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Liquid chromatography–tandem mass spectrometric assays for salinomycin in mouse plasma, liver, brain and small intestinal contents and in OptiMEM cell culture medium

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Abstract

Fast and sensitive liquid chromatography–tandem mass spectrometric assays for the determination of salinomycin in mouse plasma, liver, brain and small intestinal contents and in OptiMEM cell culture medium, were developed and validated using simple sample pre-treatment procedures. Tissue samples were homogenized with phosphate buffered saline or, for high levels in liver, with human plasma. After addition of monensin as the internal standard to plasma, homogenate or culture medium and acetonitrile extraction for tissue and plasma, the diluted medium or the supernatant was directly injected into the isocratic chromatographic system using a polar embedded reversed-phase column and formic acid in water–acetonitrile as the eluent. The eluate was completely led into an electrospray interface with positive ionization and the analytes were quantified using triple quadrupole mass spectrometry. The assays were successfully validated in the ranges 10–2000 ng/ml for OptiMEM cell culture medium, 1–2000 ng/ml for plasma and 3–2000 ng/g in liver brain and small intestinal contents. At the lowest levels, the intra-day precisions were ≤9%, inter-day precisions were ≤14% and accuracies were between 91 and 112%. The analytes were chemically stable under all relevant conditions and the assays were applied in different *in vitro* transport studies and in pharmacokinetic and tissue distribution studies with salinomycin in mice.

Keywords: Salinomycin; Monensin; LC/MS/MS; OptiMEM cell culture medium; Mouse tissues

1. Introduction

Salinomycin (Fig. 1a), a polyether antibiotic drug belonging to the group of ionophores, is produced by fermentation by the *Streptomyces albus* strain. Salinomycin is extensively used as a coccidiostat in poultry and other livestock and is also commonly fed to ruminant animals to improve feeding efficiency [1,2]. However, salinomycin is also known to cause severe intoxications when accidentally fed to animals in relatively high doses, as is described for chickens [3–5], turkeys [6–8], cats [9], pigs [10–12] and horses [13,14]. From the perspective of sensitivity of animals to salinomycin upon oral exposure, we recently studied active transport of salinomycin by multi-drug transporters of the ATP Binding Cassette (ABC) transporter family *in vitro* and we confirmed our findings *in vivo* by performing plasma pharmacokinetic studies and tissue distribution in mice. To support this study, we needed sensitive analytical assays, especially since the size of plasma and cell culture medium samples was very small (20 μl).

Sensitive analytical methods (lower limit of quantification (LLQ) ≤ 10 ng/g) that have been reported for salinomycin to date, generally use liquid chromatography coupled to tandem

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mass spectrometry (LC/MS/MS) and have been employed for eggs [15–21], tissues (liver, muscle and fat) [15–17,19,21,22], manure [23], surface water [24,25], soil [26] and sediment [25]. In addition, a time resolved fluorescence immunoassay for egg and muscle [27] and an LC–MS method for chicken egg, tissues and plasma [28] has been described. All these assays were combined with the determination of other ionophores [15–28].

To obtain low detection levels in biological samples complex labour-intensive extraction procedures have been employed, especially the LC–MS method using multiple liquid–liquid extractions followed by solid-phase extraction (SPE) [28]. A frequently used sample preparation procedure for LC/MS/MS assays for biological samples containing salinomycin is an extraction with acetonitrile followed by SPE [15,17–19,21,22],

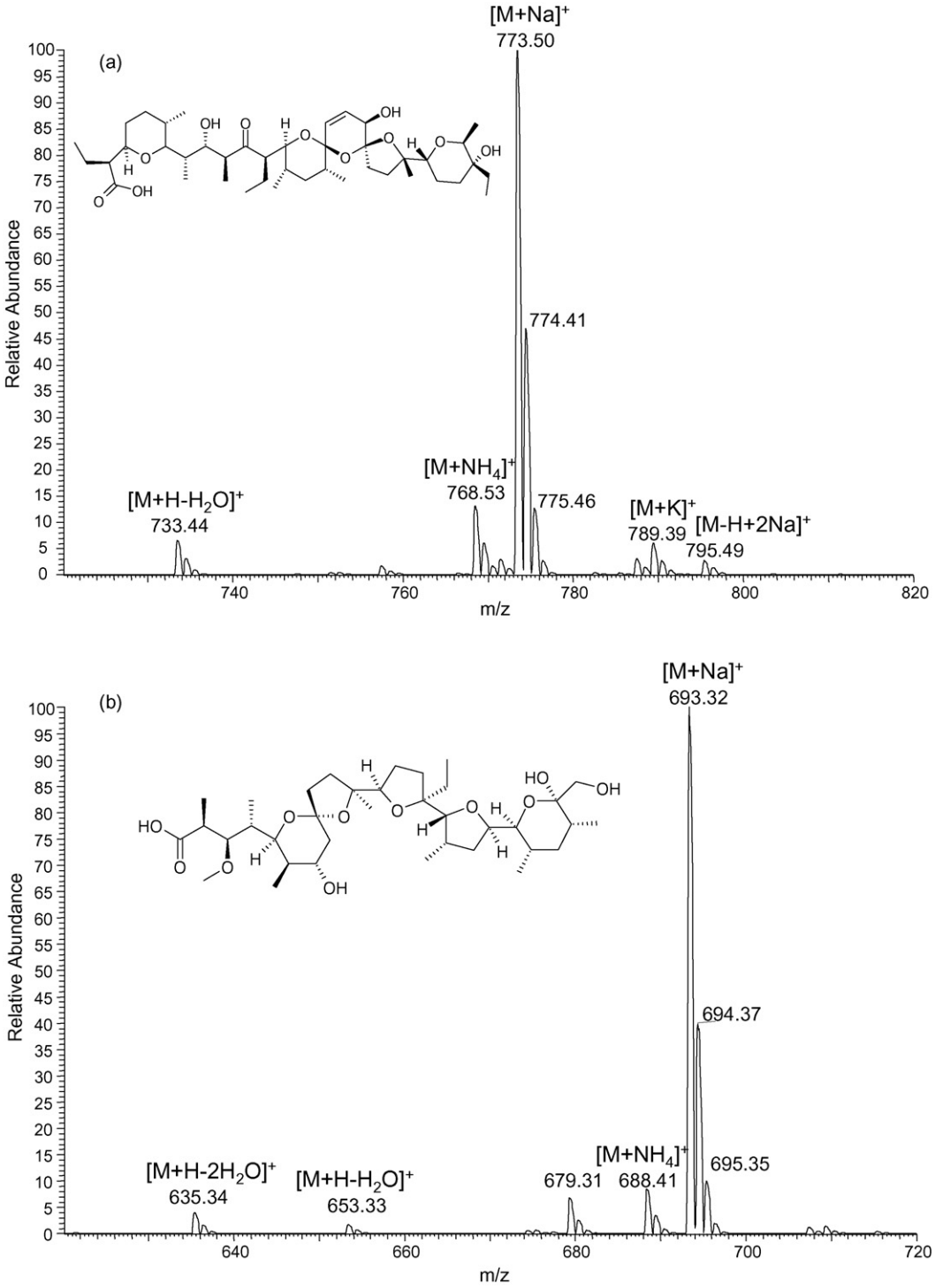

Fig. 1. Electrospray spectra with chemical structures of (a) salinomycin, single quadrupole, (b) monensin, single quadrupole, (c) salinomycin, triple quadrupole, (parent ion *m/z* 773.45; collision energy = −49 V) and (d) monensin, triple quadrupole, (parent ion *m/z* 693.36; collision energy = −49 V).

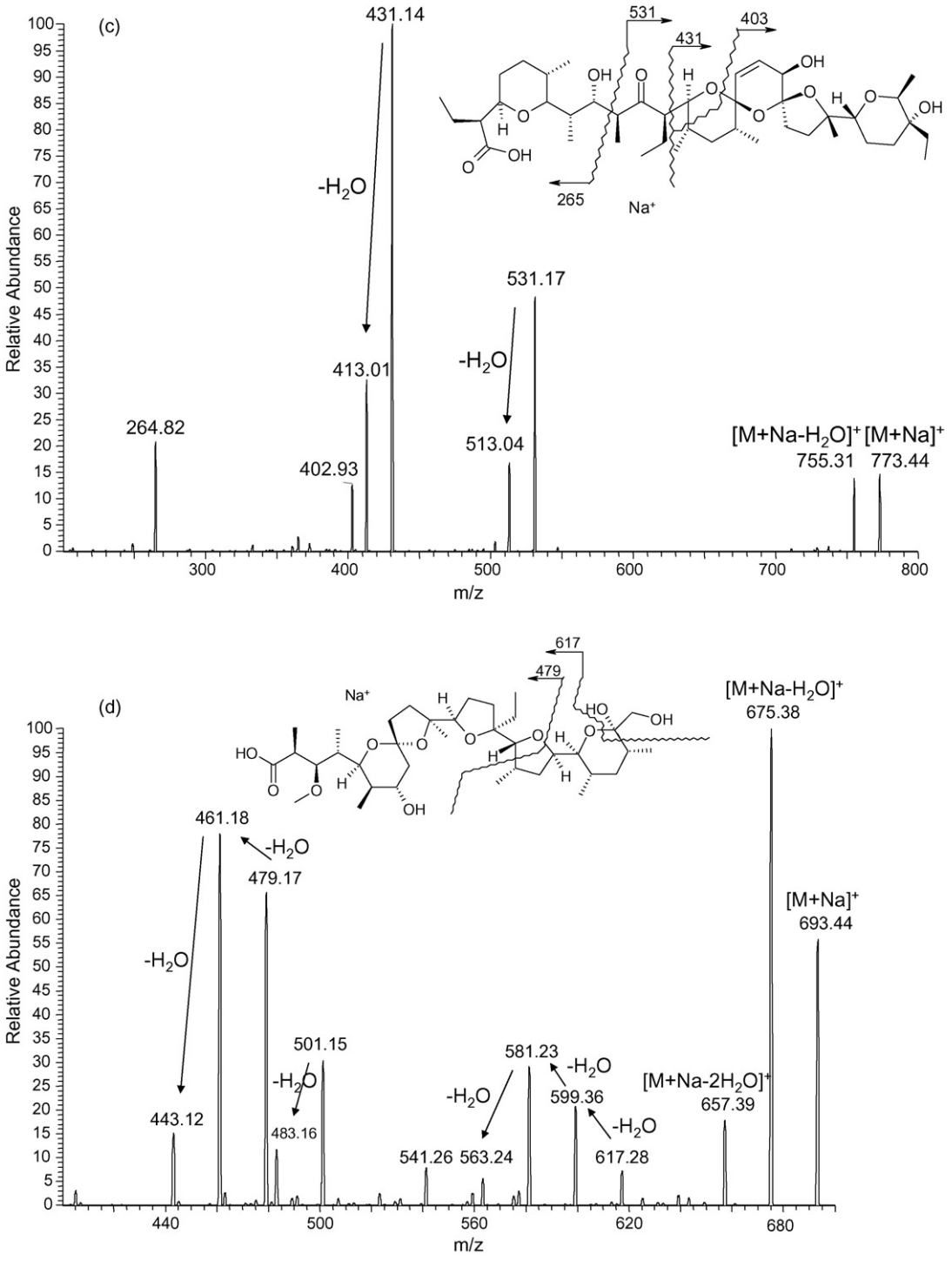

Fig. 1. (*Continued*).

typically using 5 g of sample. For the surface water assays, the use of only SPE sufficed [24,25]. For egg samples, an LC/MS/MS assay using only acetonitrile extraction was reported [20]. This simple sample pre-treatment procedure was not yet reported for plasma or tissue samples. Finally, all chromatographic assays take at least 10 min analytical rum time [15,17–26,28], with the 4 min of Rosen [16] as an exception. We therefore developed and validated sensitive and fast chromatographic salinomycin assays with a simple pre-treatment procedure using electrospray-MS/MS as detection technique for small amounts (20 μl or mg, less than 1% of the sample amounts in all other known bioanalytical LC/MS/MS assays for salinomycin [15–23]) of OptiMEM and mouse plasma, liver, brain and small intestinal contents samples. The final aim was to support *in vivo* pharmacokinetic studies in wild-type and genetically modified mice lacking one or more ABC-drug transporters.

2. Experimental

2.1. Animals

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were male wild-type mice of a FVB genetic background, between 9 and 15 weeks of age. Animals were kept in a temperature-controlled environment with a 12-h light/12-h dark circle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

2.2. Chemicals

Salinomycin SV sodium salt pentahemihydrate (Vetranal®; 94.4%) and monensin sodium salt (Vetranal®; 94.2%) both originated from Riedel-de Haën (Sigma–Aldrich, Seelze, Germany). Water of LC–MS quality, methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were obtained from Biosolve (Valkenswaard, The Netherlands) and other water used was home purified by reversed osmosis on a multi-laboratory scale. Formic acid and phosphate buffered saline were of analytical grade and originated from Merck (Darmstadt, Germany). Blank, drug-free human plasma, containing citrate, phosphate and dextrose as anti-coagulants, and red blood cells were obtained from the Sanquin Bloedbank (Utrecht, The Netherlands). Pooled mouse plasma was obtained from Biomeda (Foster City, CA, USA) and plasma of individual mice was kindly provided by Mr. A.E. van Herwaarden, MSc (The Netherlands Cancer Institute, Division of Experimental Therapy, Amsterdam, The Netherlands). Opti-MEM I (OptiMEM) cell culture medium originated from Invitrogen, (Carlsbad, CA). Blank tissue homogenates were prepared by weighing and pooling four portions of tissue from different mice in a glass tube and adding a four-fold volume (v/w) of PBS. The closed tube was vigorously shaken, manually and by vortex mixing for 1 min, and then treated in an ultrasonic bath for 15 min. After centrifugation at $3500 \times g$ for 5 min, the remaining solid fraction was discarded.

2.3. Equipment

The LC/MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-μ pumps (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer with electrospray ionization (Thermo Electron, Waltham, MA, USA). Data were recorded on and the system was controlled by a Dell Optiplex GX270 personal computer, equipped with the Finnigan Xcalibur software (version 1.4, Thermo Electron).

2.4. LC/MS/MS conditions

Partial-loop injections (3 or 5 μl) were made on a Polaris 3 C18-A column (50 mm × 2 mm, $d_p = 3$ μm, average pore diameter = 10 nm, Varian, Middelburg, The Netherlands) with a Polaris 3 C18-A pre-column (10 mm × 2 mm, $d_p = 3$ μm, Varian). The column temperature was maintained at 40 °C and the autosampler was maintained at 15 °C. The eluent comprised 10% (v/v) of 1% formic acid (v/v) in water and 90% (v/v) acetonitrile for the OptiMEM cell culture medium and plasma assays, for the tissue assay an 18–82% (v/v) solvent ratio was used. The eluent flow rate was 0.5 ml/min and the eluate was led into the electrospray probe, starting at 0.5 min after injection by switching the MS inlet valve. Tuning the ion spray at 90% (v/v) acetonitrile and 10% (v/v) of 1% formic acid (v/v) in water, operated in the positive ionization mode, for salinomycin resulted in a 4300 V spray voltage and a 324 °C capillary temperature with the nitrogen sheath, ion sweep and auxiliary gasses set at 43, 2 and 6 arbitrary units, respectively; the up-front collision induced dissociation was set off. The calibration of the quadrupoles was performed using phosphoric acid and three of its clusters ($n = 6$, 11 and 14) at m/z 98.98, 588.87, 1078.75 and 1372.68. The selected reaction monitoring (SRM) mode was used with argon as the collision gas at 2.1 mTorr. Compound dependent parameters are reported in Table 1. For both compounds, the signals of the three most prominent daughter ions were added up to obtain the highest signal to noise ratios. The mass resolutions were set at 0.7 full width at half height (unit resolution) for both quadrupoles and a 1.5-min run time was used for the 90% (v/v) acetonitrile containing eluent and 2.2 min for the 82%. The retention time of both compounds was ca. 0.8 min at 90% acetonitrile and ca. 1.2 min at 82%.

Table 1
Compound dependent mass spectrometric settings

Compound	Tube lens off set	Parent ion	Collision energy (V)	Daughter ions
Salinomycin	165	773.45	49	431.12
			47	531.28
			52	413.20
Monensin	132	693.36	35	675.36
			45	461.22
			49	479.21

Daughter ions are in order of decreasing abundance.

2.5. Sample pre-treatment

To a 20-μ1 OptiMEM sample, pipetted into a glass micro vial, 40 μl of internal standard (IS; 100 ng/ml monensin in acetonitrile) was added; the vial was closed and shaken manually. Three microliters of the sample were injected onto the column.

To a 20-μl plasma sample, pipetted into a 1.5-ml polypropylene tube, 20 μl of the IS (100 ng/ml monensine in 50% (v/v) methanol) was added. The sample was shaken shortly on a vortex mixer and next, 50 μl acetonitrile was added. The tube was closed and shaken vigorously for ca. 5 s using vortex mixing. After centrifugation at $10 \times 10^3 \times g$ for 1 min, the supernatant was pipetted into a 250-μl glass insert placed in an injection vial. Five microliters of the sample were injected onto the column.

Tissues were homogenized using a scalpel blade 11 on a glass plate. To 16–24 mg tissue, weighed into a 0.5-ml polypropylene tube 100 μl of phosphate buffered saline were added. After vigorous vortex mixing and a 15-min treatment in an ultrasonic bath, 20 μl of the IS (100 ng/ml monensin in 50% (v/v) methanol) were added. The sample was shaken shortly on a vortex mixer and next, 200 μl of acetonitrile were added. The tube was shaken vigorously for ca. 5 s using vortex mixing. After centrifugation at $10 \times 10^3 \times g$ for 1 min, the supernatant was pipetted into a 250-μl glass insert placed in an injection vial. Three microliters of the sample were injected onto the column.

From liver samples containing high levels (>2 μg/g) of salinomycin 16–24 mg of homogenized tissue were weighed into a 1.5-ml polypropylene tube and diluted and homogenized into 980 μl of human plasma using vortex mixing and a 15-min treatment in an ultrasonic bath. Next, 20 μl was pipetted and treated further like a plasma sample.

2.6. Validation

2.6.1. Calibration

Stock solutions of Salinomycin SV sodium salt pentahemihydrate were prepared at 1 and 2 mg/ml and monensin sodium salt at 1 mg/ml, both in methanol, and were stored at −30 °C. The monensin (IS) solution was diluted to 100-ng/ml solutions in both acetonitrile and 50% (v/v) methanol, both stored at −30 °C. The 1-mg/ml salinomycin working solution was used to prepare calibration samples in OptiMEM and in pooled human plasma, originating from four different volunteers, at 2000 ng/ml, daily yielding additional calibration samples at 1000, 200, 100, 20 and 10 ng/ml salinomycin in OptiMEM or 1000, 200, 100, 20, 10, 2 and 1 ng/ml salinomycin in human plasma. For the calibration of the tissue assay, a 20-μl plasma calibration sample was supplemented with 100 μl of 20% (v/v) red blood cells in PBS. The OptiMEM calibration samples and the 1, 2 and 2000-ng/ml plasma samples were processed in duplicate, the others only once. Least-squares linear regression using the individual samples and a weighting factor x^{-2} (reversed square of concentration) were employed to define the calibration curves using the ratios of the peak area of the analyte and IS in each calibration sample.

2.6.2. Precision and accuracy

The 2 mg/ml salinomycin stock solution was used to obtain validation (quality control (QC)) samples in OptiMEM at 1500 (QC-high), 200 (QC-med), 25 (QC-low) and 10 ng/ml (QC-LLQ), in pooled mouse plasma at 1500 (QC-high), 100 (QC-med), 3 (QC-low) and 1 ng/ml (QC-LLQ) and in pooled human plasma (used for the tissue assay) at 1500 (QC-high), 100 (QC-med), 10 (QC-low) and 3 ng/ml (QC-LLQ) salinomycin. Precisions and accuracies were determined by sextuple (OptiMEM and plasma), quintuple (liver homogenate, but not for QC-low) or triplicate (brain and small intestinal contents homogenates) analysis of each validation sample in three analytical runs on three separate days for all QCs (total: $n = 18$, 15 or 9). The human plasma validation samples for the tissue assay were, prior to analysis, supplemented with 100 μl of homogenized tissue (20% (w/v)) in PBS. Relative standard deviations were calculated for both, the intra-day precision (repeatability) and the inter-day precision (reproducibility). For the plasma assay, precision and accuracy were assessed additionally in OptiMEM/human plasma (2:1, v/v) and mouse liver/human plasma (1:49, w/v), both on 1 day.

2.6.3. Selectivity

Six individual mouse plasma, liver, brain and small intestinal contents samples were processed to test the selectivity of the assays. These samples were processed without IS and with IS after spiking with 1 ng/ml (for plasma) or 3 ng/g (for tissue) salinomycin, respectively.

2.6.4. Recovery

The overall recovery (both, extraction yield and ion suppression) was determined in quadruplicate by comparing processed samples (QC-high, -med and -low) with "academic" solutions, water–acetonitrile (1:2, v/v for OptiMEM samples, water–methanol–acetonitrile (3:1:5, v/v/v) for both, mouse and human plasma samples and water–methanol–acetonitrile (11:1:22, v/v/v) for the tissue samples (only for QC-med).

The IS was tested identically at the concentration used in the assay but not in human plasma. In addition, potential ion-suppression by 1500 ng/ml salinomycin (spiked into the biological matrix) was investigated because both compounds almost co-elute.

2.6.5. Stability

The stability of the analyte was investigated in QC-high and -low OptiMEM and mouse plasma samples. Quadruple analysis of these samples was performed after storage at ambient temperature, three additional freeze–thaw cycles, and −30 °C, respectively. The stability of salinomycin in mouse tissues was studied using samples of treated animals. Individual mouse liver or pooled mouse brain or small intestinal contents samples were divided in two portions. One portion was stored for 24 h at ambient temperature and the other was kept at −30 °C. Both portions were analyzed in quadruplicate in one analytical run afterwards. Furthermore, samples from OptiMEM, plasma and tissue validation runs were re-injected after additional storage of the extracts at 15 °C, ambient temperature, 4 °C and −30 °C, respectively.

2.6.6. Samples

A wild-type mouse was treated with 1 mg/kg salinomycin i.v. and blood samples were collected in heparinized capillary tubes (Oxford Labware, St. Louis, USA) from the tail vein at 7.5, 15 and 30 min and at 1, 2, 4 and 6 h after injection. After centrifugation at $2100 \times g$ for 5 min at 4 °C plasma was pipetted and stored at −30 °C. In addition, four wild-type mice were treated with 1 mg/kg salinomycin i.v. and after 180 min blood, liver, brain and small intestinal contents were collected, plasma was isolated, and the samples were stored at −30 °C. Samples were analyzed using the methods described herein.

Table 2
Regression parameters for the weighted linear regression of the salinomycin assays

Method	Intercept	Slope	Regression coefficient (r^2)	n
OptiMEM[a]	−0.004 ± 0.004	0.0033 ± 0.0005	0.992 ± 0.005	4
OptiMEM[b]	−0.0008 ± 0.0014	0.00173 ± 0.00009	0.989 ± 0.004	4
Plasma	0.0012 ± 0.0009	0.0107 ± 0.007	0.996 ± 0.002	6
Tissue	−0.002 ± 0.003	0.0117 ± 0.002	0.996 ± 0.002	5

[a] Before cleaning the ion tube of the MS.
[b] After cleaning the ion tube.

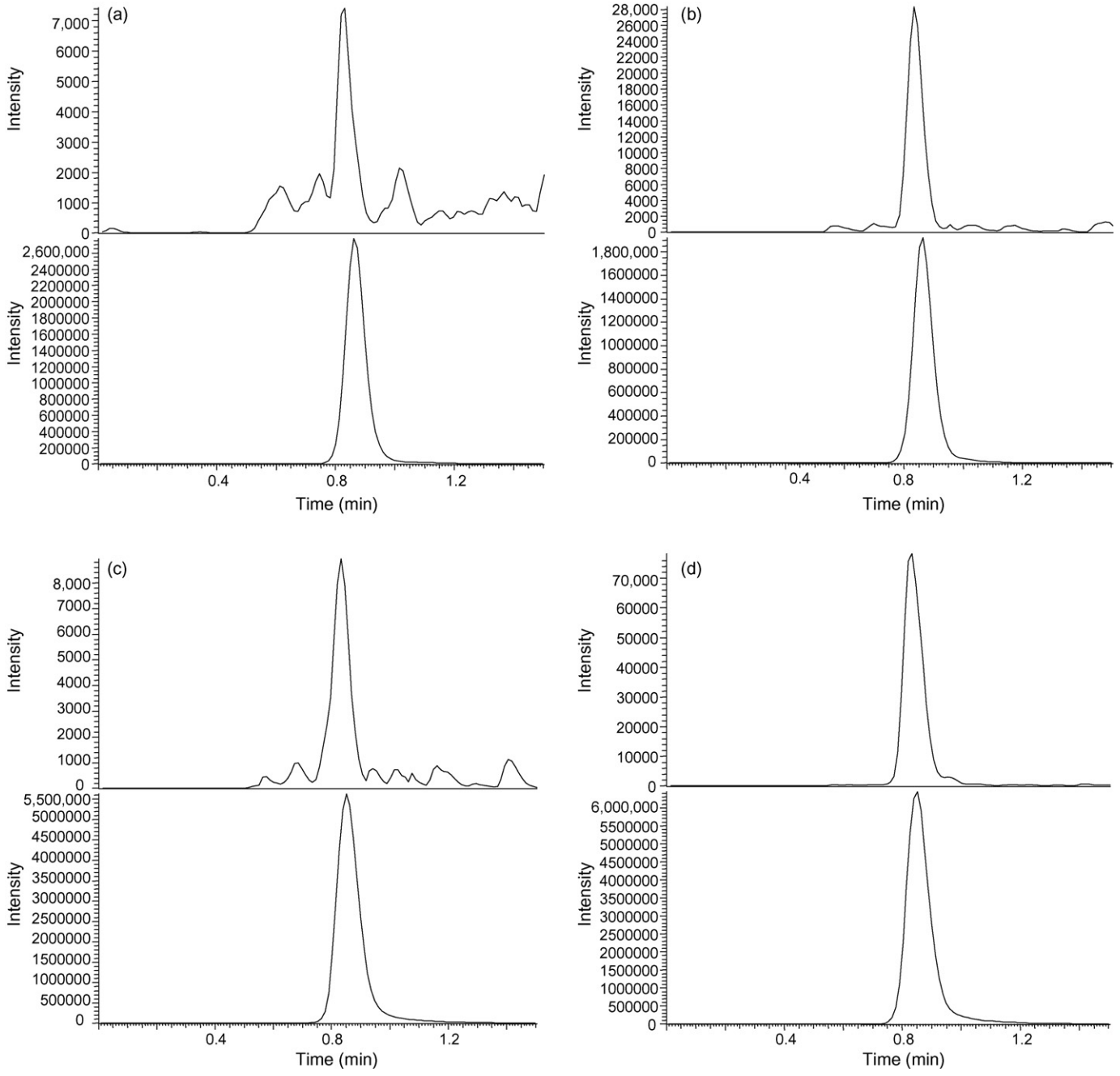

Fig. 2. MS/MS Chromatograms with the salinomycin traces (773.45 → 431.12 + 531.28 + 413.20) depicted above the monensin traces (693.36 → 675.36 + 461.22 + 479.21) of (a) a blank OptiMEM sample, (b) OptiMEM spiked with 10 ng/ml salinomycin (10 pg on column), (c) a blank plasma sample, (d) a plasma sample spiked with 1 ng/ml salinomycin (1.1 pg on column), (e) a blank liver sample, (f) a liver sample spiked with 3 ng/g salinomycin (0.5 pg on column), (g) a blank brain sample, (h) a brain sample spiked with 3 ng/g salinomycin (0.5 pg on column), (i) a blank small intestinal contents sample, (j) a small intestinal contents sample spiked with 3 ng/g salinomycin (0.5 pg on column).

3. Results and discussion

3.1. Method development

Because of the high sensitivity of the MS/MS detection, a simple pre-treatment procedure using a small sample volume could be developed. For the chromatographic separation, the modifier content (90%, v/v, acetonitrile) of the eluent and eluent flow (0.5 ml/min) were adjusted to obtain a short run time, allowing the combination of a fast analysis, co-elution of analyte and IS, and the absence of ion suppression by the plasma and OptiMEM matrices. However, under these conditions, the tissue matrices showed significant ion suppression. This suppression was observed both, by comparing the salinomycin response of tissue extracts with plasma extracts at different injection volumes (5, 1 and 0.2 μl) and by injection of blank extracts (plasma and liver) while the eluate was mixed with an infused salinomycin solution (5 μl/min; 1500 ng/ml; data not shown). The problem was simply solved by using sufficient dilution, increasing the retention time of the analytes by using 82% (v/v) acetonitrile in the eluent and choosing the appropriate (small) injection volume.

The injection of the OptiMEM samples caused a slow blockage of the entrance of the ion tube resulting in a decreasing response. Regular cleaning or replacement of the ion tube was

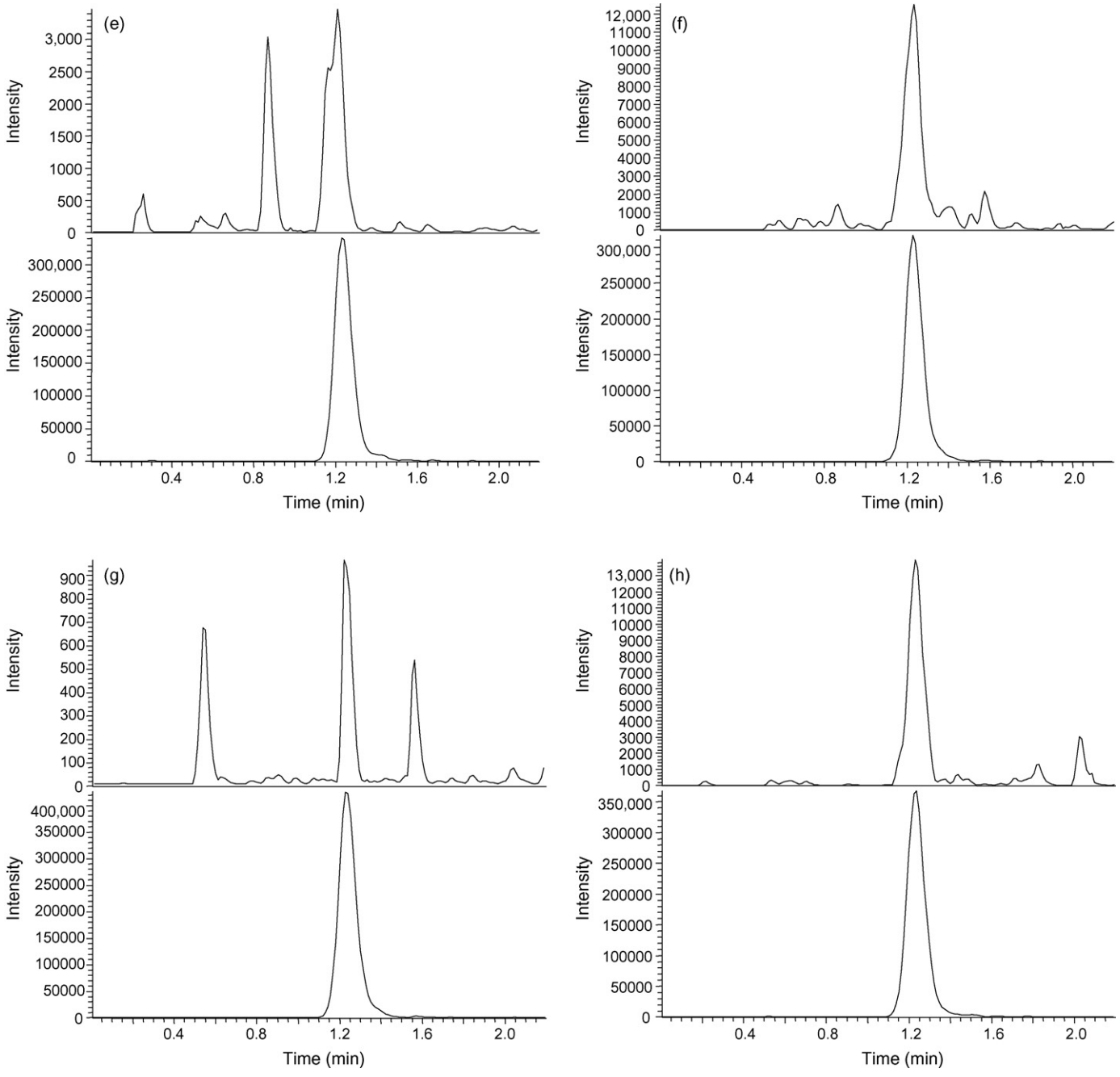

Fig. 2. (*Continued*)

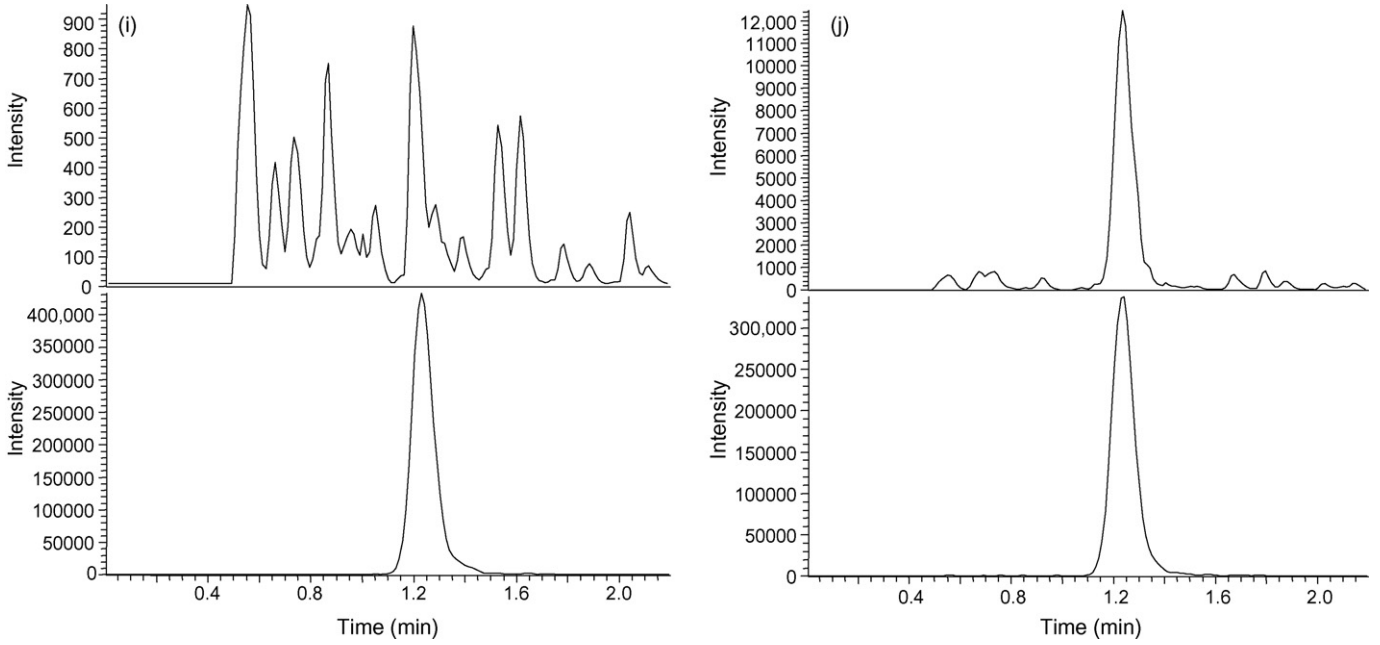

Fig. 2. (*Continued*).

therefore required during use of this method. Both other methods did not show this problem.

The performance of the OptiMEM assay was significantly affected by the choice of the container used for dilution and storage of the samples and therefore additional tests ware executed. Fifty microliters samples of 100 ng/ml salinomycin in OptiMEM were pipetted into a polypropylene reaction tube or in a glass injection vial. After vortex mixing, 20 μl of the remaining solution was pipetted into a glass insert, IS was added and the LC/MS/MS response was compared with samples which did not undergo the additional pipetting. The recovery of the salinomycin after this test procedure ($n = 2$) was ca. 80% from Sarsted polypropylene reaction tubes (1.5 ml; Nümbrecht, Germany), 20–40% from three other tubes (Sarsted 0.5-ml and Eppendorf (Hamburg, Germany) 1.5- and 0.5-ml) and ca. 55% from a glass injection vial. This test showed inter-tube type differences analogous to other method development experiments (data not shown). The choice of the 1.5-ml Sarstedt tubes, to be used for the dilution and storage of all OptiMEM samples, clearly resulted from the remarkably different performances. The performance of the OptiMEM assay was also dependent on the monensin solvent and its volume added tot the sample. Forty μl acetonitrile was chosen after comparing first OptiMEM, methanol/water (1/1, v/v) and acetonitrile as potential solvents and next 20, 40 and 80 μl volumes of acetonitrile.

3.2. Mass Spectrometry

In most previously reported LC/MS/MS methods for salinomycin, electrospray is almost exclusively used as the ionization technique, only Schlusener et al. [23,26] reported the use of atmospheric pressure chemical ionization (APCI). We, however, observed a ca. 90% lower response when using APCI compared to electrospray.

During ionization, both salinomycin and monensin form sodium adduct ions (Fig. 1a and b) as the main product. These sodium adduct ions have also been used as precursor ions in all previous reported LC/MS/MS assays because of their high abundance [15–26]. Collision induced dissociation (CID) resulted for salinomycin (Fig. 1c) in two main fragments (*m/z* 531 and 431) and a water loss (*m/z* 755) from the parent ion and for monensin (Fig. 1d) in one main fragment (*m/z* 479) and water loss from both, this fragment (*m/z* 461) and the parent ion (*m/z* 675). The electrospray ion formation and CID of these and other ionophores have been thoroughly investigated by Volmer and Lock [29].

3.3. Validation

Chromatograms of salinomycin at the LLQ levels and the IS in different matrices are depicted in Fig. 2. The blank chromatograms show a minor salinomycin peak due to carry over for OptiMEM and plasma samples and due to an endogenous interference for liver samples. The blank brain and small intestinal contents chromatograms only show detector noise after switching the inlet valve at 0.5 min.

3.3.1. Calibration

The assay was linear in the whole concentration ranges 10–2000 for OptiMEM and 1–2000 ng/ml for both plasma and tissue (red blood cells) samples, respectively. For all matrices, the concentrations were back calculated from the ratio of the peak area using the calibration curves and no deviations higher than 10% were observed (data not shown). The regression parameters of the weighted linear regression functions are reported in Table 2. These data show a higher variation for the OptiMEM assay, probably caused by the interaction of the analyte with polypropylene and glass surfaces, this interaction will

Table 3
Assay performance data ($n = 18$ on 3 days) for salinomycin quality control (QC) samples in OptiMEM

Nominal concentration (ng/ml)	Concentration found (ng/ml)	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)
1500	1408	7	10	94
200	217	4	6	108
25	24.6	6	6	98
10	11.21	6	10	112

likely be suppressed by the proteins present in samples for the other methods. The variation of the tissue assay is not increased by the extra dilution and lower injection volume compared to the plasma assay.

3.3.2. Precision and accuracy

Assay performance data are reported in Tables 3–5. No intra-day variations higher than 14% and no deviations of the accuracy higher than 12% were observed. Therefore, the upper limit of the calibration can be assigned to the upper limit of quantification [30–32]. Precisions and deviations of the accuracy meet the required ±15% (±20% for the lower limit of quantification) [30–32] for all methods. Tissue homogenates were alternatively analyzed using a 10-μl injection volume. This increased injection volume resulted at all levels in an improved precision but unfortunately also a lower accuracy (data not shown), probably due to ion suppression by the tissue constituents. The dilution of OptiMEM with human plasma and using the plasma assay this way for the analysis of OptiMEM samples seems to be a suitable approach for the improvement of the precision when assaying OptiMEM samples.

3.3.3. Selectivity

The analysis of six batches of blank control plasma and tissue samples showed a small peak for most of the analyses, the corresponding salinomycin response together with the response for the LLQ spiked samples are reported in Table 6. Only for liver the blank response exceeds 20% of the investigated LLQ, the real LLQ is therefore 5 ng/g [30–32] for liver samples. The IS response in the double blank chromatograms was <0.5% for the tissue samples and <0.1% for the plasma samples, both well below acceptable values [30–32].

Table 4
Assay performance data ($n = 18$ on 3 days) for salinomycin quality control (QC) samples in plasma ($n = 18$ on 3 days), in OptiMEM/human plasma (1:2) ($n = 6$ on 1 day) and in 2% (v/v) liver in human plasma ($n = 5$ on 1 day)

Nominal concentration (ng/ml)	Concentration found (ng/ml)	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)
Plasma				
1500	1392	3.7	4.7	93
100	106.3	3.2	6.4	106
3	2.91	3.4	6.1	97
1	0.965	7.8	10.4	96
OptiMEM/human plasma (1:2)				
1500	1396	4.5		93
100	91.8	1.0		92
2% (w/v) liver in human plasma				
1500	1484	2.0		99
100	85.5	0.9		96
3	2.93	3.8		98

Table 5
Assay performance data (on 3 days) for salinomycin quality control (QC) samples in mouse tissue homogenates

Nominal concentration (ng/ml)	Concentration found (ng/ml)	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)
Liver ($n = 15$)				
1500	1414	2.3	6.5	96
100	94.5	3.0	4.8	94
3	3.05	9.8	10.9	102
Brain ($n = 9$)				
1500	1396	2.5	4.6	93
100	92.9	2.9	3.3	93
10	10.58	4.4	5.4	106
3	2.82	5.5	12.4	94
Small intestinal contents ($n = 9$)				
1500	1327	3.1	8.5	88
100	88.0	4.9	5.1	88
10	10.83	6.4	8.7	108
3	2.72	9.1	14.0	91

Table 6
Salomycin response (±SD) for six independent blank and LLQ spiked samples of plasma and three tissues

Sample	Blank response	LLQ investigated	LLQ response
Plasma (ng/ml)	0.09 ± 0.09	1	1.16 ± 0.09
Liver (ng/g)	0.85 ± 0.35	3	3.21 ± 0.24
Brain (ng/g)	0.22 ± 0.08	3	3.24 ± 0.40
Small intestinal contents (ng/g)	0.28 ± 0.09	3	2.83 ± 0.17

Table 7
Overall recovery (±SD, $n=4$) of salinomycin for different concentrations and matrices without IS correction

Matrix	Concentration (ng/ml)	Recovery (%)
OptiMEM	1500	107 ± 29
OptiMEM	25	71 ± 12
Mouse plasma	1500	98 ± 6
Mouse plasma	100	104 ± 19
Mouse plasma	3	98 ± 19
Human plasma	1500	102 ± 7
Human plasma	3	104 ± 23
Liver	100	114 ± 16
Brain	100	115 ± 14
Small intestinal contents	100	112 ± 20

3.3.4. Recovery

All recovery experiments did not show any significant extraction loss or ion suppression for both salinomycin (Table 7) and monensin (Table 8).

3.3.5. Stability

Recoveries of salinomycin under different storage conditions in different matrices are shown in Table 9 for OptiMEM and plasma samples. The recoveries of salinomycin from tissues ($n=4$) during storage at ambient temperature for 24 h were 87 ± 15% at 25 ± 3 μg/g and 94 ± 14% at 3.8 ± 0.3 μg/g from liver, 103 ± 4% at 169 ± 3 ng/g and 106 ± 12% at 9.5 ± 0.9 ng/g from brain and 100 ± 6% from 267 ± 8 ng/g from small intestinal contents. The low recovery of the high-QC level in OptiMEM at −30 °C may also be caused by the interaction of the analyte with the tube as previously reported. Injection of acetonitrile diluted OptiMEM samples and plasma and tissue extracts after additional storage resulted again in successful performances after the times reported in Table 10 and was only limited for the acetonitrile diluted OptiMEM samples. Overall, salinomycin showed to be stable under all relevant conditions. Stability studies of salinomycin have not been performed as part of the validation of an LC/MS/MS assay for salinomycin until now [15–26].

Table 8
Overall recovery (±SD, $n=4$) of monensin (IS) at 100 ng/ml for different matrices

Matrix	Recovery (%)
1500 ng/ml salinomycin in OptiMEM	108 ± 10
25 ng/ml salinomycin in OptiMEM	101 ± 17
1500 ng/ml salinomycin in mouse plasma	93 ± 18
3 ng/ml salinomycin in mouse plasma	90 ± 20
100 ng/g salinomycin in liver	105 ± 15
100 ng/g salinomycin in brain	93 ± 8
100 ng/g salinomycin in small intestinal contents	110 ± 17

Table 9
Stability data (±SD; $n=4$) of salinomycin under different storage conditions, reporting the percentage of the initial concentration (1500 and 25 ng/ml in OptiMEM; 1500 and 3 ng/ml in plasma)

Matrix	Conditions/sample	QC-high	QC-low
OptiMEM	18 h at ambient temperature	85 ± 4	83 ± 5
	3 freeze–thaw cycles	102 ± 15	90 ± 3
	6 months at −30 °C	76 ± 2	96 ± 2
Plasma	24 h at ambient temperature	98 ± 2	98 ± 4
	3 freeze–thaw cycles	99 ± 2	92 ± 4
	5 months at −30 °C	107 ± 3	114 ± 8

Table 10
Storage of acetonitrile diluted OptiMEM samples and plasma and tissue extracts and the results of re-injection of a run including calibration and QC samples

Assay	Temperature	Time to successful re-injection	Time to unsuccessful re-injection
OptiMEM	Ambient	21 h	
	15 °C	18 h	2 Days
	4 °C	3 Days	
	−30 °C		7 Days
Plasma	Ambient	3 Days	
	4 °C	4 Days	
	−30 °C	4 Months	
Tissues	15 °C	22 h	
	4 °C	8 Days	

For the tissue assay QC samples from all validated tissues (liver, brain and small intestinal contents) were included.

3.3.6. Samples

A concentration–time curve of salinomycin in plasma in a wild-type mouse is shown in Fig. 3. The plasma concentration–time curve shows a biphasic decline after i.v. bolus administration of salinomycin. Concentrations observed could all be assessed using the validated assay. Tissue levels of salinomycin (±SD) in wild-type mice ($n=4$), 180 min after i.v. administration of 1 mg/kg was 72.5 ± 3.6 ng/ml in plasma, 9.2 ± 0.7 ng/g in brain, 209 ± 55 ng/g in small intestinal con-

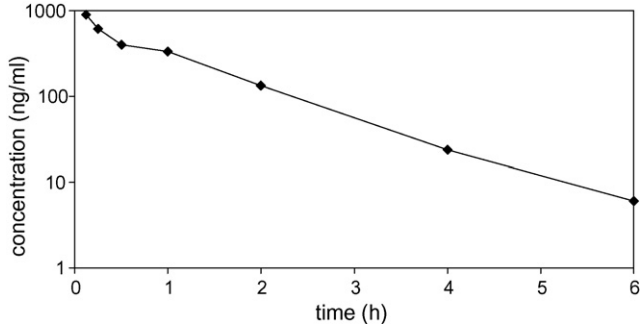

Fig. 3. Plasma concentration–time curve of salinomycin after administration of 1 mg/kg i.v. to a WT mouse.

tents and 7.4 ± 1.0 μg/g in liver, respectively. Therefore, also for tissue samples analysis the validated assays were well suitable.

4. Conclusions

We reported novel LC/MS/MS assays for the quantitative analysis of salinomycin in OptiMEM cell culture medium and in mouse plasma, liver, brain and small intestinal contents, which can be used for pharmacokinetic and tissue distribution studies and for *in vitro* transport studies. The assays are fast, use a simple sample pre-treatment and a small sample amount, meet common validation criteria and are, relative to the sample amount, by far the most sensitive salinomycin assays reported hitherto.

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