Determination of Menbutone: Development and Validation of a Sensitive HPLC Assay according to the European Medicines Agency Guideline

Cristina López, Raquel Díez *, José M. Rodríguez, Matilde Sierra, Juan J. García, Nélida Fernández, M. José Díez and Ana M. Sahagún

Abstract: Menbutone is often used to stimulate the hepato-digestive activity in case of digestive disorders and hepatic insufficiency in different species (cattle, sheep, goats, pigs, horses, dogs) because it has choleretic and cholagogue effects and increases gastric and pancreatic juices. The objective was to develop and validate an HPLC method to quantify menbutone in sheep plasma using sparfloxacin as internal standard. The HPLC mobile phase consisted of acetonitrile:monopotassium phosphate solution. The method was validated according to EMA guideline (EMEA/CHMP/EWP/192217/2009). Mean retention times of menbutone and sparfloxacin were 4.5 and 2.2 min, respectively. The method met all specifications of the EMA guideline, being selective and linear in the range of 0.2–100 µg/mL ($R^2 \geq 0.99$). The within-run precision range was 0.19–8.21%, with an accuracy of 102.99–119.52% for the lower limit of quantitation (LLOQ). For the other values (LOW, MED, HIGH) the precision range was 0.01–4.77%, with an accuracy of 85.17–109.67%. The LLOQ was 0.2 µg/mL, and no interference from the biological matrix was found. Stability of menbutone in the biological matrix at different storage conditions was also demonstrated. Thus, the method can be used to determine menbutone concentrations in plasma sheep in different types of studies.

Keywords: EMA guideline; HPLC; menbutone; validation

1. Introduction

Menbutone (MB), or genabilic acid, is a derivative of oxobutyric acid, named according to the International Union of Pure and Applied Chemistry (IUPAC) rules as 4-(4 methoxynaphthalene-[1])-4-oxobutyric acid [1].

This compound promotes the transit and assimilation of food. It acts as a liver detoxifier and improves the normal activity of the stomach, bile and duodenum. It is a powerful choleretic and cholagogue agent, and causes a rapid improvement in appetite; the digestive process and gastroenteric movements.

MB is used in the European Union (EU) to stimulate the hepato-digestive activity in the case of digestive disorders and hepatic insufficiency by increasing pancreatic juices, bile and peptic secretion between 2 to 5 times the normal levels, maintaining this effect for 2–3 h after administration [2–4]. In the last decade, several veterinary medicines containing this active ingredient have been approved for use in most EU countries (by national authorization or mutual-recognition procedures) [5,6]. It is marketed for veterinary use in different animal species including cattle, sheep, goats, pigs, horses and dogs, under several trade names [7]. MB is administered by parenteral routes (intravenous or intramuscular), with doses ranging between 2.5 mg and 10 mg/kg [7].
As explained before, MB is used mainly in food-producing animals. The European Commission has established that no maximum residue limit (MLR) is required for this drug as it is rapidly eliminated from the body [8].

A higher use of this drug is expected due to the widening of its availability in the EU countries. As far as we know, up to date, few methodologies have been reported to determine MB. These methods include spectrophotometry [4], HPLC-UV detection [3,9,10], HPLC-MS [3,11] and Micellar Liquid Chromatography (MLC) [12], and none of them have been carried out in sheep nor validated in accordance with the European Medicines Agency (EMA) guideline on bioanalytical method validation [13]. Moreover, these methods show a low sensitivity, are tedious and sometimes require specific instruments not always available. On this basis, it is necessary to carry out and validate a more efficient bioanalytical method to determine and quantify MB in sheep plasma.

Thus, the objective of this study was to develop and validate a simple and fast high-performance liquid chromatography method for MB determination and quantification in sheep plasma using an appropriate extraction procedure.

2. Materials and Methods
2.1. Chemicals and Reagents

MB was purchased from Sigma-Aldrich (Schnelldorf, Germany) with a purity of 98.5%. Sparfloxacin was used as internal standard (IS) (purity 98%, Sigma-Aldrich (Schnelldorf, Germany). All reagents and solvents used were of HPLC grade: Acetonitrile (HiPerSolv CHROMANORM®, Radnor, PA, USA), methanol (LiChrosolv Merck, Madrid, Spain), monopotassium phosphate (AnalaR NORMAPUR®, Radnor, PA, USA), sodium hydroxide 1N (Panreac Quimica S.A., Barcelona, Spain) and acetic acid 10% (Chromanorm VWR Chemicals, Radnor, PA, USA). HPLC grade water was used for the extraction and quantification procedures. For the solid phase extraction (SPE) Oasis HLB 1cc 30 mg cartridges (Waters Corporation, Mildford, MA, USA) were employed.

2.2. Animals and Experimental Procedures

Six non-lactating healthy female Spanish Churra sheep (4–5 years old) weighing 80 ± 7 kg were used. The study was carried out in the experimental farm of the Veterinary Faculty of the University of Leon. Animals were determined to be clinically normal by physical examination. Sheep were allowed to acclimatize to their environment before starting the experiment, and maintained in an adequately ventilated building. They were provided a diet of hay and pelleted feed concentrate twice a day with water and saltlick ad libitum. The study was approved by the Ethics Committee of the University of Leon (OEBA-ULE-004-2019).

Blood samples were collected from the jugular veins into heparinized tubes (Vacutainer, BD, Plymouth, UK). Samples were centrifuged at 1500 rpm for 20 min, and plasma stored at −20 °C until analysis.

2.3. Preparation of Stock, Calibration and Quality Control Working Solutions

MB stock solution (10 mg/mL) was prepared in HPLC grade water, also adding 100 µL NaOH 1N. IS (1 mg/mL) was also dissolved in HPLC grade water and 100 µL NaOH 1N. Calibration working solutions containing both MB (2, 5, 10, 50, 100, 500 and 1000 µg/mL) and IS (20 µg/mL) were then prepared by diluting an appropriate volume of the stock solution in 10 mL of HPLC grade water.

Quality control working solutions (QC) were obtained by adding an aliquot of each stock solution to obtain final concentrations of 0.2 µg/mL (QC1: LLOQ, lower limit of quantitation), 0.6 µg/mL (QC2: LOW, three times the LLOQ), 30 µg/mL (QC3: MED, between 30% and 50% of the calibration curve range) and 75 µg/mL (QC4: HIGH, 75% of the upper calibration curve range).

Stock solutions, calibration working solutions and quality control working solutions were daily prepared.
2.4. Preparation of Analysis Samples

The following types of samples were used:

1. Blank samples: Biological matrix without MB and IS (1 mL).
2. Zero samples: Biological matrix (0.9 mL) with 0.1 mL IS (20 µg/mL).
3. Calibration standards: 0.9 mL plasma was spiked with 100 µL of each calibration working solution to obtain calibration curves. Thus, concentration calibration samples were 0.2, 0.5, 1, 5, 10, 50 and 100 µg/mL for MB and 2 µg/mL for IS.
4. Quality control samples were also prepared in plasma (0.9 mL) at concentrations of 0.2, 0.6, 30 and 75 µg/mL for MB (0.1 mL) and 2 µg/mL for IS (0.1 mL).

All samples were fully thawed at room temperature.

2.5. Extraction Method

To carry out the extraction procedure, plasma samples were deproteinized with 1 mL 10% acetic acid, shaken for 1 min and centrifuged at 3000 rpm for 10 min. Supernatant was then transferred into the SPE cartridge. Cartridges were previously conditioned with 1 mL methanol and then with 1 mL HPLC grade water. After washing twice with 1 mL HPLC grade water, the cartridge was properly dried and eluted with 1 mL mobile phase; 20 µL of eluate was injected into the HPLC system. All procedures were performed at room temperature.

2.6. HPLC System and Conditions

The samples were analyzed by reverse phase high performance liquid chromatography (HPLC) in an HPLC system Waters Alliance e2695 equipped with photodiode array detector (model 2998) (Waters Corporation, Milford, MA, USA).

Chromatographic separation was performed at room temperature with an Xbridge BEH C18 column (5 µm, 4.6 × 250 mm) (Waters® Corporation, Milford, MA, USA). The mobile phase consisted of a mixture of acetonitrile and monopotassium phosphate buffer (1.36 g/L) 49:51 (v/v). The flow rate was 1.2 mL/min, and the wavelength was set at 236 and 297 nm. The injection volume was 20 µL. Sparfloxacin was used as the internal standard. The limit of detection (LOD) was estimated integrating the baseline noise of the HPLC system in the area covering the mean retention time of MB in six plasma samples spiked with the IS and defined as the mean baseline noise/IS peak area ratio plus three standard derivations.

Photodiode array detectors provide three-dimensional information that allows an accurate assessment of peak identification, purity and quantitation in a single run. We used our spectral library to establish peak homogeneity and identity. The study was conducted under the Good Laboratory Practice (GLP) regulations at our GLP-compliant laboratory LAFARLE (University of Leon, Spain), certified by the Spanish Agency of Medicines and Medical Devices (AEMPS) [14].

2.7. Method Validation Procedure

The validation of the method was carried out by using the following parameters: Selectivity, carry-over, lower limit of quantification, calibration curve, precision, accuracy and stability, in accordance to the Guideline on Bioanalytical Method Validation of the European Medicines Agency (EMA/CHMP/EWP/192217/2009) [13].

2.7.1. Selectivity

Selectivity was evaluated by comparing the chromatograms of the blank plasma samples from six different individual sources of this blank matrix, zero sample (biological matrix with IS) and biological matrix with MB and IS.
2.7.2. Carry-Over

Carry-Over was assessed by injecting blank samples after a high concentration calibration standard sample (100 \( \mu \)g/mL) and a quality control of the highest concentration QC4 (75 \( \mu \)g/mL) in each run.

2.7.3. LLOQ and Calibration Curve

LLOQ was defined by analysing blank samples spiked with the lowest calibration concentration (0.2 \( \mu \)g/mL). Calibration curves included a blank sample, a zero sample and seven calibration samples from 0.2 \( \mu \)g/mL (LLOQ) to 100 \( \mu \)g/mL for MB and 2 \( \mu \)g/mL IS. These samples were analysed in three different runs carried out in duplicate on three different days. The linear regression analysis was carried out on known concentrations of MB against the ratio of area of MB vs. IS. The determination coefficient (R\(^2\)), slope and intercept of the resulting calibration curves were determined. The blank and zero samples were not considered to calculate the calibration curve parameters.

2.7.4. Accuracy and Precision

Intra-day and inter-day accuracy (ratio between mean found and nominal concentrations) and precision were evaluated through the four QC levels (QC1 or LLOQ; QC2 or LOW; QC3 or MED; QC4 or HIGH). For the intraday (within-run) assay, five replicates of each QC level were processed the same day. For the inter-day (between-run) assay, each QC level was processed five times on 3 different days. Precision was expressed as the coefficient of variation (CV).

2.7.5. Stability

Stability was evaluated using QC2 (LOW) and QC4 (HIGH) samples at different storage conditions.

2.8. Method Application

To explore the applicability of the method in the clinical practice, MB concentrations were measured in plasma samples obtained from 2 sheep. MB was administered intramuscularly into the deep gluteal muscle of the right hind limb at a dose of 10 mg/kg. Blood samples were alternately collected from the jugular veins into heparinized tubes (Vacutainer, BD, Plymouth, UK) at 30 min, and 1, 1.5, 2, 4, 8 and 24 h. Samples were centrifuged at 1500 rpm for 20 min, and plasma was stored at \(-20^\circ\)C until analysis. Plasma samples (1 mL) were spiked with 20 \( \mu \)L IS before analysis.

Animal procedures and management protocols were authorized in advance by both the Ethics Committee of the University of Leon and the regional authorities (OEBA-ULE-004-2019). No invasive procedure was involved beyond blood sampling.

2.9. Data Analysis

The HPLC Empower 3 (Waters Corporation, Milford, MA, USA) software was employed for data acquisition and processing. A descriptive statistic (mean and standard deviation) was carried out on data values using SPSS Statistical Software V. 26.0 (IBM Corporation, Armonk, NY, USA).

3. Results and Discussion

Under the chromatographic conditions described previously, the retention time of MB was 4.5 min, and 2.2 for the IS. The three-dimensional chromatogram is shown in Figure 1.
Figure 1. Representative three-dimensional chromatogram of a plasma sample containing menbutone (4.5 min) and sparfloxacin (IS) (2.2 min).

3.1. Selectivity

After having tested blank plasma from six different individual sources, no endogenous interferences were observed in MB and IS retention times, as shown in Figure 2. Mean retention times of MB and IS were 4.524 min (λ = 236 nm) and 2.150 min (λ = 297 nm), respectively. Therefore, the method used meets the selectivity criterion indicated by the European validation guideline [13]. Moreover, the LOD calculated was 0.08 µg/mL.

Figure 2. Representative HPLC chromatogram of: (A) Blank plasma sample; (B) zero sample (IS, 2 µg/mL); (C) plasma sample fortified with menbutone (5 µg/mL) and IS (2 µg/mL).
3.2. Carry-Over

Carry-Over was assessed by injecting a mobile phase sample after the highest concentration calibration sample (100 µg/mL), a high concentration QC (75 µg/mL) and the two highest concentrations in working solutions (calibration and quality control) dissolved in the mobile phase. No signal was observed at the retention times of MB and IS. Therefore, the quality parameter was also met.

3.3. Lower Limit of Quantification (LLOQ)

LLOQ was determined by analysing plasma samples with the lowest calibration concentration (0.2 µg/mL). According to the guideline [13], to define this concentration as LLOQ, precision and accuracy conditions must be met (precision as coefficient of variation (CV) not exceeding ±20% and accuracy between 80% and 120%). Inter-day precision (CV) of the assay was 0.96% and accuracy 112.66% (Table 1). So, the precision (CV) and accuracy in the inter-day runs were within the criteria established [13].

Table 1. Data from accuracy and precision for LLOQ.

| Nominal Concentration (µg/mL) | Intra-Day | Inter-Day |
|-------------------------------|-----------|-----------|
|                               | Batch 1   | Batch 2   | Batch 3   | CV (%) | Accuracy (%) | CV (%) | Accuracy (%) |
| 0.2                           | 0.14      | 115.40    | 2.49      | 114.63 | 1.34         | 111.43 | 0.96         | 112.66 |
| 0.58                          | 114.96    | 0.83      | 112.86    | 0.38   | 106.70       |        |              |

3.4. Calibration Curve

Calibration curves showed to be linear within the range of 0.2–100 µg/mL. Table 2 summarizes the results of the regression analysis with respect to the linearity of the method between 0.2 and 100 µg/mL (MB: 0.2, 0.5, 1, 5, 10, 50 and 100 µg/mL and IS: 2 µg/mL), showing a good linearity and high coefficients of determination (R² > 0.999).

Table 2. Data from linear regression analysis of calibration curves.

| Slope | Intercept | R² | p     |
|-------|-----------|----|-------|
| Calibration curve 1 | 0.816 | 0.026 | 0.999 | < 0.001 |
| Calibration curve 2 | 0.730 | 0.013 | 1.000 |       |
| Calibration curve 3 | 0.815 | 0.031 | 1.000 |       |

Back calculated values of MB concentration in calibration samples are presented in Table 3. All samples in each analytical run fulfilled the criteria of being within ±15% of the nominal value, and ±20% for LLOQ. On the other hand, the mean recovery of MB was 91.12 ± 9.25%.

Table 3. Back-calculated values of menbutone in calibration samples.

| Nominal Concentration (µg/mL) | Calibration Curve 1 | Calibration Curve 2 | Calibration Curve 3 |
|-------------------------------|---------------------|---------------------|---------------------|
| 0.2                           | 115.18              | 113.75              | 109.06              |
| 0.5                           | 94.09               | 96.70               | 104.19              |
| 1                             | 90.61               | 96.37               | 102.59              |
| 5                             | 110.68              | 102.78              | 96.32               |
| 10                            | 103.85              | 98.81               | 103.48              |
| 50                            | 96.97               | 100.00              | 99.21               |
| 100                           | 100.69              | 100.01              | 100.17              |
3.5. Accuracy and Precision

Accuracy and precision were determined for the four QC levels (QC1, QC2, QC3 and QC4). The intra-day and inter-day precision and accuracy of the method are shown in Table 4. Within-run precision ranged from 0.19 to 8.21%, with an accuracy from 102.99 to 119.52% for LLOQ. On the other hand, precision ranged for QC2, QC3 and QC4 from 0.01 to 4.77%, with an accuracy from 85.17 to 109.67%. Between-run precision ranged from 0.28 to 2.68%, and the accuracy from 92.91 ± 5.64 to 109.09 ± 5.27%. Therefore, the method used meets the accuracy and precision criterion described in the European guideline on bioanalytical method validation [13].

Table 4. Intra- and inter-day accuracy and precision for the quality controls (QC).

| Nominal Concentration (µg/mL) | Sample Number | Intra-Day | Inter-Day |
|------------------------------|---------------|-----------|-----------|
|                              |               | Batch 1   | Batch 2   | Batch 3   |           |           |
|                              |               | CV (%)    | Accuracy (%) | CV (%)    | Accuracy (%) | CV (%)    | Accuracy (%) |
| QC1 0.2                      | 1             | 2.64      | 109.05     | 3.34      | 111.17     | 2.13      | 105.99      |
|                              | 2             | 8.21      | 105.45     | 0.85      | 111.30     | 0.19      | 109.46      |
|                              | 3             | 6.35      | 104.18     | 0.47      | 117.67     | 2.71      | 103.56      |
|                              | 4             | 4.59      | 102.99     | 0.66      | 113.02     | 0.58      | 103.79      |
|                              | 5             | 6.01      | 119.52     | 0.99      | 113.99     | 0.48      | 109.29      |
| QC2 0.6                      | 1             | 0.71      | 88.03      | 0.48      | 95.19      | 1.45      | 95.67       |
|                              | 2             | 0.43      | 86.08      | 3.69      | 97.77      | 0.67      | 94.50       |
|                              | 3             | 1.09      | 86.79      | 4.17      | 105.61     | 0.12      | 93.24       |
|                              | 4             | 0.31      | 85.17      | 0.28      | 93.77      | 0.69      | 93.45       |
|                              | 5             | 0.88      | 85.84      | 0.42      | 94.97      | 4.77      | 97.63       |
| QC3 30                       | 1             | 0.36      | 85.68      | 0.19      | 93.22      | 2.88      | 105.39      |
|                              | 2             | 0.09      | 86.94      | 0.00      | 92.63      | 0.07      | 105.10      |
|                              | 3             | 0.18      | 88.18      | 0.07      | 93.52      | 3.35      | 103.80      |
|                              | 4             | 1.29      | 97.54      | 0.07      | 93.84      | 3.32      | 104.70      |
|                              | 5             | 0.29      | 89.39      | 0.11      | 94.61      | 0.29      | 97.70       |
| QC4 75                       | 1             | 0.03      | 87.95      | 0.03      | 93.55      | 2.91      | 104.92      |
|                              | 2             | 0.10      | 88.66      | 0.01      | 93.20      | 0.09      | 108.21      |
|                              | 3             | 0.10      | 88.00      | 0.03      | 94.24      | 0.02      | 107.97      |
|                              | 4             | 0.05      | 92.76      | 0.23      | 100.26     | 0.21      | 108.80      |
|                              | 5             | 0.01      | 92.94      | 0.10      | 97.52      | 0.26      | 109.67      |

3.6. Stability

The stability of MB and IS in plasma at different storage conditions was tested using LOW (QC2) and HIGH (QC4) quality control concentrations (0.6 µg/mL and 75 µg/mL, respectively). Concentrations were calculated by using the calibration curve obtained on the day of the analysis (Table 5). According to the guideline [13], three freeze–thaw cycles from −20 °C to room temperature were carried out. In all cases the acceptance criteria were fulfilled, with an accuracy ranging from 85.40 to 104.35 %.

Table 5. Stability of quality controls (QC2 and QC4) at different storage conditions.

| Temperature (°C) | Time | QC2 | QC4 |
|------------------|------|-----|-----|
|                  |      |     |     |
| −20              | 24 h | 0.44 | 85.40 |
|                  | 48 h | 0.40 | 85.94 |
|                  | 72 h | 1.13 | 92.52 |
| 4                | 24 h | 1.42 | 89.57 |
|                  | 48 h | 1.89 | 87.56 |
|                  | 72 h | 1.07 | 89.54 |
| 25               | 24 h | 3.40 | 93.95 |
|                  |     |     | 1.81 | 87.75 |
Table 5. Cont.

| Temperature (°C) | Time          | QC2 CV (%) | QC2 Accuracy (%) | QC4 CV (%) | QC4 Accuracy (%) |
|------------------|---------------|------------|------------------|------------|------------------|
| −20              | 7 days        | 0.24       | 93.92            | 0.08       | 97.54            |
|                  | 15 days       | 3.69       | 86.96            | 0.06       | 93.36            |
|                  | 1 month       | 1.13       | 88.31            | 1.45       | 89.54            |
| 4                | 24 h after extraction | 0.30       | 87.82            | 0.14       | 104.35           |
|                  | 48 h after extraction | 1.96       | 91.63            | 0.54       | 91.62            |
| 25               | 24 h after extraction | 0.81       | 88.84            | 0.16       | 102.96           |
|                  | 48 h after extraction | 1.56       | 94.45            | 3.98       | 89.87            |

3.7. Method Application

The application of the method was tested by analyzing plasma samples obtained from two sheep after MB intramuscular administration. The drug was detected in all samples without interferences, as shown in Figure 3.

![Representative HPLC chromatogram of sheep plasma samples after intramuscular MB administration (10 mg/kg) at time sampling: (A) 4 h and (B) 24 h.](image-url)

Figure 3. Representative HPLC chromatogram of sheep plasma samples after intramuscular MB administration (10 mg/kg) at time sampling: (A) 4 h and (B) 24 h.

4. Conclusions

In the present study, a new, sensitive, simple and fast analysis HPLC method for menbutone quantification in sheep plasma was successfully developed and validated for the first time, and it may be used to perform pharmacokinetic studies in this animal species.

The HPLC method was validated for selectivity, carry-over, lower limit of quantification (LLOQ), calibration range, accuracy, precision and stability according to the European Medicines Agency Guideline on Bioanalytical Method Validation [13], always fulfilling the criteria established. As additional advantages, its low cost, the low volume of sample needed or the simple instrumentation required should also be considered. Moreover, the
chromatographic runtime is about 6 min, which allows a large number of samples to be processed in a short time.

**Author Contributions:** Conceptualization, M.S. and J.J.G.; methodology, C.L., J.M.R. and A.M.S.; software, R.D.; validation, M.J.D.; formal analysis, R.D. and N.F.; investigation, C.L., R.D., J.M.R., N.F. and A.M.S.; resources, R.D.; data curation, N.F.; writing—original draft preparation, C.L.; writing—review and editing, M.S. and J.J.G.; visualization, A.M.S.; supervision, M.J.D.; project administration, A.M.S.; funding acquisition, A.M.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** The animal study protocol was approved by the Ethics Committee of the University of Leon (OEBA-ULE-004-2019).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. O’Neil, M. *The Merck Index*, 15th ed.; Royal Society Chemistry: Whitehouse Station, NJ, USA, 2013; ISBN 9781849736701.
2. Symonds, H.W. The choleretic effect of menbutone and clanobutin sodium in steers. *Vet. Rec.* **1982**, 110, 423–425. [CrossRef] [PubMed]
3. Belal, F.; El-Razeq, S.A.-M.A.; Fouad, M.M.; Fouad, F.A. Spectrofluorimetric analysis of menbutone in veterinary formulations: Application to residue determination in bovine meat and milk. *Eur. J. Chem.* **2016**, 7, 156–160. [CrossRef] [PubMed]
4. El-Bagary, R.I.; Elkady, E.F.; Ayoub, B.M. Spectrophotometric methods for the determination of sitagliptin and vildagliptin in bulk and dosage forms. *Int. J. Biomed. Sci.* **2011**, 7, 55–61. [PubMed]
5. Heads of Medicines Agencies (EMA). VMRI (Veterinary Mutual Information Recognition). Product Index. Available online: https://www.hma.eu/vmriproductindex.html (accessed on 15 January 2022).
6. European Medicines Agency (EMA). CMDv/GUI/032 Guidance for Link to National Databases of Authorised Products. Available online: https://www.hma.eu/fileadmin/dateien/Veterinary_medicines/CMDv_Website/Procedural_guidance/General_info_on_applications/GUI-032-06_Link_to_national_databases_of_authorised_products.pdf (accessed on 15 January 2022).
7. European Medicines Agency (EMA). Veterinary Medicines. Available online: https://www.medicinesinfo.eu/select-language?destination=/node/210934 (accessed on 7 March 2022).
8. European Commission. Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Off. J. Eur. Union* **2010**, L15, 1–72.
9. Zhou, L.; Zhang, P.; Wu, L.; Wu, X.; Luo, L.; Xu, X.; Luo, Y. Determination of menbutone residuals in edible swine tissues based on solid-phase extraction and RP-HPLC. *Lat. Am. J. Pharm.* **2019**, 38, 464–471. [CrossRef]
10. Luo, L.; Wang, L.Z.; Luo, Y.H.; Wang, X. Improved Synthesis Method of Menbutone. CN Patent CN104370734A, 25 February 2015. Available online: https://patents.google.com/patent/CN104370734A/en (accessed on 17 January 2022).
11. Hirosh, M.; Kouhei, F.; Toshiaki, T. Application to residue determination in bovine meat and milk. *Kumamoto-Ken Hoken Kankyo Kagaku Kenkyusho* **2010**, 39, 21–25.
12. Belal, F.; El-Razeq, S.A.A.; Fouad, M.M.; Zayed, S.; Fouad, F.A. Determination of menbutone in bovine milk and meat using micellar liquid chromatography: Application to injectable dosage forms. *Food Anal. Methods* **2016**, 9, 638–645. [CrossRef]
13. European Medicines Agency (EMA). Guideline on Bioanalytical Method Validation (EMEA/CHMP/EWP/192217/2009). Available online: http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/document/document_detail.jsp?webContentId=W500109686%26mid=W500109686 (accessed on 15 January 2022).
14. Spanish Agency of Medicines and Medical Devices (AEMPS). List of Laboratories Certified for Good Laboratory Practice Compliance [Listado de Laboratorios Certificados para el Cumplimiento de Buenas Prácticas de Laboratorio]. Available online: https://www.aemps.gob.es/industria-farmaceutica/buenas-practicas-de-laboratorio/listadolab-bpl/ (accessed on 7 March 2022).