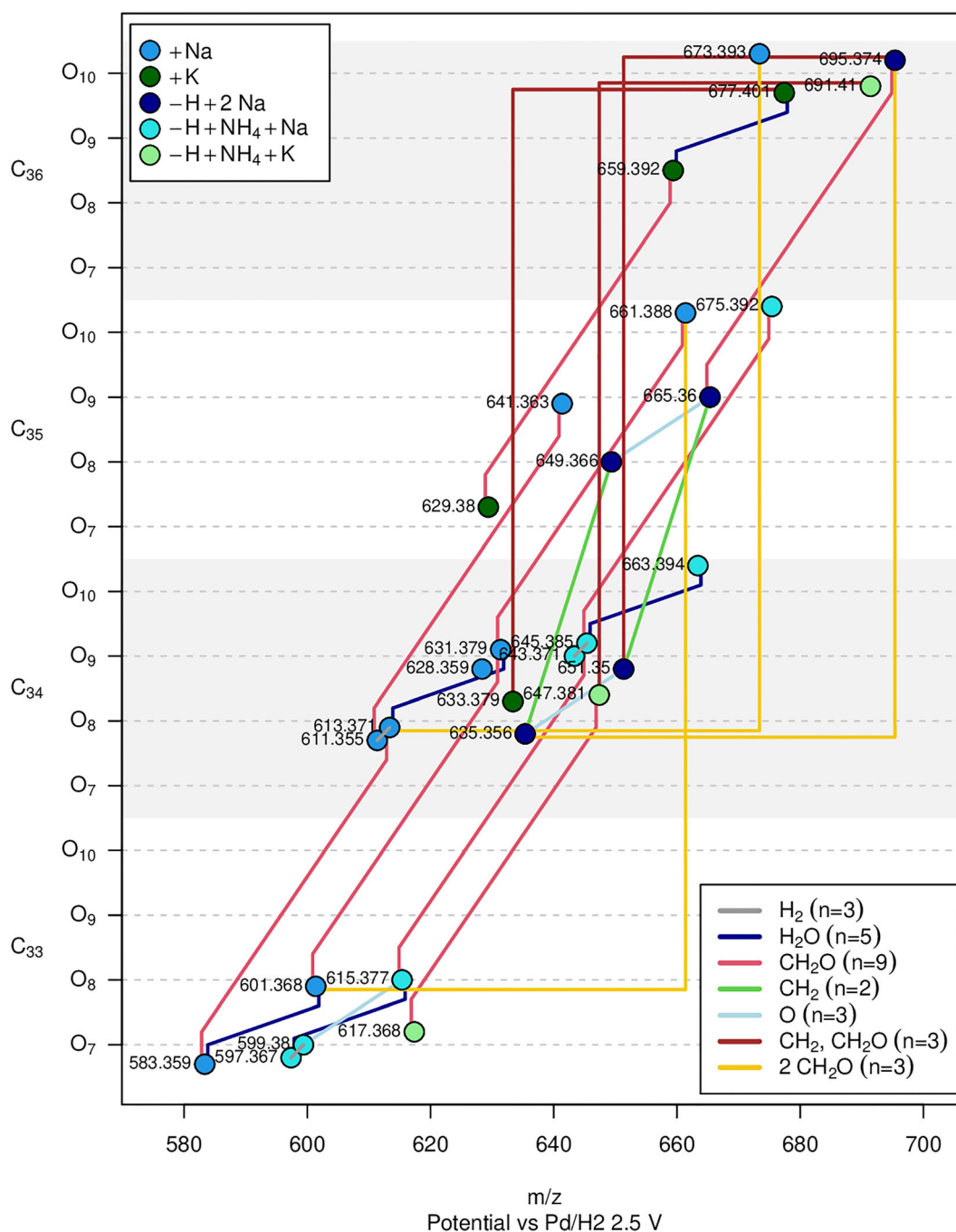


FIGURE 3 The identified online-detected transformation products (TPs) of Lasalocid (LAS) generated at a potential around 2.5 V are given. The TPs are ordered as a mass axis (x-axis). The y-axis orders the online electrochemistry (EC)-TPs according to the C and O atom number of the calculated sum formulas. The color of the TP dot is visualizing the complexed-cation; see legend top left. Identified modification reactions were displayed at the legend bottom right; the concerned TPs were connected by a line [Color figure can be viewed at wileyonlinelibrary.com]

of the online EC-TPs will be considered. If the intensity course correlates well with the intensity course of LAS, the modification will occur during ESI ionization process; for example, see Figure 2: the disodium complex (m/z 635.353) has a potential-dependent intensity course similar to LAS (m/z 613.372). If the online-formed TPs occur at a small potential range with a different potential-dependent intensity course, they are probably formed by the applied potential inside the ECR (e.g., see Figure 2: m/z 633.376). Furthermore, starting from a

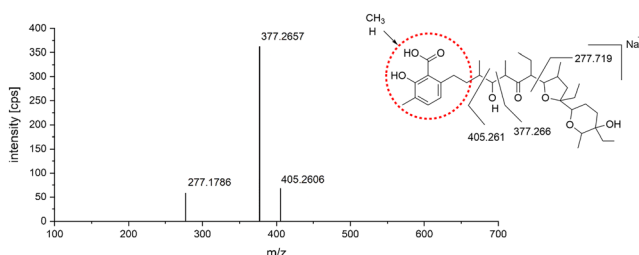
built online EC-TP, another EC-TP can also be built during the ESI ionization process, and then both EC-TPs show a similar potential-dependent intensity course. The absence of most m/z traces at the offline measurements can be justified by a low stability of the TPs and loss of the adduct-formation during storage. As a consequence, LAS or the offline EC-TPs are detected as products of reconversion processes. The ammonia adducts of offline EC-TPs were not detected in online experiments. Figure 5 shows proposed generation routes of

TABLE 2 HRMS data of detected offline EC-TPs of LAS as ammonia adducts

| Offline EC-TP | <i>m/z</i> calc. | Retention time (s) | Sum formula | Modification reaction | Further adducts |
|---------------|------------------|--------------------|---|--------------------------|---|
| 1 | 624.448 | 161.49 | C ₃₅ H ₆₂ O ₈ N | -Na; + CH ₈ N | 645.376 - C ₃₅ H ₅₈ O ₈ K 652.478 - C ₃₅ H ₆₇ O ₈ NNa |
| 2 | 624.448 | 164.90 | C ₃₅ H ₆₂ O ₈ N | -Na; + CH ₈ N | 629.402 - C ₃₅ H ₅₈ O ₈ Na 645.376 - C ₃₅ H ₅₈ O ₈ K 652.478 - C ₃₅ H ₆₇ O ₈ NNa |
| LAS | 613.372 | 181.66 | C ₃₄ H ₅₄ O ₈ Na | | 608.416 - C ₃₄ H ₅₈ O ₈ N 637.451 - C ₃₄ H ₆₄ O ₈ NNa |

Notes: Further identified adducts were given. The mass determination error for the calculation of the sum formula was ± 5 ppm.

Abbreviations: EC-MS, electrochemistry-mass spectrometry; LAS, lasalocid; TP, transformation products.

**FIGURE 4** MS/MS spectrum of offline EC-TP-1 (*m/z* 624.447).

The structure of lasalocid (LAS) is presented for comparison, together with associated fragmentation. Methylation and dehydrogenation are proposed to occur at the red-marked circle [Color figure can be viewed at wileyonlinelibrary.com]

the two offline EC-TPs based on rearrangement by selected online EC-TPs. The online EC-TPs that show methylation or adduct formation with methanol serve as a starting point. The loss of hydrogen, methanol, or water occurs during storage. Both offline EC-TPs show the same modification reaction; only the retention time is different. The offline EC-TPs can be constitutional (structural) isomers as well as stereoisomers to each other. The offline EC-TPs were detected as ammonia-ion complex(es), but further ion complexes are visible at the mass spectra, which include the sodium complex, the sodiated-ammonia complex, and the potassium complex. All ion complexes show the same retention time, so the formation of different ion complexes is proposed by ESI ionization effects. The EC-treated solution contained 1 mM of ammonium formate, which is why ammonia complexes occurred with the highest intensity. The LAS solution was prepared from a standard provided as a sodium complex and using solvents containing traces of potassium. Literature studies of LAS complexes showed that potassium has a higher affinity than sodium, and potassium complexes were easily detected.

Ion exchange in TPs simulated by EC of the ionophore antibiotics monensin³¹ and salinomycin³⁴ is already known. Monensin and its TPs were detected as sodium ion and as ammonium ion. Further metal ions were not detected. This is in accordance with literature, where sodium complexes of monensin were mentioned with highest affinity/stability. Methanol adduct formation was identified as the modification reaction of an EC-TP of monensin. For salinomycin, TPs were detected as sodium ions and sodiated-ammonia ions.

3.4 | HLM/RLM assay

LAS was incubated separately with HLM and RLM, and TPs were analyzed using LC/HRMS. The HLM data showed seven HLM-TPs, listed in Table 3. The results of the RLM data showed a lower conversion rate and fewer TPs (three) (Table 3; Table S2 [supporting information]). All three RLM-TPs were also found in HLM incubation. In conclusion, the RLM incubation is dispensable because it has a lower conversion rate (intensity and number of TPs) and produces redundant TPs that are found in HLM. Exact masses were used to determine sum formulas and modification reactions as described earlier. The most frequent modification reaction is oxidation (+O) leading to four TPs. Oxidation can be either hydroxylation (+OH) or epoxidation (+O). Dehydrogenation (-H) and dehydration (-H₂O) were also found as modification reactions. After the changes were visible at the molecule structure by redox processes, changes in the complexation with cations were visible. Some LAS-TPs were disodium complexes, and for some TPs the sodium was exchanged by potassium. The HLM-TP-6 shows the highest intensity, and during evaluation two modification reactions with the same intensity were observed, first oxidation (*m/z* 629.367) and second the sodium was exchanged by potassium (*m/z* 645.341). The Na-K exchange occurred in combination with oxidation for all four TPs: HLM-TP-1 showed di-oxidation, HLM-TP-2 showed oxidation and di-dehydrogenation. HLM-TP-4 was a shoulder peak of HLM-TP-6 (both oxidation, Na-K exchange) with lower intensity, indicating stereochemical isomers. The disodium complex-TPs were HLM-TP-3 and HLM-TP-5, both showing dehydration and dehydrogenation; the difference is the loss of one more oxygen atom at HLM-TP-3. The last TP HLM-TP-7 showed di-dehydrogenation.

MS/MS data of the HLM-TPs are only partially available due to the low intensity of most HLM-TPs. The MS/MS-spectra of HLM-TP-6 is presented in Figure 6 together with the chemical structure and fragmentation pattern (based on the results of Lopes et al.³²). The suggested position of the hydroxylation at a tertiary C atom is in accordance with the obtained fragmentation pattern. Tertiary C atoms show a higher reactivity for hydroxylation than secondary or primary C atom.³⁵ The second possibility for hydroxylation of LAS is marked by "x."

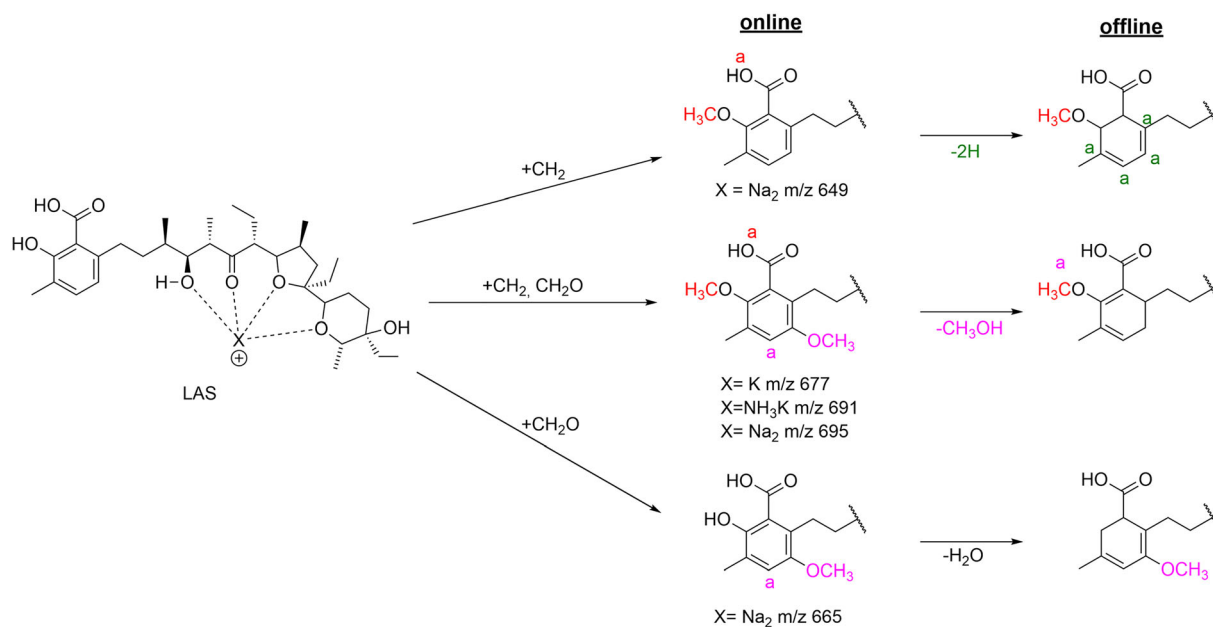


FIGURE 5 Possible formation pathways of offline EC-TP-1 and EC-TP-2 considering the online-measured TPs. The final offline EC-TPs were formed due to reconversion processes during storage, including a cation (X) - exchange. The red letter “a” indicates equivalent C atoms where the modification reaction could occur [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Detected TPs of LAS in a liver microsome assay using HLM and RLM

| LM-TP | m/z calc. | Retention time [s] | Sum formula | Modification reaction |
|----------------|----------------|--------------------|--|------------------------|
| 1 | | 60.9 | $C_{34}H_{54}O_{10}K$ | $-Na; +2 O; + K$ |
| 2 | 643.325 | 72.7 | $C_{34}H_{52}O_9K$ | $-2 H; - Na; + O; + K$ |
| 3 | 617.343 | 77.0 | $C_{34}H_{51}O_7Na_2$ | $-3 H; - O; + Na$ |
| 4 [§] | 645.341 | 80.3 | $C_{34}H_{54}O_9K$ | $-Na; + O; + K$ |
| 5 [§] | 601.348 | 80.7 | $C_{34}H_{51}O_6Na_2$ | $-3 H; -2 O; + Na$ |
| 6 [§] | 629.367645.341 | 84.1 | $C_{34}H_{54}O_9Na$ $C_{34}H_{54}O_9K$ | $+ O-Na; + O; + K$ |
| 7 | 611.356 | 99.3 | $C_{34}H_{52}O_8Na$ | $-2 H$ |
| LAS | 613.372 | 125.3 | | |

Notes: The three detected RLM-TPs are § marked. The mass determination error for the calculation of the sum formula was ± 5 ppm.

Abbreviations: EC-MS, electrochemistry-mass spectrometry; HLM, human liver microsomes; LAS, lasalocid; LM, liver microsomes; RLM, rat liver microsomes; TP, transformation products.

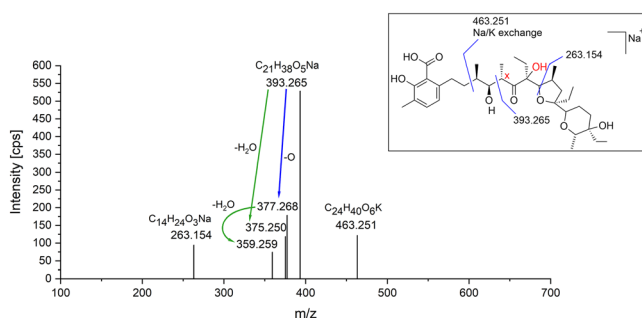


FIGURE 6 MS/MS spectrum of the HLM-TP-6 (K complex) with specification of the sum formulas. Top right has the structure of lasalocid (LAS), the hydroxylation is marked in red, the “x” shows the equivalent position for the hydroxylation. Associated fragmentation of LAS is shown [Color figure can be viewed at wileyonlinelibrary.com]

The LM assay led to the synthesis of seven HLM-TPs, all due to oxidation/reduction of LAS. The HLM-TPs were detected as sodium, disodium, and potassium complexes. A comparison between the EC-TPs and the HLM-TPs showed no matching results. The HLM-TP-7 is similar to the online EC-TP (m/z 611.355), showing di-dehydrogenation. Oxidation (+O) as modification reaction occurred thrice for the online EC results at 2.5 V and was also a main modification reaction for HLM-TPs. The comparison of our study results with the literature on metabolites of LAS^{6–8} shows hydroxylation as the main reaction. The potassium-based TPs cause confusion with hydroxylation, based on the small mass difference of 0.0021 Da between potassium-based and hydroxylated TPs. Without high mass accuracy the risk is enhanced to determine hydroxylation instead of an ion-exchange. Potassium-based TPs of ionophore

antibiotics were already identified in liver microsome TPs of salinomycin.³⁴

4 | CONCLUSION

This study demonstrates the use of EC to simulate oxidative TPs of LAS. Additional HLM/RLM assays were performed. The online EC/HRMS measurements led to formation of several TPs, and after typical EC modification reactions (+/- H; + CH₂; + H₂O; + CH₂O), different ion complexes were observed. In accordance to the divalent character of LAS disodium, sodiated-ammonia, and potassiated-ammonia complexes were identified, besides monovalent complexes with sodium and potassium. Interestingly, only two stable EC-TPs based on the methylation reaction were found when the collected EC eluate was stored and subjected to LC/HRMS analysis subsequently. Three different formation pathways starting from the online TPs were proposed. Finally, the region of methylation of LAS at the aromatic ring system was identified. The microsomal assays allowed us to detect three RLM-TPs and seven HLM-TPs, although all the three RLM-TPs were identical to three HLM-TPs. Oxidation/hydroxylation and dehydrogenation were identified as modification reactions, in accordance with literature. In addition, potassium-based TPs were found. The comparison between the EC-TPs and HLM-TPs shows no significant overlap. Although EC settings applied to this work are not well suited to simulate microsomal transformation reactions, they provide complementary information. In general, the probability that the methylated offline EC-TPs are relevant as metabolic or environmental TP is low. A slight overlap between the results of EC and microsomal TPs is that LAS can form various ion complexes (EC: Na⁺, 2 Na⁺, K⁺; NaNH₄⁺, KNH₄⁺; HLM/RLM: Na⁺, K⁺). For future investigations on TPs of LAS, the correct identification of occurring ion complex is as important as the correct identification of the modification reaction. The results of our study contribute to a better understanding of bio- or environmental transformation process of LAS. In particular, the understanding of different ion complexes of LAS is important to improve the residue analysis of LAS.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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