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Development and validation of an LC-MS/MS assay for the quantification of cintirorgon (LYC-55716) in mouse plasma and tissue homogenates

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ABSTRACT

Cintirorgon (LYC-55716) is a promising first-in-class antitumor agent as a RORγ agonist in the treatment against various types of cancer. To support preclinical mouse studies, a bioanalytical method was developed and successfully applied for quantification of cintirorgon in mouse plasma and tissue homogenates using LC-MS/MS. The method was fully validated in mouse plasma and partial validation was performed in eight different homogenates originating from brain, kidney, liver, lung, small intestine, small intestine content, spleen, and testis. Sample preparation was performed using 96-well plates for fast and efficient analysis. Protein precipitation was done by addition of 20 µL acetonitrile containing monensin as internal standard to 10 µL sample. Chromatographic separation was achieved on a Polaris 3 C18-A column using gradient elution with 0.2% (v/v) formic acid and 0.2% (v/v) ammonium hydroxide in water (A) and methanol (B) as eluents. The total run time was 3 min. Detection was carried out with a triple quadrupole mass spectrometer with electrospray ionization operated in the positive ion-mode. Quantification could be accomplished within a linear validated concentration range of 5–4,000 ng/mL (10–4,000 ng/mL in brain homogenates) with an intra- and inter-day precision between 4.6–14.7% and 5.1–15.6% (including the LLOQ), respectively, and accuracies between 89.1%–111.2%. The method was successfully applied to a preclinical study with cintirorgon in mice.

1. Introduction

Cintirorgon (LYC-55716) is a promising first-in-class immunotherapeutic agent in cancer treatment as mono- and combination therapy. It has successfully undergone phase 1/2 A studies as monotherapy in adults with locally advanced or metastasized cancer, such as non-small cell lung cancer, renal cell carcinoma, ovarian carcinoma, and urothelial carcinoma [1]. Currently, a phase 1b combination therapy of cintirorgon and pembrolizumab trial is ongoing to assess the safety and tolerability in adults with metastatic non-small cell lung cancer [2].

Cintirorgon has agonistic properties to the retinoic acid receptor–related orphan receptor γ (RORγ), a transcription factor encoded by the *RORC* genes [3]. This receptor modulates Type 17 effector T cells that contribute to antitumor responses by concurrently increasing immune activation and decreasing immune suppression. Increased immune activation by RORγ is established by enhancing multiple processes that play a role in tumor resistance, such as the number of effector T cells in tumors, the cytolytic activity, and the co-stimulatory molecules (CD137, CD27, CD226). The production of pro-inflammatory cytokines, chemokines and prolonged survival of anti-tumor lymphocytes are also increased by RORγ activation. On the other hand, immune suppression is decreased by reduction of regulatory T cells, CD39 and CD73 enzymes that generate extracellular adenosine, PD-1, and other co-inhibitory molecules e.g. TIM3, LAG3, or TIGIT [3–6].

Abbreviations: Cps, counts per second; EMA, european Medicines Agency; FDA, food and drug administration; h, Hour; LC-MS/MS, liquid chromatography tandem mass spectrometry; LLOQ, lower limit of quantification; *m/z*, mass-to-charge ratio; min, minutes; ms, millisecond; Mw, molecular weight; PK, pharmacokinetic; QC, quality control; ROR, retinoic acid receptor–related orphan receptor; RT, room temperature; SRM, selected reaction monitoring

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To the best of our knowledge, there are no bioanalytical methods for the analysis of cintirorgon reported so far. Given the importance of mouse knockout and tumor models in the development of anticancer drugs like cintirorgon, we developed our assay for various mouse matrices. To support preclinical pharmacokinetic (PK) studies in mice, an LC-MS/MS method was developed for its quantification in mouse plasma and eight different homogenates (brain, kidney, liver, lung, spleen, small intestine, small intestine contents, and testis). Since preclinical PK studies often generate many samples, a simple and fast method was developed using a 96-well format with protein precipitation as sample pretreatment. Full method validation was performed in mouse plasma and partial validation for all eight other matrices in compliance with the FDA and EMA international guidelines [7,8]. The method was successfully applied in a preclinical study with cintirorgon in mice, demonstrating its applicability.

2. Materials and methods

2.1. Chemicals and reagents

The reference standards cintirorgon (> 99.5%, Mw = 603.5 g/mol) and monensin sodium (> 94.2%, Mw = 692.85 g/mol) were purchased from ChemieTek (Indianapolis, IN, USA) and Sigma-Aldrich (Seelze, Germany), respectively. Ammonium hydroxide solution in water (ACS reagent, 28.0–30.0% NH_3 basis) was also purchased from the latter. Methanol (HPLC grade), acetonitrile (HPLC-S grade), and water (ULC-MS grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid (analytical grade) originated from Merck (Darmstadt, Germany). Lithium heparin plasma of mouse (mixed gender) and human (mixed gender) sources were supplied by Sera Laboratories (Heywards Health, West Sussex, UK).

2.2. Analytical instruments

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). The MS was controlled using the Finnigan Xcalibur software (version 1.4, Thermo Electron). The same software was used to record data.

2.3. LC-MS/MS conditions

Partial-loop injections (10 μL) were done on a Polaris 3 C18-A column (50 × 2 mm, d_p = 3 μm, average pore diameter = 10 nm, Varian, Middelburg, The Netherlands) with a corresponding pre-column (10 × 2 mm). The column temperature was maintained at 40 °C and the sample rack compartment at 4 °C. A gradient program with a flow rate of 0.5 mL/min was used with 0.2% formic acid and 0.2% ammonium hydroxide in water as eluent A and methanol as eluent B. After injection, the percentage of methanol was increased linearly from 70% to 90% (v/v) during 1.5 min. Next, the column was flushed with 100% (v/v) methanol for 0.3 min and finally, the column was reconditioned at the starting conditions (70% (v/v) B) for 1.2 min resulting in a total run time of 3 min. The whole eluate was then transferred into the electrospray probe between 0.7 and 2.2 min after injection by switching the MS divert valve. Positive electrospray settings of the assay were a 5,000 V spray voltage and a 320 °C capillary temperature. The nitrogen sheath, ion sweep, and auxiliary gasses were set at 60, 0 and 25 arbitrary units, respectively. The skimmer voltage was set off. The SRM mode was used with argon and the collision gas was set at 1.7 mTorr. Compound-dependent parameters are reported in Table 1. Mass resolutions were set at 0.7 full width at half height (unit resolution) for both separating quadrupoles.

Table 1
LC-MS/MS parameters of individual compounds.

Parameter	Cintirorgon	Monensin
Precursor ion (*m/z*)	621.2	693.4
Product ion (*m/z*)	395.2; 282.1	461.2
Tube lens off set (V)	122	132
Collision energy (V)	-30; -40	-45
Dwell time (ms)	100	100
Retention time (min)	1.5	1.7

2.4. Stock and working solutions

The IS working solution of 200 ng/mL was prepared by dilution of the reference standard in methanol to a concentration of 1 mg/mL and it was then further diluted in acetonitrile to obtain the intended concentration of 200 ng/mL. This IS working solution also functioned as precipitation solvent. Stock solutions were prepared by diluting the cintirorgon reference standard in methanol to a concentration of 0.2 mg/mL. For the preparation of the calibration standards, the stock solution was further diluted to a concentration of 4,000 ng/mL in mouse lithium heparin plasma, which was used as the highest calibrator. The remaining calibrators were prepared by subsequent dilution of the highest calibrator in mouse lithium heparin plasma to obtain a total of eight calibrators with concentrations of 2, 4, 20, 40, 200, 400, 2,000, and 4,000 ng/mL. Least-squares weighted linear regression using the ratio of the peak areas of cintirorgon and the internal standard (IS), monensin, was employed to define the calibration with a weighing factor of $1/x^2$, where x is the analyte concentration. Quality control (QC) samples in mouse lithium heparin plasma were prepared from a separate stock solution at 3,000 (QC-H), 100 (QC-M), 10 (QC-L), and 5 (QC-LLOQ) ng/mL cintirorgon.

2.5. Sample preparation

Ten μL of plasma samples were pipetted into a 200-μL polypropylene 96-well plate with conical bottom and 20 μL of 200 ng/mL IS in acetonitrile was added. The 96-well plates were closed with a silicone mat and were vortex mixed for 10 s at maximum capacity. Next, the plate was centrifuged for 5 min at 2643×g and 20 μL of the supernatant was transferred to a 1-mL 96-deep well plate with round bottom. After adding 200 μL of 50% (v/v) methanol in water, samples were mixed by gentle manual shaking. The prepared plate was placed in the autosampler rack without the silicone mat for injection. Finally, 10 μL of the final sample was injected onto the analytical column for analysis.

2.6. Bioanalytical method validation

Full validation was performed for mouse plasma and partial validation for eight homogenates using the latest United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [7,8].

2.6.1. Calibration

Calibration standards were prepared in mouse lithium heparin plasma. A double blank (no IS), a blank (IS only), and eight non-zero calibration standards were analyzed in three independent runs in duplicate (n = 6). A linear regression of $1/x^2$ was used to express the area ratio between cintirorgon/IS against the corresponding cintirorgon concentration (x). At least 75% of the non-zero calibration standards should be within ± 15% deviation of the nominal concentration to accept the analytical run.

2.6.2. Accuracy and precision

The assay performance was determined in three independent runs in 6-fold (n = 18) at QC-LLOQ, QC-L, QC-M, and QC-H level in

mouse lithium heparin plasma. For the eight homogenates, the performance was determined at QC-M level only (n = 18). The accuracy should be between 85%–115% (80–120% for the LLOQ) accuracy to meet the criteria. The overall inter- and intraday precision was considered acceptable if variations were within ± 15% standard deviation (± 20% for the LLOQ) [7,8].

2.6.3. Selectivity

Singular analysis of six individual blank mouse lithium heparin plasma and four individual mouse homogenates was used to assess selectivity and specificity at LLOQ level. The response from each spiked sample and its corresponding double blank were compared. The interference in the double blank samples at the retention time of the analyte and IS should be < 20% and < 5%, respectively [7,8].

2.6.4. Recovery and matrix effect

The matrix effect (n = 4, at QC-L, QC-M, and QC-H level), relative matrix effect (n = 6, at QC-L and QC-H level), and extraction recoveries (n = 4, at QC-L, QC-M, and QC-H level) were determined for cintirorgon in mouse lithium heparin plasma. For the homogenates, the relative matrix effect (n = 4) was determined at QC-L and QC-H levels. To determine the matrix effect, two sets of samples were prepared, a matrix present and a matrix absent set of samples. Matrix present samples were prepared by spiking 20 µL blank extracts with 10 µL working solution in 50% methanol at QC-L, QC-M, or QC-H level. Matrix absent samples were prepared by spiking 10 µL working solution in 50% methanol at QC-L, QC-M, or QC-H level to 10 µL acetonitrile and then diluting it with 200 µL 50% methanol. The absolute area response of the matrix present samples should be between 85%–115% of the matrix absent samples [7,8]. The relative matrix effect was determined in individual mouse plasma and individual homogenates by dividing the area ratio of the matrix present samples with the area ratio of the matrix absent samples. The area ratio of the matrix present samples should be within 85–115% of the matrix absent samples. The extraction recoveries were calculated by dividing the area ratio of the extracted (and diluted) spiked plasma samples by the area ratio of the matrix present samples.

2.6.5. Stability

Various stability experiments in mice plasma and eight homogenates were performed including final extract autosampler (4 °C) stability, freeze (−30 °C)/thaw (room temperature) stability, short-term stability (5 h at room temperature). Long-term stability was tested in mouse plasma (−30 °C, 9 months).

2.6.6. Incurred samples reanalysis

Seven mouse homogenates (there were no study samples of lungs) from six different mice and the last time point of the plasma samples from of the same mice were reanalyzed after 1 week to verify the reliability of the reported cintirorgon concentrations in the study samples. The difference between the reanalyzed and original samples concentrations was divided by the mean concentration of the two results. Two thirds of the reanalyzed results should be within ± 20% of the mean to meet the criteria.

2.7. Pharmacokinetic study in mice

For the preclinical study, 50 mg/mL cintirorgon was prepared in dimethyl sulfoxide (DMSO), which was diluted in polysorbate 80/ethanol (1:1, v/v) and then further diluted in 5% glucose water (w/v) to obtain a final concentration of 4 mg/mL cintirorgon. Thus, the concentrations of DMSO, polysorbate 80, ethanol, and glucose in the dosing solution in water were 8%, 4%, 4%, and 4.2% (v/v/v/w), respectively. The housing and handling of the 6 wild-type (FVB/NRj) male mice were according to the institutional guidelines complying with the Dutch and EU legislation. After oral administration of cintirorgon, blood was sampled via the tail vein at t = 0.05, 0.125, 0.25, 0.5, and 1 h, using heparin-containing tubes (Sarstedt, Nümbrecht, Germany). At t = 2 h, the mice were anesthetized with isoflurane and blood was again collected in heparin-containing tubes, but this time by cardiac puncture. The mice were then sacrificed by cervical dislocation to collect the brain, kidney, liver, spleen, small intestine, and testis. The small intestine was separated from the small intestine content, which was also collected, and washed with cold saline to remove any remaining feces. Plasma was isolated from the whole blood samples by centrifugation at 9,000 ×*g* for 6 min at 4 °C. The brain, kidney, liver, spleen, small intestine, small intestine content, and testis were weighed and 1, 2, 3, 1, 3, 2, and 1 mL of 2% (w/v) bovine serum albumin was added, respectively. As a final step, the samples were homogenized with the Fast Prep-24™ 5 G (MP Biomedicals, Santa Ana, CA, USA) homogenizing device. All samples were stored at −30 °C until analysis. PK parameters were calculated using the PKSolver add-in for excel [9].

3. Results and discussion

3.1. Method development

Preclinical PK studies in mice usually generate many samples of which a majority has low sample volume. For this reason, a simple and fast 96-well format was used for the protein precipitation step which also enables working with low sample volumes. Because of the lack of a commercially available stable isotopically-labeled IS, several compounds (e.g. monensin, salinomycin, and diclofenac) were tested as IS alternatives [10,11]. The retention time of monensin was most similar to that of cintirorgon, with a difference of only 0.2 min, and was thus selected for further use as IS (Fig. 1).

ESI-mass spectrometric parameters for cintirorgon were optimized by direct infusion. Abundant Q1 peaks for cintirorgon after positive ionization were the ammonium and sodium adducts (Fig. 2A) in ammonium hydroxide solution and only the sodium adduct in formic acid solution, after negative ionization the protonated molecule was found. Collision-induced dissociation (CID) revealed the most abundant product ions for the ammonium adduct Fig. 2B). The two most abundant product ions of this precursor were selected for quantification. For monensin, ESI-MS/MS optimization was described previously by our group resulting in the use of the sodium adduct and its CID product for quantification [11].

For chromatographic optimization, alkaline versus acidic conditions were compared with the latter showing better peak shapes. This might be caused by free silanol groups that become protonated in an acidic environment. However, since better MS responses were observed when ammonia was present to facilitate detection of the ammonium adduct, ammonium hydroxide was added to the acidic mobile phase with formic acid. Next, acetonitrile was compared to methanol as organic modifier. Better responses were observed with methanol. This was probably because of the proton-donating properties of methanol at high concentrations and cintirorgon eluting at a concentration of 70% methanol.

At first, injection volumes of 30 µL were used but the accuracy and precision at the initial LLOQ level of 2 ng/mL did not meet the criteria for the homogenates for some tissues (Supplemental Table 1). In contrast, the performance in mouse lithium heparin plasma with injection volumes of 30 µL was acceptable. The poor performance in the homogenates was most likely because of matrix effects. It was therefore decided to lower the injection volume from 30 to 10 µL, exposing less matrix onto the system which resolved the issue. However, because of the decreased sensitivity, it was necessary to increase the LLOQ from 2 to 5 ng/mL, as 2 ng/mL failed to meet the criteria (Table 2). For the sake of simplicity of the method, the LLOQ was increased for all matrices as no results below LLOQ

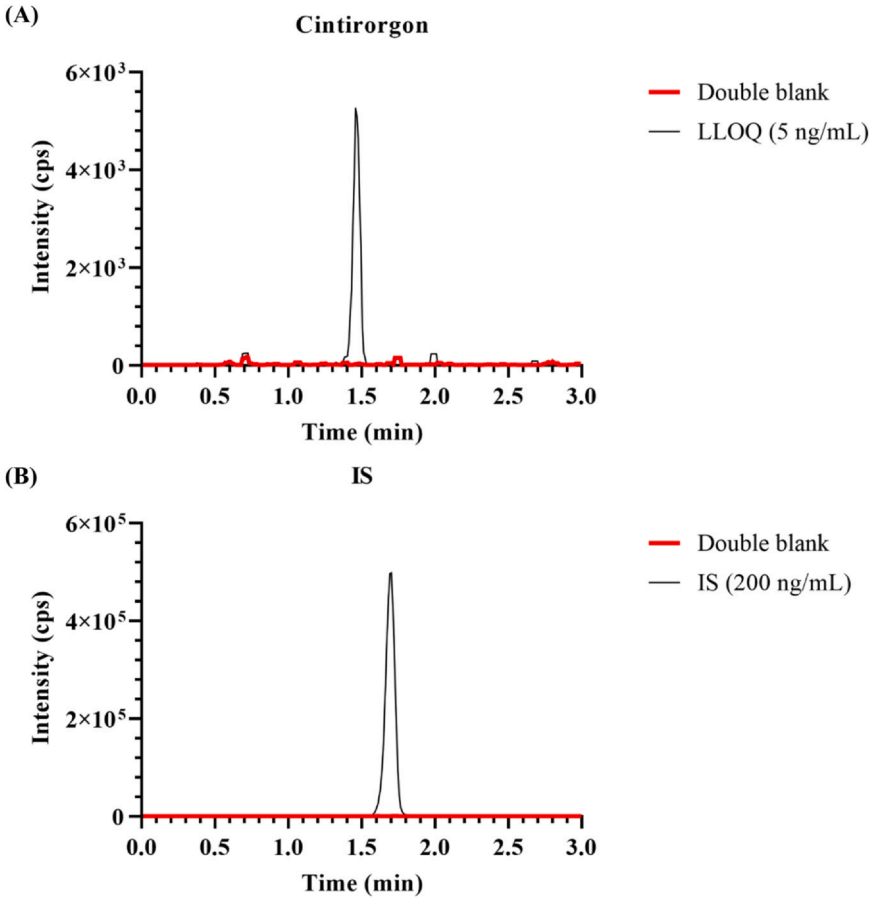

Fig. 1. Representative LC-MS/MS chromatograms in mouse lithium heparin plasma of a double blank (red) and cintirorgon (black) at LLOQ level (A) and a double blank (red) and IS (black) at the concentration used for quantification (B, 200 ng/mL).

were found for cintirorgon concentrations in the study samples. However, to meet the criteria for the selectivity experiments, it was necessary to increase the LLOQ of the brain homogenates further from 5 to 10 ng/mL. This decreased the deviation from the nominal concentration from −22.6% to −11.6%.

3.2. Validation

3.2.1. Calibration

Calibration curves were linear within the validated concentration range of 2–4,000 ng/mL where a linear regression of $1/x^2$ was used to express the area ratio cintirorgon/IS against the corresponding cintirorgon concentration (x). At least 75% of the non-zero calibration standards were within ± 15% standard deviation. The regression parameters were y = −0.0004 (± 0.0048) +0.0028 (± 0.0003)x, with y being the area ratio cintirorgon/IS and x being the cintirorgon concentration in ng/mL. The regression coefficient was r = 0.9942 (± 0.0013).

3.2.2. Accuracy and precision

Results of the accuracy and precision experiments are reported in Table 2. All four concentration levels tested in mouse lithium heparin plasma and the single concentration level in all eight homogenates were within ± 15% (± 20% at LLOQ level) deviation, as required by the guidelines [7,8].

3.2.3. Selectivity

The selectivity of this method was considered acceptable as the responses in the double blank samples at the retention time of cintirorgon and the IS in all matrices were ≤4.67% and ≤ 0.49% of the corresponding LLOQ (5 ng/mL) responses, respectively. The average measured concentration in the plasma and homogenates that were spiked at LLOQ level were all within ± 20% deviation, except for the brain (Table 3). To meet the criteria, the LLOQ for the brain homogenates was increased from 5 to 10 ng/mL, which improved the deviation from −22.6% to −11.6%. The highest deviation was found in the liver homogenates and was −15.8%.

3.2.4. Recovery and matrix effect

Matrix effects in mouse lithium heparin plasma at QC-L, QC-M, and QC-H level were between 100.2%–109.0%. The extraction recoveries at the same levels were between 90.6%–112.4% (Table 4). The relative matrix effect of the 9 different tested matrices at QC-L and QC-H level were between 91.6%–107.5% (Table 5). Based on these results, (relative) matrix effects and recovery losses were considered negligible.

3.2.5. Stability

Stability results of cintirorgon in all tested conditions are reported in Table 6. In plasma, QC-L and QC-H levels were tested. At least 24 h at room temperature and 9-month storage at 30 °C showed cintirorgon to be stable. At least three freeze (−30 °C)/thaw (room temperature) cycles were acceptable, and the recovered responses of the final extracts after 9 days at 4 °C were within 85–115%. Lastly, the recovered responses of the homogenates at QC-M level at room temperature after 5 h were also within 85–115%.

3.2.6. Incurred samples reanalysis

The acceptance criteria for the singular reanalysis of the plasma samples (n = 6) and homogenates (n = 42) were met. Only 1 out of 6

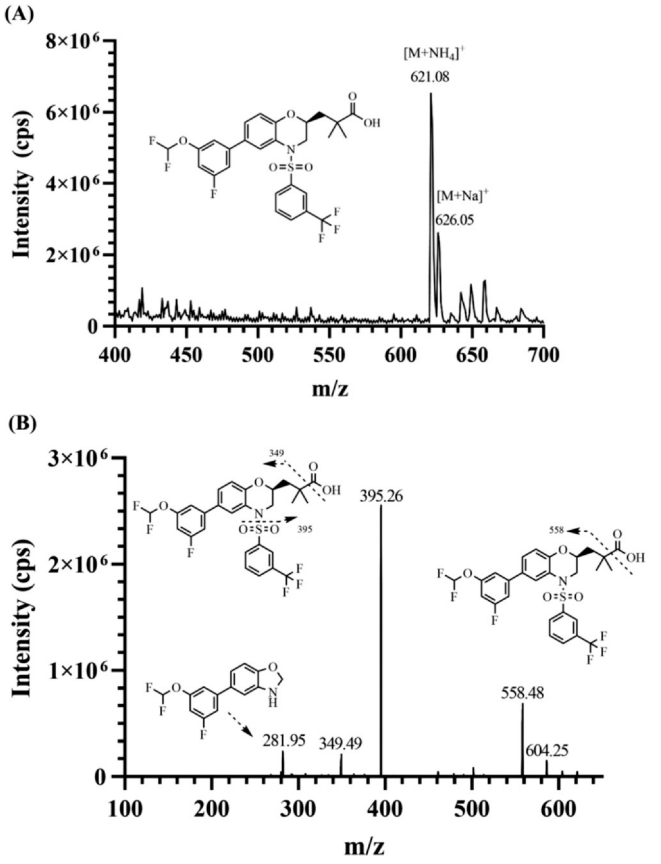

Fig. 2. ESI spectrum of cintirorgon (A) and product ion spectrum of cintirorgon with its proposed fragment ions (B) with 0.4% ammonium hydroxide/methanol (1:1, v/v) as solvent.

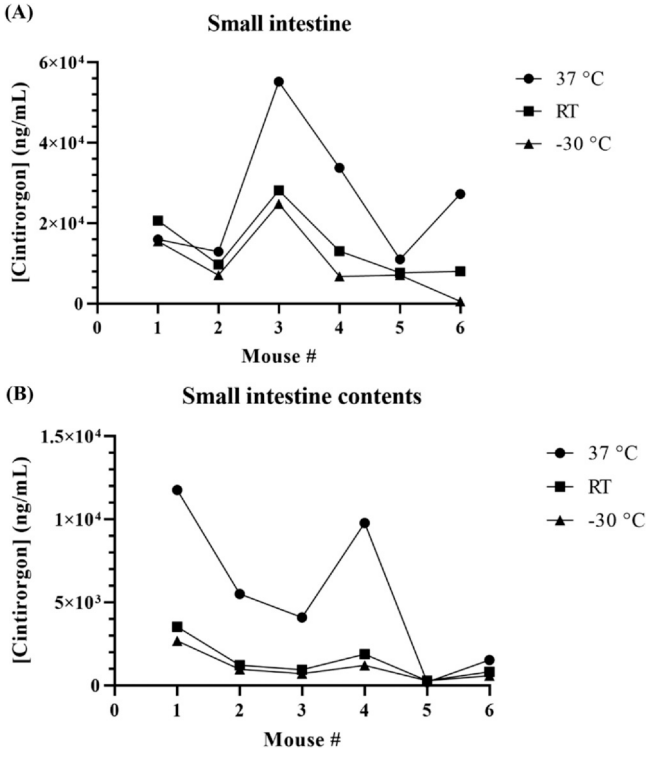

Fig. 3. Cintirorgon concentrations (ng/mL) in small intestine (A) and small intestine content (B) of six individual mice after 4-h storage at three different conditions.

Table 2
Assay performance data for cintirorgon in mouse lithium heparin plasma and mouse homogenates (n = 18 on 3 different days).

Matrix	Level (ng/mL)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
Plasma	2	100.3	23.9	25.9
	5	97.0	14.7	15.6
	10	99.3	7.5	10.4
	100	92.8	4.6	5.1
	3,000	89.1	4.5	7.9
Brain	100	111.2	9.2	10.3
Kidney	100	92.3	8.1	9.5
Liver	100	96.8	7.6	10.7
Lung	100	96.1	11.1	12.7
Small intestine	100	88.6	10.0	13.4
Small intestine contents	100	109.4	7.3	9.5
Spleen	100	100.8	9.1	9.8
Testis	100	101.0	5.9	13.9

Table 3
Selectivity data in mouse plasma and homogenates.

Matrix	Spiked concentration (ng/mL)	Mean measured concentration (ng/mL)	Deviation (%)	n
Brain	5	3.87	-22.6	4
Brain	10	8.84	-11.6	4
Kidney	5	4.76	-4.8	4
Liver	5	5.79	15.8	4
Lung	5	4.56	-8.8	4
Plasma	5	5.28	5.6	6
Small intestine	5	4.46	-10.8	4
Small intestine contents	5	4.21	-15.8	4
Spleen	5	4.55	-9.0	4
Testis	5	5.21	4.2	4

Table 4
Matrix effect and recovery data in mouse lithium heparin plasma.

Level	Matrix effect (%)	Recovery (%)
QC-L	109.0 ± 12.9	93.7 ± 2.7
QC-M	107.1 ± 4.7	90.6 ± 4.7
QC-H	100.2 ± 9.9	112.4 ± 8.9

Table 5
Relative matrix effect in mouse lithium heparin plasma (n = 6) and homogenates (n = 4).

	Relative matrix effect at QC-H level (ratio, %)	Relative matrix effect at QC-L level (ratio, %)
Brain	101.23 ± 2.73	94.80 ± 12.20
Kidney	97.04 ± 1.81	91.53 ± 6.03
Liver	96.65 ± 2.41	107.48 ± 7.79
Lung	100.13 ± 0.99	102.66 ± 5.39
Plasma	93.42 ± 4.81	101.27 ± 12.87
Small intestine	101.13 ± 1.55	102.84 ± 12.61
Small intestine contents	104.54 ± 2.60	102.12 ± 13.51
Spleen	101.05 ± 0.59	97.41 ± 17.68
Testis	105.82 ± 2.61	95.45 ± 11.17

reanalyzed plasma samples exceeded the ± 20% requirement. For the homogenates, none of the reanalyzed samples exceeded the requirements except for the small intestine and small intestine contents. Interestingly, these homogenates had higher cintirorgon concentrations than initially measured. During analysis of the study samples, there was an extra observed peak with a lower retention time than cintirorgon. It was hypothesized that this extra peak was a metabolite and that it was enzymatically hydrolyzed to form

Table 6
Stability data of cintirorgon in mouse lithium heparin plasma and homogenates at short term conditions at room temperature (RT, 24 h or 5 h), long term conditions (−30 °C, 9 months), three freeze (−30 °C)/thaw (RT) cycles, and autosampler conditions (4 °C).

Matrix	Condition	Level (ng/mL)	Recovery (%)	n
Brain	RT, 5 h	100	92.4 ± 2.6	4
Kidney	RT, 5 h	100	97.6 ± 2.6	4
Liver	RT, 5 h	100	100.5 ± 3.0	4
Lung	RT, 5 h	100	99.2 ± 5.0	4
Plasma	3 F/T	3,000	113.2 ± 12.1	4
		5	98.2 ± 5.3	4
	RT, 24 h	3,000	107.1 ± 7.9	4
		5	105.3 ± 10.3	4
	-30 °C, 9 months	3,000	110.8 ± 4.1	4
		5	104.7 ± 12.7	4
Plasma extract	4 °C, 9 days	3,000	90.1 ± 3.4	3
		10	105.7 ± 8.7	6
SI	RT, 5 h	100	101.8 ± 6.8	4
Small intestine contents	RT, 5 h	100	105.7 ± 4.7	4
Spleen	RT, 5 h	100	99.8 ± 4.7	4
Testis	RT, 5 h	100	92.7 ± 3.1	4

*Abbreviations: F/T = freeze (−30 °C)/thaw (RT), RT = room temperature, h = hours.

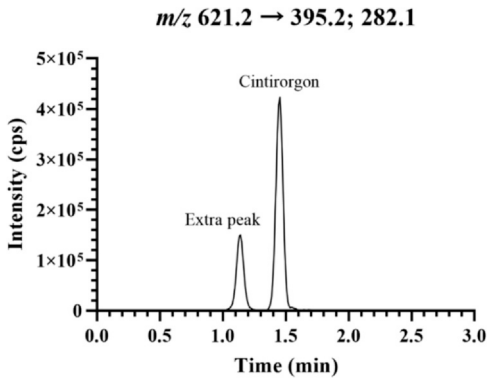

Fig. 4. Chromatogram of the small intestine content from a wild-type mouse where an extra peak was observed.

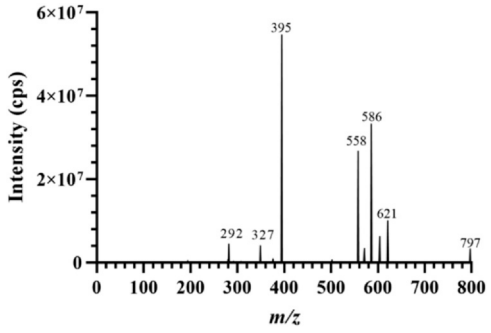

Fig. 5. Product-ion scan between 1.1 and 1.14 min (m/z 797.3 → 621.2; 395.2).

cintirorgon. An experiment where the small intestine and its contents were stored separately for 4 h at three different conditions (at −30 °C, room temperature, and 37 °C) showed results in line with this hypothesis. As shown in Figure 3, slightly higher cintirorgon concentrations were measured for the samples that were stored at room temperature compared to the ones that were stored at −30 °C. At 37 °C, cintirorgon concentrations were significantly increased in most samples. Since glucuronidation is an important pathway in drug metabolism, it was tested if the extra peak originated from cintirorgon-glucuronide that undergoes in-source fragmentation and would therefore be observed in the cintirorgon mass transition.

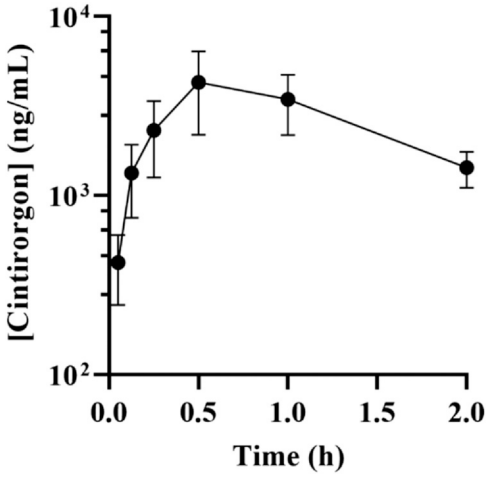

Fig. 6. Plasma curve of cintirorgon in wild-type mice up to t = 2 h after 40 mg/kg oral administration (n = 7).

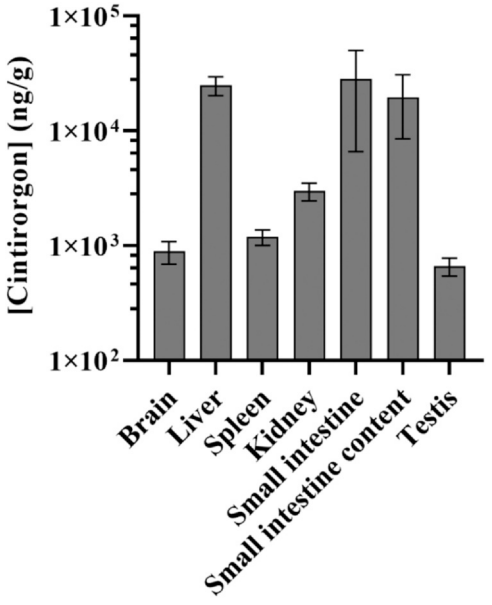

Fig. 7. Measured tissue concentrations of cintirorgon in wild-type mice at t = 2 h after 40 mg/kg oral administration (n = 7).

A product-ion spectrum was recorded at the retention time of the extra peak between 1.1 and 1.14 min (Fig. 4 and 5). The mass of cintirorgon-glucuronide ammonium adduct (*m/z* 797.3), cintirorgon ammonium adduct (*m/z* 621.2), and the product ion (*m/z* 395.2) were all present in the spectrum which supports the hypothesis that the extra peak is indeed cintirorgon-glucuronide. (Fig. 5).

3.3. Preclinical application

The applicability of the method was successfully shown in a PK study with mice. A representative plasma PK curve up to 2 h after 40 mg/kg cintirorgon oral administration is shown in Figure 6. Figure 7 shows the calculated cintirorgon concentrations in the tissues at t = 2 h. The T_{max} was reached at 0.86 ± 0.56 h, the C_{max} was 4109 ± 2033 ng/mL, and the $AUC_{0-2\,h}$ was 5,355 ± 1,906 ng·mL^{-1}·h^{-1}. The elimination phase parameters need further attention and are therefore not mentioned here. In this study, the peak concentration of cintirorgon (4,109 ± 2,033 ng/mL) in mice with oral 20 mg/kg cintirorgon were of the same order as seen in patients treated

with cintirorgon at the clinically recommended dose (C_{max} = 3,340–12,200 ng/mL), albeit at the low end [6] (Supplementary Table 1).

4. Conclusion

To the best of our knowledge, this is the first validated bioanalytical method reported for the analysis of cintirorgon. Full method validation was performed in mouse plasma and partial validation for eight different mouse homogenates (brain, kidney, liver, lung, small intestine, small intestine content, spleen, and testis) based on the current international guidelines [7,8]. Because of the large number of samples usually produced by PK studies, a fast and simple sample preparation method was established using a 96-well format. This enabled analysis of a single sample within 3 min. The method was successfully applied in a preclinical PK study with mice where cintirorgon concentrations were quantitively determined in plasma and seven homogenates (brain, kidney, liver, small intestine, small intestine content, spleen, and testis).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2021.114421.

References

[1] Study of LYC-55716 in adults subjects with locally advanced or metastatic cancer - Tabular View - ClinicalTrials.gov, (n.d.). ⟨https://www.clinicaltrials.gov/ct2/show/record/NCT02929862?term=lyc-55716&draw=2&rank=2⟩ (accessed March 18, 2021).
[2] D.R. Camidge, S.M. Gadgeel, H.J. Wilkins, G. Weems, R. Santana-Davila, M. Johnson, RORγ agonist LYC-55716 in combination with pembrolizumab to treat metastatic non-small cell lung cancer: an open-label, multicenter phase Ib trial, Ann. Oncol. 29 (2018) viii417, https://doi.org/10.1093/annonc/mdy288.042
[3] A. Ladurner, P.F. Schwarz, V.M. Dirsch, Natural products as modulators of retinoic acid receptor-related orphan receptors (RORs), Nat. Prod. Rep. 38 (2021) 757–781, https://doi.org/10.1039/D0NP00047G
[4] A. Young, D. Mittal, J. Stagg, M.J. Smyth, Targeting cancer-derived adenosine: new therapeutic approaches, Cancer Disco 4 (2014) 879–888, https://doi.org/10.1158/2159-8290.CD-14-0341
[5] novel_immuno_oncology_targets.png (1179×768), (n.d.). ⟨https://lycera.com/wp-content/uploads/2016/03/novel_immuno_oncology_targets.png⟩ (accessed January 21, 2021).
[6] D. Mahalingam, J.S. Wang, E.P. Hamilton, J. Sarantopoulos, J. Nemunaitis, G. Weems, L. Carter, X. Hu, M. Schreeder, H.J. Wilkins, Phase 1 open-label, multicenter study of first-in-class RORγ agonist LYC-55716 (cintirorgon): safety, tolerability, and preliminary evidence of antitumor activity, Clin. Cancer Res. 25 (2019) 3508–3516, https://doi.org/10.1158/1078-0432.CCR-18-3185
[7] US Food and Drug Administration (FDA), Bioanalytical method validation guidance for industry, 2018. ⟨https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf⟩ (accessed September 10, 2021).
[8] European Medicines Agency (EMA), Guideline on Bioanalytical Method Validation. Committe for Medicinal Produts for Human Use and European Medicines Agency., Londen. (2011).
[9] Y. Zhang, M. Huo, J. Zhou, S. Xie, PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel, Comput. Methods Prog. Biomed. 99 (2010) 306–314, https://doi.org/10.1016/j.cmpb.2010.01.007
[10] R.W. Sparidans, J.S. Lagas, A.H. Schinkel, J.H.M. Schellens, J.H. Beijnen, Liquid chromatography-tandem mass spectrometric assay for diclofenac and three primary metabolites in mouse plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 872 (2008) 77–82, https://doi.org/10.1016/j.jchromb.2008.07.012
[11] R.W. Sparidans, J.S. Lagas, A.H. Schinkel, J.H.M. Schellens, J.H. Beijnen, Liquid chromatography-tandem mass spectrometric assays for salinomycin in mouse plasma, liver, brain and small intestinal contents and in OptiMEM cell culture medium, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 855 (2007) 200–210, https://doi.org/10.1016/j.jchromb.2007.05.008