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Development and validation of a high-performance liquid chromatography method for levothyroxine sodium quantification in plasma for pre-clinical evaluation of long-acting drug delivery systems

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Levothyroxine (LEVO) sodium is an FDA-approved drug that is used to treat underactive thyroid (hypothyroidism) and other conditions. It is generally used as a thyroid-stimulating hormone administered orally. However, this approach has some drawbacks such as this drug should be taken every day 30 min to 1 h prior to breakfast with an empty stomach, moreover, some food interactions must be monitored. Thus, alternative innovative approaches capable of providing sustained LEVO release should be developed. Our research was designed to establish a simple quantitative determination method for LEVO in rat plasma for pre-clinical evaluation of long acting formulations using a high-performance liquid chromatography method, to validate the analytical method according to ICH guidelines and to characterise its pharmacokinetic behavior in rats. After simple protein precipitation with acetonitrile, LEVO was eluted on a Xselect CSH™ C18 column (Waters, 3.0 × 150 mm) with a particle size of 3.5 μm using a mobile phase of water and acetonitrile at a ratio of 65 : 35% v/v, including 0.1% v/v of trifluoracetic acid. The calibration standards used for plasma ranged between 0.5–1000 ng mL^{-1} with a correlation coefficient (r^2) of ≥0.998. The limit of detection was 0.44 ng mL^{-1} and the lower limit of quantitation was 1.33 ng mL^{-1}. The extraction recovery of LEVO in rat plasma samples by this method was between 80 and 85%. The method was selective, sensitive, accurate and precise for detecting and quantifying LEVO in a pharmacokinetic study carried out in rats for pre-clinical evaluation of long acting formulations. The validated HPLC method meets the ICH established requirements and therefore offers a wide range of potential applications in pre-clinical therapeutic drug monitoring, pharmacokinetics and toxicology.

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1. Introduction

Hypothyroidism is a common disease caused by inadequate function of the thyroid gland.[1] This condition is characterised by lower endogenous secretion of triiodothyronine (T3) and thyroxine (T4). In order to preserve the function of the cardiovascular system, and mental and physical health, sufficient production of these thyroid hormones is vital. Moreover, these hormones are also important in the growth and development of children through the regulation of the transcription of genes.[2,3] To treat this disease, levothyroxine (LEVO) sodium is generally used as a thyroid-stimulating hormone administered orally.[4]

When administered orally, LEVO shows a bioavailability of 65%. However, this drug should be taken 30 min to 1 h prior to breakfast with an empty stomach. Moreover, this tablet should be taken with a full glass of water as LEVO forms complexes with products made from soybean, calcium, sodium and other minerals.[5,6] Additionally, LEVO should be taken every day and the treatment requires extra assistance of doctors and pharmacists.[3] Additionally, untreated hypothyroidism can result in symptoms such as weight gain, chronic fatigue and cold intolerance. Moreover, if this condition remains untreated for a long time more serious symptoms may appear (myxoedema coma among others) that can lead to premature death due to cardiovascular complications.[7,8] Accordingly, there is a clear need for more efficient drug delivery systems for LEVO administration. A potential alternative to solve these limitations is the use of LEVO loaded implantable devices.

In order to develop new drug delivery systems, such as implantable devices, robust analytical methods need to be developed and validated to evaluate *in vitro* and *in vivo* drug

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release.[9,10] Determination of drug pharmacokinetics in an animal model is critical before any drug delivery system can move to clinical trials.[11,12] Accordingly, a reliable HPLC method to quantify LEVO in plasma is required. Previously described HPLC methods require the use of mass spectrometry or complicated extraction steps.[13–15] The use of complex equipment/extraction procedures will limit the applicability of the method. Accordingly, there is a clear need for simple methods to quantify LEVO in plasma.

In the present work, a simple method for the quantification of LEVO in plasma for pre-clinical evaluation of long acting formulations has been described and validated according to the International Council for Harmonisation (ICH) guidelines.

2. Experimental section

2.1 Chemicals and materials

LEVO sodium pentahydrate (purity: ≥99%) was obtained from Enke Pharma-tech (Cangzhou, China). Trifluoroacetic acid (TFA) (purity: ≥99%) and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich Pte Ltd, (Singapore). HPLC column Xselect CSH™ C_{18} (Waters, 3.0 × 150 mm, 3.5 μm particle size) was obtained from Waters (Dublin, Ireland). Dichloromethane (DCM) was purchased from Merck (Darmstadt, Germany). Poly(caprolactone) (PCL) (CAPA 6506; MW = 50 000 Da) was provided by Perstorp (Malmö, Sweden). All other reagents were of analytical grade and obtained from standard commercial suppliers.

2.2 Preparation of stock, calibration standard and quality control samples

A stock solution of LEVO was prepared by solubilizing 50 mg of LEVO in 50 mL ethanol in a volumetric flask. The final concentration of the stock solution was 1 mg mL^{-1}. Subsequently, the working standard solution preparation was carried out by appropriate dilution of the stock solution using the mobile phase. The preparation of the calibration standard and the quality control (QC) solutions was carried out by consecutive dilution of the working standard solutions. The solutions used to prepare the calibration plot in plasma were prepared by spiking 20 μL of the standard working solution with 180 μL of blank rat plasma to obtain a series of solutions with concentrations ranging between 0.5 ng mL^{-1} and 1000 ng mL^{-1}. Moreover, three different concentrations of the QC solution were also prepared in plasma utilizing a similar method. In this study, the concentrations of the QC solution used were 5 ng mL^{-1}, 100 ng mL^{-1} and 750 ng mL^{-1} for low QC, medium QC and high QC, respectively.

2.3 Sample preparation and analyte extraction

To extract LEVO from plasma samples, a one-step protein precipitation method was applied. Specifically, acetonitrile was used as an appropriate solvent. In addition, to obtain the highest extraction efficiency, the effect of the solvent volume on the extraction efficiency of levothyroxine from plasma was investigated by using different solvent volumes, namely 300 μL, 500 μL, 700 μL and 900 μL. The solvent was used to extract LEVO from 100 μL of plasma samples. Following the addition of the extraction solvent, the mixture was vortexed for 10 min. Subsequently, to precipitate the protein, the samples were further centrifuged at 14 000×*g* for 15 min at 4 °C. The supernatant was collected in a glass vial. To obtain the LEVO dry extract from plasma samples, the solvent was evaporated in a fume hood for 3 h. Afterwards, the extract obtained was reconstituted using 100 μL of the mobile phase and the mixture was vortexed for 10 min. Then, the samples were centrifuged at 14 000×*g* for 15 min. To determine the amount of LEVO in the plasma samples, the clear supernatant was collected and injected into the HPLC column and analyzed using HPLC-UV.

2.4 Instrumentation and development of HPLC-UV conditions

To quantify the amount of LEVO in plasma samples, a HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan) system with a DAD detector was used. As the stationary phase for analyte separation, an Xselect CSH™ C18 column (waters, 3.0 × 150 mm) with a particle size of 3.5 μm was used. As the mobile phase, a mixture of 0.1% v/v trifluoracetic acid in water and acetonitrile at a ratio of 65 : 35% v/v was used. The quantification of LEVO was carried out at a flow rate of 0.5 mL min^{-1} with a UV detector at 225 nm, an injection volume of 20 μL and at 25 °C.

2.5 Validation of the bioanalytical method

The bioanalytical method for LEVO quantification in plasma samples was validated according to the guidelines from the ICH and the US FDA.[16,17] Selectivity, linearity, lower limits of quantification (LLOQ), carry over, dilution integrity, accuracy, precision, extraction recovery and stability were all evaluated as the validation parameters.

2.5.1 Selectivity. The blank plasma and the plasma spiked with LEVO were injected into the HPLC and analyzed using the method developed. The sample preparations were performed as per the selected extraction procedure developed previously.

2.5.2 Linearity, LOD and LLOQ. To evaluate the linearity of the analytical method, the calibration curve of LEVO in plasma samples was prepared in the concentration range described earlier. Specifically, the calibration curve consisted of seven consecutive concentrations and all working solutions were analyzed at three different times.

To calculate the lower limit of quantitation (LLOQ) and the limit of detection (LOD), the standard deviation (SD) of the response and the calibration curve slope were first calculated. Afterwards, the LLOQ and LOD were determined using the following equations.

$$\mathrm{LOD} = 3.3\sigma/S \quad (1)$$

$$\mathrm{LLOQ} = 10\sigma/S \quad (2)$$

Where σ = the SD of the response of the data and S = the calibration curve slope.[18]

2.5.3 Accuracy and precision. Accuracy and precision evaluations of the analytical method were performed in six replicates for the sample concentrations of LLOQ, low QC, medium QC and high, as stated previously. To evaluate the accuracy and precision of the method, the relative standard deviation (RSD) of the responses and the relative errors (RE) of all solutions tested were calculated, respectively. Particularly, the intra-day and inter-day accuracy and precision were assessed. The accuracy and precision were considered to fulfil the criteria when the RSD and RE of all values were ±15%.[16,17]

2.5.4 Carry-over and dilution integrity. High concentrations of QC samples were injected to ascertain the absence of carry-over. Then, a blank plasma sample was injected. The chromatogram of blank plasma solution was evaluated, and, when observed, the area of LEVO detected in the blank samples should not be more than 20% of the area of the sample solution at the LLOQ concentration.[17]

The dilution integrity was evaluated by spiking the blank plasma with LEVO with the concentrations beyond the highest concentration of calibration standard solutions. The spiked plasma samples were diluted 5 and 10 times with blank plasma. LEVO was extracted and calculated. The accuracy and precision of all samples were assessed.

2.5.5 Extraction recovery. The extraction recovery of LEVO from plasma samples was calculated by equating the concentration of LEVO analyzed from plasma samples at the concentrations of LLOQ, low QC, medium QC and high QC with the concentration of LEVO analyzed from samples prepared in the mobile phase.

2.6 Assessment of the pharmacokinetics following subcutaneous administration of LEVO implants

The bioanalytical method was applied to the evaluation of pharmacokinetic profiles of LEVO after the administration of implants to rats. The implants were prepared using PCL and LEVO sodium as described previously.[19] In brief, a mixture of PCL and LEVO sodium (PCL/LEVO = 40/60) was dissolved in DCM (2 g of PCL/LEVO and 5 g of DCM). The resulting implants were cylindrical rods (2.5 × 40 mm) containing 67 ± 8 mg of LEVO sodium. These implants were cut into 4 equal parts. Each rat was implanted with one of these smaller implants. The *in vivo* study was officially approved by the ethical Committee of the Faculty of Medicine, Hasanuddin University (protocol number: UH20110635). Before the experiments, healthy male Wistar rats with a mean mass of 221 ± 12 g ($n = 5$) were adapted for 1 week to the laboratory situation. In this study, implants containing 15 mg of LEVO were administered subcutaneously to the rats. Prior to implant insertion, the hair from the dorsal part of the rats was shaved under sedation using ether. Then, an antiseptic solution was applied, and the hairless area was incised around 20 mm in length. Finally, the implants were applied at the cut area. Following administration of the implants, blood samples were taken *via* the tail vein at 2 hours, 4 hours, 8 hours, 1 day, 2 days, 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks, and placed in an Eppendorf tube containing 3.8% w/v of sodium citrate to avoid coagulation of blood. To collect the plasma samples, the blood samples obtained were centrifuged at 4 °C for 10 min at 3000×*g*. The plasma was frozen at −20 °C before analysis. The amount of LEVO was determined using the validated method.

2.7 Statistical analysis

Microsoft® Excel® 2016 (Microsoft Corporation, Redmond, USA) was used to calculate the mean, S.D. RSD and RE. All data were presented as mean ± standard deviation (S.D.) and were statistically analysed using GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA). The pharmacokinetic parameters were calculated using PKSolver (add-in program of Microsoft Excel).[20]

3. Results

3.1. Sample preparation and analyte extraction

In order to extract LEVO from plasma samples, a simple and rapid protein precipitation method was followed using acetonitrile.[21] Initially, different volumes of acetonitrile were used, namely 300 μL, 500 μL, 700 μL and 900 μL to extract LEVO sodium in 100 μL of plasma samples. This step was performed in order to achieve the highest extraction efficiency with the minimum handling time. The extraction efficiencies were found to be 67.98 ± 4.32%, 83.81 ± 7.31%, 84.91 ± 8.28% and 89.87 ± 9.08% for solvent volumes of 300 μL, 500 μL, 700 μL and 900 μL, respectively. According to this result, 500 μL was the optimal volume for LEVO sodium extraction. Higher acetonitrile volumes (700 μL and 900 μL) did not improve the extraction efficiency (p >0.05). Therefore, 500 μL was selected as the optimum volume.

In order to investigate the effect of endogenous compounds in rat plasma on the separation of LEVO sodium, a selectivity study was conducted. This test was performed by observing the chromatograms of blank plasma and plasma samples spiked with LEVO sodium solution. The chromatograms of these samples are presented in Fig. 1. It was found that LEVO sodium eluted at 7.6 minute, and, essentially, the elution times of endogenous substances were not found at the elution time of LEVO sodium, confirming the selectivity of the method.

3.2. Linearity, LOD, and LLOQ

To investigate the LOD and LLOQ of the HPLC method for LEVO sodium quantification a calibration curve was constructed. The calibration plot equation, the LOD and LLOQ are presented in Table 1.

The linearity of the curve was evaluated from the obtained r^2 value (Table 1). The obtained value was 0.998 when analyzing concentrations ranging between 0.5 ng mL^{-1} and 1000 ng mL^{-1}. Following the examination, the LOD and LLOQ values were calculated to be 0.44 ng mL^{-1} and 1.33 ng mL^{-1}, respectively.

3.3. Accuracy and precision

The precision and accuracy of the HPLC method were evaluated by measuring QC samples on the same day and over different days. The results of these experiments are shown in Table 2.

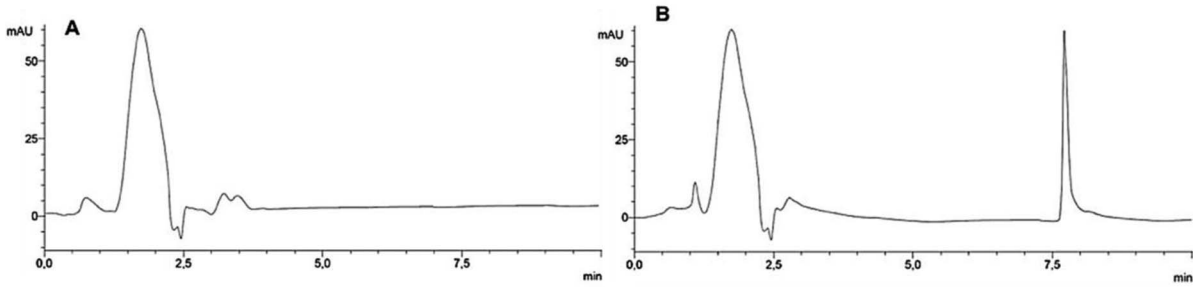

Fig. 1 Representative HPLC-UV chromatograms of blank plasma (A) and plasma spiked with a standard solution of LEVO sodium (500 ng mL^{-1}).

Table 1 Properties of the calibration curve for quantification of LEVO with LOD and LLOQ values

Concentration range	r^2	y-intercept	Slope	LOD (ng mL^{-1})	LLOQ (ng mL^{-1})
0.5–1000	0.998	196.3	1561.5	0.44	1.33

Following these evaluations, the RSD values ranged between 6.47% and 14.48% for intra-day measurements and between 8.66% and 12.10% for inter-day measurements. Furthermore, % RE values were between −3.76 and 12.12 and −4.51 and 6.73 for intra-day and inter-day measurements, respectively. According to these results, the % RSD and % RE were within the range of ±15% which is considered acceptable, suggesting that the developed method was accurate and precise.

Table 2 Precision and accuracy data for the HPLC method for quantification of LEVO in rat plasma (means ± S.D., $n = 3$)

Intra-day precision and accuracy				
Replication	Concentration added (ng mL^{-1})	Concentration found (ng mL^{-1}) ± SD	Precision (% RSD)	Accuracy (% RE)
1	1.33	1.37 ± 0.12	8.76	3.01
	5	4.91 ± 0.32	6.52	−1.80
	100	112.12 ± 9.19	8.20	12.12
	750	743.02 ± 54.18	7.29	−0.93
2	1.33	1.28 ± 0.19	14.84	−3.76
	5	5.13 ± 0.34	6.63	2.60
	100	109.41 ± 10.02	9.16	9.41
	750	755.43 ± 81.31	10.76	0.72
3	1.33	1.39 ± 0.09	6.47	4.51
	5	5.21 ± 0.41	7.87	4.20
	100	104.09 ± 9.01	8.66	4.09
	750	742.33 ± 77.28	10.41	−1.02
Inter-day precision and accuracy				
Day	Concentration added (ng mL^{-1})	Concentration found (μg mL^{-1}) ± SD	Precision (% RSD)	Accuracy (% RE)
1	1.33	1.41 ± 0.17	12.06	6.02
	5	5.18 ± 0.46	8.88	3.60
	100	100.93 ± 9.17	9.09	0.93
	750	759.68 ± 72.01	9.48	1.29
2	1.33	1.39 ± 0.16	11.51	4.51
	5	5.03 ± 0.49	9.74	0.60
	100	104.67 ± 9.82	9.38	4.67
	750	761.05 ± 80.08	10.52	1.47
3	1.33	1.27 ± 0.11	8.66	−4.51
	5	5.04 ± 0.61	12.10	0.80
	100	106.73 ± 11.27	10.56	6.73
	750	752.47 ± 68.18	9.06	0.33

Table 3 Mean extraction recoveries of LEVO in rat plasma (means ± S.D., $n = 3$)

Concentration added (μg mL^{-1})	% extraction recovery ± SD	% RSD
1.33	83.21 ± 7.32	8.80
5	80.19 ± 8.01	9.99
100	84.32 ± 6.92	8.21
750	82.93 ± 7.43	8.96

3.4. Carry-over and dilution integrity

In our study, we did not observe any carry-over effect in the chromatogram of blank plasma samples following the injection of LEVO sodium samples at high QC concentration. The chromatograms of blank samples did not show any peaks at 7.6 min which was the retention time of LEVO sodium. Accordingly, it could be established that there was no effect for higher LEVO sodium concentrations. Furthermore, bearing in mind that the concentration of LEVO sodium in plasma could not be predicted, dilution integrity evaluation was carried out. This step was performed to investigate the effect of dilution on the actual concentration of LEVO sodium in plasma matrices. In this study, the dilution factors were 5 and 10. The results showed that following the determination of LEVO sodium concentration for these dilution factors, the recovery percentages were in the range of 98.27 ± 9.01%–101.81 ± 11.92% with a precision of 8.09–13.98%. Considering the acceptable precision of ±15%, the dilution integrity evaluation of this method was found to meet the ICH established requirements.

3.5. Extraction recovery

In order to investigate the recovery of the drug from plasma samples, recovery was determined at different LEVO concentrations. Table 3 shows the extraction efficiency and % RSD values obtained in this evaluation. It was observed that the extraction recovery values were between 80.19 ± 8.01% and 84.32 ± 6.92% with % RSD between 8.21% and 9.99%. When analyzed statistically, there was no significant difference in the extraction efficiency values of LEVO sodium from plasma at all concentrations. Moreover, because the RSD values were less than 15%, the extraction process of the method was reproducible and precise.

Table 4 Pharmacokinetic parameters of LEVO sodium after subcutaneous administration of implant formulation in Wistar rats (means ± SD, $n = 3$)

Parameter	Value
C_{max} (ng mL^{-1})	4.72 ± 0.74
T_{max} (day)	14
AUC_{0-t} (ng per mL per day)	89.84 ± 15.38
$T_{1/2}$ (day)	12.64 ± 2.09

3.6. Assessment of the pharmacokinetics following subcutaneous administration of LEVO implants

Following the validation of the developed HPLC method, the validated method was applied to study the pharmacokinetics of LEVO sodium administered as subcutaneous implants in rats. These implants were prepared using poly(caprolactone). The resulting implants can be seen in Fig. 2A. The implant size and design were similar to previously reported implants and commercially available products, such as Nexplanon®.[22] In order to study drug release *in vivo*, these full-size implants were cut into 4 equal pieces. Each animal received one of these smaller size implants.

The HPLC method described in previous sections was used to quantify LEVO sodium levels in plasma. Furthermore, the pharmacokinetic parameters such as C_{max}, t_{max}, AUC0-4 week and $t_{1/2}$ were calculated. The concentration of LEVO sodium in rat plasma after the 4 week application period of the implant is shown in Fig. 2B. Furthermore, the calculated pharmacokinetic parameters of LEVO sodium are depicted in Table 4. The maximum plasma concentration of LEVO sodium was found to be 4.72 ± 0.74 ng mL^{-1}. This concentration was achieved after 14 days following the implantation of the PLC implant. Furthermore, the AUC value was 89.84 ± 15.38 ng per mL per day with a mean half-life of 12.64 ± 2.09 days. Accordingly, this validated method was successfully applied to quantify LEVO sodium following *in vivo* delivery of the subcutaneous implant over a 4 week period.

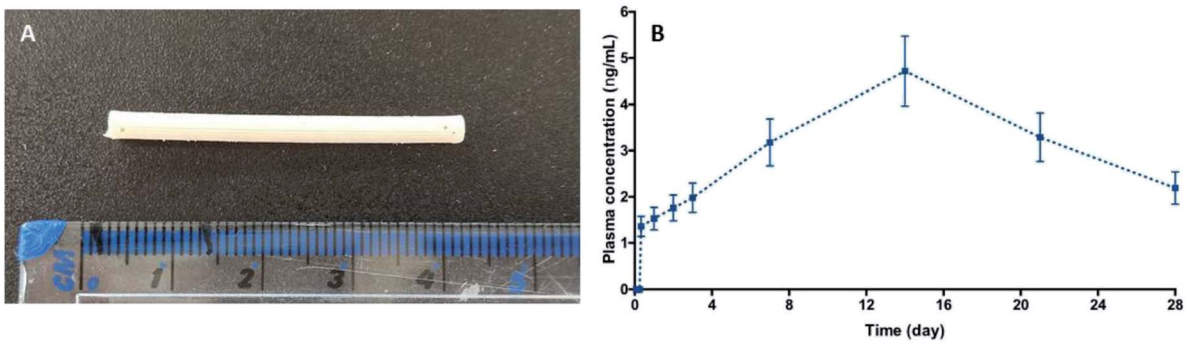

Fig. 2 Image of a full size LEVO sodium implant (A). LEVO sodium plasma concentration as a function of time after subcutaneous administration of an implant in Wistar rats (B) (means ± SD, $n = 3$).

4. Discussion

Previously described methods to measure LEVO sodium *in vivo* require the use of complex extraction procedures coupled with the use of equipment that is not readily available in every lab such as electrochemical detectors or mass spectrometry detectors.[23,24] In this work, we have reported the use of simple HPLC coupled with UV-visible detection that can be used to measure plasma levels of LEVO sodium. Instead of using complex and more expensive sample preparation methods, such as solid phase extraction,[25] we propose the use of a simple protein precipitation technique using acetonitrile. The addition of organic solvents to precipitate proteins has been described extensively.[21,26–29] The method was validated according to ICH guidelines. The simplicity of the bioanalytical method developed in this study provides broad applications in all types of laboratories.

The method described in this work achieved a lower LOD than previously described methods used to quantify LEVO in biological fluids using HPLC coupled with UV-vis detection (0.44 ng mL^{-1} *vs.* 2 ng 20 μL^{-1}).[25] It is obvious that the LLOQ is higher than basal LEVO levels (0.018 ng mL^{-1}).[30,31] However, this method has not been developed to evaluate basal LEVO levels but to be used in pre-clinical drug delivery experiments. Rats are normally the first animal species that are used to check if a drug is released from implantable devices. This method can be highly valuable for preclinical evaluation of LEVO-containing subcutaneous implants or alternative drug delivery systems. Researchers and pharmaceutical companies are interested in the development of long-acting drug delivery systems for the treatment of chronic conditions.[32,33]

Hypothyroidism is a chronic condition requiring daily oral LEVO sodium administration. As described in the introduction section, oral LEVO administration presents certain challenges. Accordingly, LEVO sodium loaded implantable devices capable of providing sustained drug release can be an alternative. The interest of pharmaceutical companies and researchers in this type of drug delivery system is increasing. Titan Pharmaceuticals (USA) is currently evaluating a subdermal implant for hypothyroidism treatment.[34] Moreover, there are some studies describing drug delivery systems designed for sustained LEVO release.[3,35] Accordingly, there is a clear need for implantable devices for LEVO sodium release.

Implantable devices can be formulated in a wide variety of ways ranging from non-covalent hydrogels to solid implantable systems.[32,36–40] In this case, the selected implants were made of poly(caprolactone) a biodegradable and biocompatible polymer that has been extensively used for drug delivery applications.[41] Stewart *et al.* described the use of similar types of implants for LEVO sodium release.[19] However, in this work only *in vitro* release was evaluated. This work described the use of PCL to prepare implants loaded with 20% (w/w) of LEVO sodium. These implants were capable of providing sustained LEVO release for periods of at least 30 days. Interestingly, the obtained levels of LEVO were up to 556 times the basal levels required for healthy humans (0.009–0.018 ng mL^{-1}).[30,31] Additionally, the average weight of a human is *ca.* 300 times the weight of a Wistar rat. Therefore, it can be estimated that the implant size required to achieve drug levels within the therapeutic range for humans should be approximately half the size of the implant administered to rats. This estimation has been done without taking into account that LEVO elimination in rats is quicker than in humans (half-life in humans 5–6 days *vs.* half-life in rats 0.5–1 day).[42] Therefore, the implant size can be reduced even more.

5. Conclusion

In the present study, a new and sensitive HPLC method using UV detection has been developed to quantify LEVO in plasma samples. The method was validated as per the ICH and FDA bioanalytical guidelines. Acetonitrile was used to extract LEVO from plasma samples using a protein precipitation technique. Importantly, the developed method was found to be valid, selective, linear, precise and accurate with 80% extraction recovery of LEVO from plasma samples. Importantly, the sensitivity of the method was confirmed by the low LOQ value obtained which was 1.33 ng mL^{-1}. Essentially, the validated HPLC method was successfully utilized to assess the pharmacokinetics of LEVO following the administration of the long acting formulation containing LEVO for 28 days in rats. Accordingly, this method could potentially provide useful applications in pre-clinical evaluation of LEVO.

Author contributions

Andi Dian Permana: conceptualization; data curation; investigation; methodology; project administration; validation; visualization; roles/writing – original draft; writing – review & editing; supervision, Sarah A. Stewart: methodology; software; writing – review & editing, Juan Domínguez-Robles: methodology; validation; visualization; writing – review; supervision, Muh. Nur Amir: methodology; validation; visualization, Muh. Akbar Bahar: methodology; validation; visualization, Ryan F. Donnelly: supervision; writing – review & editing, Eneko Larrañeta: conceptualization; funding acquisition; project administration; supervision; writing – review & editing.

Conflicts of interest

The authors declare no conflicts of interest.

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