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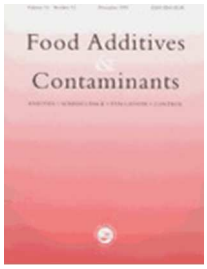

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	stored at 4 °C. In muscle tissue stored at -18 °C no severe degradation of the tested antibiotics was observed with the exception of the penicillins. The presented stability data are useful as a reference for laboratories carrying out validation studies of analytical methods for antibiotic (residue) detection. It saves them time needed for long term stability testing of solutions and samples.

The stability of antibiotics in matrix and reference solutions determined using a straight-forward procedure applying mass spectrometric detection

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Abstract

The stability of an antibiotic is a very important characteristic, especially in the field of antibiotic residue analysis. During method development or validation, the stability of the antibiotic has to be demonstrated no matter if the method is used for screening, confirmation, qualitative or quantitative analysis. A procedure for testing the stability of antibiotics in solutions and food samples using LC-MS/MS is described here. The procedure is based upon the assumption that the antibiotics are stable when stored at -70 °C. Representative solutions or spiked samples containing the antibiotic are stored at the temperature to be tested (-18 or 4 °C) and at -70 °C. After a selected storing time samples are moved from the chosen storage temperature to -70 °C. At the end of the study, all samples -per class of antibiotic- are analysed in one batch. By applying statistical models it is finally concluded at which circumstances the antibiotic is stable. The stability of 60 antibiotics belonging to the classes of tetracyclines, sulphonamides, quinolones, penicillins, macrolides and aminoglycosides are tested. The stability of solutions containing tetracylines and penicillins is only guaranteed for three months while stored at -18 °C. Solutions of all other antibiotics tested are stable for at least 6 or 12 months when stored at 4 °C. In muscle tissue stored at -18 °C no severe degradation of the tested antibiotics was observed with the exception of the penicillins. The stability data reported here are useful as a reference for laboratories carrying out validation studies of analytical methods for antibiotic (residue) detection. The data should save time needed for long term stability testing of solutions and samples.

Keywords: stability, degradation, antibiotics, mass spectrometry

Introduction

Nowadays, many antibiotics are used in animal production, particularly in intensive animal rearing like pigs, poultry and veal calves. For food safety and prevention of antibiotic resistance only the use of registered antibiotics is allowed and maximum residue limits (MRLs) in food products are established in EU/37/2010 (2010) to protect the consumer from being exposed to antibiotics. To monitor at these MRLs sensitive quantitative analytical methods are needed. In most cases, for the quantitative analysis, solutions of antibiotics are used as reference standards. To obtain a correct quantification, it is very important that knowledge is available on the stability of the antibiotic in the solution.

Next to the stability of the antibiotics in reference solutions it is also important to have knowledge regarding the stability of the antibiotic in the sample material. Only then suitable storage conditions can be chosen in case the analysis cannot take place immediately

after sample collection. To obtain information on the stability of antibiotics in matrix a suitable quantitative analytical method has to be available. Additionally a statistical procedure for the data evaluation is mandatory.

It is obvious that characterisation of the method of analysis is very important. For that reason the validation of analytical testing methods is a primary requirement ISO 17025 (2005) accreditated laboratories. Guidelines for the validation of the analysis of antibiotics in food matrices are established in EC/2002/657 (2002) and these state that for qualitative and quantitative methods, may it be either screening or confirmatory, the stability of the analyte in solution and in matrix are main characteristics to be determined. Analyte stability information has to be obtained either from experimental data or from literature. Unfortunately stability testing can be time-consuming and only limited literature is available about antibiotic stability.

Some approaches for stability testing of reference solutions and samples are reported (Okerman 2007, Croubels 2003, Jiménez 2004). Okerman *et al.* (2007) apply microbial techniques to determine the decrease in microbial activity over a period of six months. Croubles *et al.* (2003) recommend ultra violet (UV) detection for analytes possessing good UV absorbing properties because the between-day variation of the UV signal is low compared to mass spectrometric (MS) detection. Jiménez *et al.* (2004) applied several detection techniques among which UV and gas chromatography in combination with MS detection of trimethylsilyl derivatives. These techniques, but especially microbial inhibition and derivatization techniques, are non-specific and thus degradation products might not be distinguishable from the native compound. Liquid chromatography coupled to triple quadrupole mass spectrometry (LC-QqQ/MS) is more likely to discriminate degradation products from the native compound and is therefore the preferred detection technique if day to day variation is overcome. Another disadvantage of the reported approaches is that analyses are carried out on different occasions (Croubels 2003, Jiménez 2004). Therefore fresh reference solutions are to be prepared often or, in case of matrix stability testing, a sample preparation has to be carried out several times. This introduces additional errors and is less time and thus cost efficient.

The publically available information on the stability of reference solutions of veterinary drugs is limited (Okerman 2007, Mathijssen 2010, Chédru-Legros 2010). The availability of stability information of antibiotics in animal matrices is even worse (Verdon 2000). According to 2002/657/EC (2002) the stability in matrix is preferably tested in incurred materials, but if these are not available the use of fortified materials is acceptable.

In this paper we present a straight-forward and time efficient procedure for stability testing of antibiotics in reference solutions and muscle matrix using LC-QqQ/MS as a specific detection technique. This method was applied to determine the stability of solutions of 60 antibiotics used in veterinary practice belonging to the tetracycline (n=4), sulfonamide (n=16),

quinolone (n=10), macrolide (n=11), penicillin (n=8) and aminoglycoside (n=11) group. Furthermore, the stability of a selection of 12 antibiotics, representative for different compound groups, in muscle matrix was studied. The results of this research can be used as a reference for the validation of testing methods aiming for the analysis of antibiotics.

Material and methods

Chemicals, reagents and solutions

HPLC grade methanol and acetonitrile (Biosolve, Valkenswaard, The Netherlands), 25% and 32% ammonia, ammonium formate, formic acid, citric acid monohydrate, disodium hydrogenphosphate dihydrate, Na_2EDTA, potassium dihydrogen phosphate, trichloro acetic adic, sodium hydroxide (Merck, Darmstadt, Germany), piperidine (>99%) and heptafluorobutyric acid (HFBA) (Sigma-Aldrich, St. Louis, MO, USA) were used. Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 18 MΩ cm^{-1} (Millipore, Billerica, MA, USA). EDTA-McIlvain buffer was prepared by mixing 500 mL citric acid solution (0.1 M, 21.0 g citric acid mono hydrate) with phosphate buffer (0.2 M, 35.6 g Na_2HPO_4 dihydrate in 1 L of water) until a pH of 4.0 is obtained. 74.4 g Na_2EDTA is added and the volume adjusted to 2 L with water. Potassiumphosphatebuffer containing EDTA and trichloro acetic acid was prepared by dissolving 1.36 g KH_2PO_4, 0.15 g Na_2EDTA and 20.0 g trichloro acetic acid in 1 L water.

The following reference standards (purity is mentioned between brackets) were all obtained from Sigma-Aldrich: T*etracyclines*: oxytetracycline (99%), tetracycline (98%), chlortetracycline (93%) and doxycycline (99%). *Sulfonamides*: sulfadiazine (99.6%), sulfathiazole (100%), sulfapyridine (>99%), sulfamerazine (99.8%), sulfamoxole (98.5%), sulfadimidine (also called sulfamethazine or sulfadimerazine, 100%), sulfamethizole (99.9%), dapsone (98.4%), sulfamethoxypyridazine (99.6%), sulfamonomethoxine (98.5%), sulfachloropyridazine (99.4%), sulfadoxine (99.9%), sulfamethoxazole (100%), sulfisoxazole (99.6%), sulfadimethoxine (>99%) and sulfaquinoxaline (>91.6%). *Quinolones*: marbofloxacin (98.8%), norfloxacin (99%), ciprofloxacin (99.9%), enrofloxacin (99.6%), danofloxacin (99.9%), sarafloxacin (97.2%), difloxacin (99.8%), oxolinic acid (99%), nalidixic acid (99.4%) and flumequine (99%). *Penicillins*: amoxicillin (85.6%), ampicillin (96.8%), penicillin G (also called benzylpenicillin, 99.9%), penicillin V (also called phenoxymethylpenicillin, 98.3%), cloxacillin (94.7%), dicloxacillin (95.6%), nafcillin (87.6%) and oxacillin (89.2%). *Aminoglycosides*: apramycin (71.7%), dihydrostreptomycin (75.1%), gentamycin (including C1, C1A, C2 and C2A, 59.5%), kanamycin (77.7%), neomycin (82.5%), paromomycin (82.5%), spectinomycin (69.7%) and streptomycin (76%). *Macrolides*:

tilmicosin (83.7%), tiamulin (99.9%), erythromycin (65.7%), tylosin A (101.3%), valnemulin (>95%), josamycin (101.5%), lincomycin (93%) and spiramycin I (92.6%). Three other macrolides were obtained elsewhere: tulathromycin (97.6%) from Pfizer (New York, NY, USA), aivlosin (also called 3-acetyl-4"-isovaleryl tylosin, 67.9%) from Eco Animal health (London, UK) and pirlimycin (90.3%) from Pharmacia and Upjohn Company (Bridgewater, NJ, USA).

Stock solutions were prepared by accurately weighing in 3 to 6 mg (±0.02 mg) of reference standard. After correction for purity and counter-ions present of the amount of reference standard taken, it was dissolved in solvent (on weight basis) to obtain the required concentration. Separate methanolic stock solutions were prepared at a concentration of 100 mg L^{-1} of tetracyclines and penicillins and at a concentration of 1000 mg L^{-1} for sulfonamides. Separate stock solutions of 1000 mg L^{-1} were prepared in acetonitrile for all macrolides and in water for the aminoglycosides. Separate stock solutions of quinolones were prepared by dissolving the analyte in 2M ammonia using sonification (30 min) to obtain a concentration of 5000 mg/L after which the solution was diluted to 100 mg L^{-1} with methanol.

Instrumentation

The liquid chromatography (LC) instrumentation used was an Acquity UPLC system (Waters, Milford, MA, USA) and detection was carried out using a triple quadrupole Quattro Premier mass spectrometer (Waters) (QqQ/MS) operating with electrospray ionization (ESI) in positive mode. The operating parameters were: capillary voltage, 2.5 kV; cone voltage, see Table 1; source temperature, 120 °C; desolvation temperature, 300 °C; cone gas flow, 200 L hr^{-1}; desolvation gas, 500 L hr^{-1}. Data were acquired and processed using MassLynx 4.1 software (Waters).

LC-QqQ/MS analysis

Several multi-compound methods were applied to determine the stability of the antibiotic substances. All analytical methods used for stability testing were fully validated as described in 2002/657/EC (2002) as a quantitative confirmatory method and fulfil the criteria established for trueness, repeatability, within-laboratory reproducibility, robustness and selectivity.

Tetracyclines

The analysis was carried out according to a previously described method (Berendsen 2006).

Sulfonamides

Muscle (1 g) was extracted with 10 mL of water. After mechanical shaking and centrifugation the aqueous extract was filtered using a 30 kD ultrafilter (Millipore) and the filtrate was transferred into a glass vial.

Chromatographic separation was established using a Waters Acquity UPLC BEH C_{18} analytical column, 50 x 2.1 mm, 1.7 µm. The gradient (mobile phase A, 5 mM formic acid in water; mobile phase B, 5 mM formic acid in water/acetonitrile (1:9, v/v), flow rate 0.8 mL min^{-1}) was: 0-0.5 min, 0% B; 0.5-5.5 min, linear increase to 15% B; 5.5-6.6 min, linear increase to 25% B; 6.6-6.7 min, linear increase to 100% B; 6.7-7.2 min, 100% B; 7.2-7.3 min, return to 0% B. The flow was split 1:1 (MS:waste). The injection volume was 40 µL. Fragmentation was carried out using collision induced dissociation (CID) with the settings presented in Table 1.

Quinolones

Muscle (1 g) was extracted with 10 mL of water. After mechanical shaking and centrifugation the aqueous extract was filtered using a 30 kD ultrafilter (Millipore) and the filtrate was transferred into a glass vial.

A chromatographic separation was established using a Waters Symmetry C_{18} analytical column, 150 x 3 mm, 5 µm. The gradient (mobile phase A, 5 mM formic acid in water; mobile phase B, 5 mM formic acid in water/acetonitrile (1:9, v/v), flow rate 0.4 mL min^{-1}) was: 0-1 min, 0% B; 1-11 min, linear increase to 100% B; 11-13 min, 100% B; 13-14 min, return to 0% B. The injection volume was 50 µL. Fragmentation was carried out using CID with the settings presented in Table 1.

Macrolides

Muscle (2 g) was extracted with 20 mL EDTA-McIlvain bufffer. After mechanical shaking and centrifugation 10 mL of the aqueous extract was transferred onto a conditioned OASIS® MCX 3CC, 60 mg solid phase extraction cartridge (Waters). The cartridge was washed with 3 mL MeOH / water / 32% ammonia (10:85:5 v/v) and dried under vacuum. The macrolides were eluted from the cartridge using 3 ml MeOH / 32% ammonia (95:5 v/v). The solvent was evaporated (45°C, N_2) and the residue reconstituted in 700 µL 2% ACN in 10 mM ammonium formate buffer, adjusted to pH=4.0 with formic acid. The extract was transferred into a glass vial.

A chromatographic separation was established using a Waters Atlantis dC_{18} analytical column, 150 x 3 mm, 5 µm. The gradient (mobile phase A, 10 mM ammonium formate buffer, pH adjusted to 4.0 with formic acid; mobile phase B, 10 mM ammonium formate buffer in water/acetonitrile (1:9 v/v), adjusted to pH=4.0 using formic acid; flow rate 0.4 mL min^{-1}) was: 0-1 min, 30% B; 1-17 min, linear increase to 100% B; 17-18 min, 100% B; 18-19 min,

return to 30% B. The flow was split 2:1 (MS:waste). The injection volume was 50 µL. Fragmentation was carried out using CID with the settings presented in Table 1.

Penicillins

Muscle samples were treated according to a previously reported method (van Holthoon 2010). Before analysis 100 µL of each mixed standard solution was transferred into a 4 mL glass container. The solvent was evaporated under nitrogen at 45°C after which 1.0 mL 2% piperidine (Sigma-Aldrich) solution in water was added to dissolve the residue. The penicillins were derivatized to penicillin piperidines at room temperature during 30 minutes after which 100 µL 10% acetic acid (Merck, Darmstadt, Germany) in water and 2.0 mL 100 mM ammonium acetate buffer, pH=6.0, were added. These solutions were injected into the previously reported LC-MS/MS system (van Holthoon 2010).

Aminoglycosides

Muscle (2 g) was extracted with 20 mL 10 mM potassiumphosphatebuffer pH=4.0 containing EDTA and trichloroacetic acid. After mechanical shaking and centrifugation the pH of the supernatant was adjusted to pH=7.7 using sodium hydroxide (30% in water). The aqueous extract was transferred onto a conditioned CBX 6CC, 500 mg solid phase extraction cartridge (Avantor, Phillipsburg, NJ, USA). The cartridge was washed with 4 mL water and dried under vacuum. The aminoglycosides were eluted from the cartridge using 10% acetic acid in MeOH. The solvent was evaporated (60°C, N_2) and the residue reconstituted in 400 µL 0,065% HFBA in water. The extract was transferred into a glass vial.

A chromatographic separation was established using a Waters Symmetry C_{18} analytical column, 150 x 3 mm, 5 µm. The gradient (mobile phase A, 0.065% HFBA in water; mobile phase B, 0.065% HFBA in methanol; flow rate 0.4 mL min^{-1}) was: 0-0.5 min, 0% B; 0.5-5.5 min, linear increase to 45% B; 5.5-17.0 min, linear increase to 60% B; 17-22 min, 60% B; 22-23 min, return to 0% B. The flow was split 1:1 (MS:waste). The injection volume was 20 µL. Fragmentation was carried out using CID with the settings presented in Table 1. Using this procedure gentamycin C2 and C2A cannot be distinguished.

Note: please insert Table 1 here

Methodology

Stability of antibiotics in solutions

Of each stock solution of quinolones, macrolides and aminoglycosides 1 mL was transferred into ten different glass containers. Of the sulfonamide stock solutions a mixed standard

solution of 10 mg L^{-1} in methanol was prepared of which 1 mL was transferred into ten different glass containers. For each solution at t=0, two containers were stored at -70 °C and 8 at 4 °C. After 1, 3, 6 and 12 months each, two containers were moved from 4 °C to -70 °C. After 12 months, all containers were placed at room temperature to warm up and the solutions were diluted to suitable concentrations with water (aminoglycosides in 0.065% HFBA in water) in duplicate (I and II) to prevent overloading of the analytical system. This experiment is schematically presented in Figure 1. These solutions were analysed using LC-QqQ/MS in the following order: I t=0, I t=12, I t=6, I t=3, II t=0, II t=12, II t=6 and II t=3. This series was repeated four times resulting in five injections per dilution. For quinolones this experiment was carried out for a six month period only. For tetracyclines stock solutions and penicillins mixed standard solutions the experiment was carried out with transfer of containers at t=1 week, t=2 weeks, t=1 month, t=2 months and t=3 months at both 4 °C and -18 °C because of expected instability.

After integration of the peaks, for each storage time, the average peak area of dilution I was compared with dilution II using a Students' t-test (n=5) to check if the dilutions were carried out correctly. If no statistical difference was observed between both dilutions the overall average peak area of dilution I and II was calculated (n=10) for each storage time. Next, the average of each storage time was compared to the average peak area of the solution stored at -70 °C from the beginning of the experiment (t=0), again using a Students' t-test. If the average peak area at a certain storage time is above 90 % of the average peak area at t=0 the compound is considered stable for that specific storage time. If it drops below 90% the solution was considered to be unstable. If a difference between dilution I and II was found, a worst case scenario was adopted by using the most deviating average of dilution I or II in the calculations.

With this approach the instability of a compound is observed by comparing a signal obtained for a solution stored at -70 °C with a solution stored at 4 °C or -18 °C. It is assumed that the antibiotics tested are stable at -70 °C (BCR/01/97 1997), however even if an analyte is unstable at -70 °C a difference in signal is expected because it will by definition be more unstable at higher temperatures.

Note: please insert Figure 1 here

Stability of antibiotics in matrix

The stability of antibiotics in muscle matrix was studied using in-house reference material that was prepared for each compound group individually. For each compound group one or more representative substances were selected. A bulk of blank muscle material was fortified with the selected antibiotics at and around the MRL (EU/37/2010, 2010). Each of the

materials was homogenized under cryogenic conditions and the homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories (Thompson 2006) and ISO 13528 (2005), taking into account the insights discussed by Thompson (2000).

Just after preparation of the materials, 12 samples of each material were randomly selected of which 6 were stored at -70 °C and 6 at -18 °C. It is assumed that the antibiotics tested are stable at these storage conditions. After the selected test period all 12 samples were analysed in one batch in random order. After integration of the peaks, the overall average peak area and relative standard deviation (VC%, n=6) were calculated for each storage time and a Students' t-test was applied to test for statistical significant differences between the samples stored at -70 °C and the samples stored at -18 °C.

Results and discussion

With the presented approach, for the analysis of the stability of antibiotics in reference solutions and muscle matrix all analyses for a specific class of antibiotics are carried out within one day using LC-MS/MS as the detection technique. Therefore, additional variation due to day to day variation of the instrumentation is prevented. Because the applied detection technique is highly selective, interference of the detector's response caused by degradation products or other interferences is unlikely and only the response of the native compound is recorded. Therefore, this method allows straight-forward, effective and cost efficient stability testing.

Stability in solution

The results of the stability study of all tested antibiotics in reference solutions, stored at 4 °C, are presented in Table 2. Stability results of the tetracycline and penicillin reference solutions, stored at -18 °C are presented in Table 3. The relative response is calculated by dividing the average response at t=I by the average response at t=0. A compound is considered instable if the relative response drops below 90 %.

Note: please insert Table 2 here

Note: please insert Table 3 here

It is observed that the results for the tetracyclines vary severely for the different storage times and as a result, for tetracycline at t=1 week and chlortetracycline at t=2 months a result is found below the established criterion whereas the next result is again above this criterion. As a result a higher uncertainty in the establishment of the maximum storage time is obtained and a conservative estimation should be made to prevent the use of degraded reference solutions.

Nevertheless, it is clearly observed that especially oxytetracycline and tetracycline are instable in methanolic solutions which was also demonstrated before (Okerman 2007). This research clearly illustrates that tetracycline and oxytetracycline stock solutions should be prepared fresh on a weekly basis if they are stored at 4 °C and every two months (tetracycline) or every three months (other tetracyclines) if stored at -18 °C. The difference in stability of oxytetracycline and tetracycline compared to chlortetracycline and doxycycline is confirmed by Okerman *et al.* (2007). They reported no loss of activity for chlortetracycline and doxycycline during 6 months storage at -20 °C whereas oxytetracycline and tetracycline loose approximately 25% of their activity in the same period. It is noted that in our experiments the degradation of oxytetracycline and tetracycline is more severe. This difference might be explained due to the fact that Okerman *et al.* (2007) monitored the total antibiotic activity (this might include metabolites) instead of the level of the native compounds or because the concentration of the reference solutions were a factor 10 lower in our experiments.

Next to the tetracyclines, the penicillins show severe instability. During storage at 4 °C at least a 45% degradation is observed within one week for all compounds. If these penicillin solutions are stored at -18 °C, the stability is much more prolonged: under these conditions ampicillin and penicillin V solutions are stable for at least two months, the other penicillin solutions for at least three months. This is not in agreement with the results of Okerman *et al.* (2007) who reported no degradation of aqueous ampicillin solutions for at least 6 months when stored at -20 °C and, in the contrary, approximately a 20% degradation of penicillin G and amoxicillin within three months. These differences might be explained due to the fact that they monitored the total antibiotic activity instead of the level of the native compounds, because the concentration of the reference solutions are a factor 10 lower in our experiments or because different solvents were used: Okerman *et al.* (2007) prepared stock solutions in water, whereas we tested the stability in methanolic solutions. The latter is most likely, because instability of penicillins in the presence of methanol was also reported by Mastovska *et al*. (2008). For oxacillin the results are in agreement with Mathijssen *et al*. (2010) who reported no degradation for aqueous oxacillin solutions during 6 months when stored at -20 °C.

The other tested antibiotics showed to be stable for the tested period of time at 4 °C indicating that quinolones are at least stable for 6 months and the other antibiotics for at least 12 months.

Stability in matrix

The results of the stability study of the antibiotics in matrix, stored at -18 °C are presented in Table 4.

Note: please insert Table 4 here

Note: please insert Table 5 here

From the results it is concluded that all tested antibiotics, with the exception of ampicillin and to less extend cloxacillin, are stable in muscle matrix when stored at -18 °C for the tested period of time. Verdon *et al.* (2000) indicated no degradation of amoxicillin in porcine muscle during storage at -20 °C for 3 months and severe degradation after 8 months of storage. From the results of the stability of solutions, for ampicillin and amoxicillin a similar degradation rate is expected and thus the quick degradation of ampicillin is unexpected. This observation might be explained by a between muscle variation and therefore we recommend to store samples containing penicillins at -70 °C even if the storage period is limited.

For the penicillin antibiotics the same experiment was carried out after stabilizing the muscle matrix by adding 2 mL phosphate buffer (0.2 M, pH=6) to 2 g minced muscle (van Holthoon 2010). The results of the stability study for the buffered muscle matrix containing ampicillin and cloxacillin are presented in Table 5. It is concluded that after addition of phosphate buffer pH=6 cloxacillin as well as ampicillin are stable in muscle matrix for at least 3 months. This is in agreement with previously reported results by Kondrat'eva *et al*. (1967) and Lu *et al*. (2008) who showed prolonged stability of penicillin G, oxacillin and penicillin V solutions at pH=6 to 7. The stabilisation of muscle samples containing penicillins is recommended if no -70 °C storage capacity is available.

Conclusions

A straight-forward procedure is reported for the stability testing of antibiotics in solution and in muscle samples using LC-MS/MS detection. The method proved to be straight-forward, effective and cost efficient. From this research stability information is obtained that can be used for the construction of a validation dossier. Severe instability at 4 °C was observed for oxytetracycline, tetracycline and all tested penicillin solutions only. When stored at -18 °C the stability of these solutions is prolonged; at least 2 months for tetracycline, ampicillin and penicillin V, and at least 3 months for oxytetracycline and the other tested penicillins. Methanolic solutions of chlortetracycline and doxycycline are stable for at least 3 months when stored at 4 °C. Methanolic solutions of the tested sulfonamides, solutions of macrolides in acetonitrile and aqueous solutions of the aminoglycosides are stable for at least 12 months when stored at 4 °C. Alkaline methanolic solutions of the tested quinolones are stable for at least 6 months when stored at 4 °C.

The tested antibiotics remain stable in muscle matrix for at least 3 months with the exception of the penicillins. A significant degradation was observed within 3 months for ampicillin and cloxacillin when stored at -18 °C. After stabilizing these muscle samples at

pH=6, the stability of the penicillins in matrix is prolonged to at least 3 months. Therefore it is recommended to store penicillin containing samples at -70 °C or to stabilize them at pH=6.

From the work presented here, a huge amount of stability data is obtained. However, stability data is never complete, because numerous compound matrix combinations (including all species) are to be studied and new antibiotic substances become available regularly. Therefore, we suggest that an open access, easy searchable database should be created in which stability data, gathered by various laboratories, can be stored.

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Figure 1. Schematic representation of the stability study.

Table 1. Precursor ions, collision energy and product ions of the antibiotics.

Table 2. Stability of antibiotics in solution stored at 4°C.

Table 3. Stability of antibiotics in solution stored at -18°C.

Table 4. Stability of antibiotics in muscle stored at -18 °C.

Table 5. Stability of penicillins in muscle stored at -18 °C after buffering at pH=6.

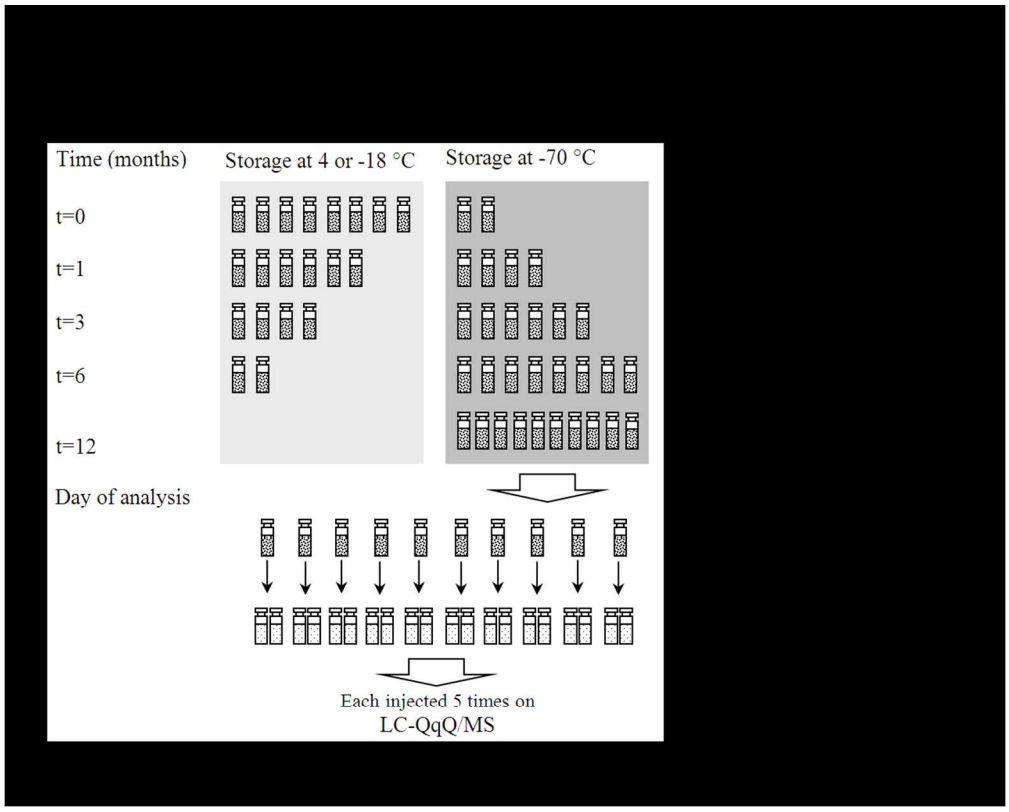

Figure 1. Schematic representation of the stability study.

Table 1. Precursor ions, collision energy and product ions of the antibiotics.

Compound	Cone (V)	Precursor ion (m/z)	Collision energy (eV)	Product ion (m/z)
Sulfonamides				
Sulfadiazine	24	251.2	15	92.2
Sulfathiazole	26	256.2	15	156.1
Sulfapyridine	26	250.2	30	92.2
Sulfamerazole	28	265.2	30	92.2
Sulfamoxole	23	268.2	15	156.1
Sulfadimidine	28	279.2	30	92.2
Sulfamethizole	24	271.1	15	156.1
Dapsone	30	249.2	15	156.1
Sulfamethoxypyridazine	25	281.2	15	156.1
Sulfamonomethoxine	30	281.2	20	156.1
Sulfachloropyridazine	23	285.2	15	156.1
Sulfadoxine	24	311.2	20	156.1
Sulfamethoxazole	22	254.2	25	92.2
Sulfisoxizole	21	268.2	15	156.1
Sulfadimethoxine	28	311.2	20	156.1
Sulfaquinoxaline	30	301.2	15	156.1
Quinolones				
Marbofloxacin	20	363.1	17	72.4
Norfloxacin	20	320.1	18	276.1
Ciprofloxacin	20	332.1	21	288.3
Enrofloxacin	20	360.2	20	316.1
Danofloxacin	20	358.2	35	340.1
Sarafloxacin	20	386.1	23	342.1
Difloxacin	20	400.1	21	356.2
Oxolinic acid	20	262.1	25	244.1
Nalidixic acid	20	233.1	20	215.2
Fumequine	20	262.1	24	244.1
Macrolides				
Tilmicosin	25	435.4	18	695.6
Tiamulin	25	494.3	28	192.2
Erythromycin	25	734.5	25	158.1
Tylosin A	40	916.6	32	174.2
Valnemulin	25	565.4	28	263.2
Josamycin	30	828.5	32	174.2
Lincomycin	28	407.2	21	126.0
Spiramycin I	20	422.4	20	100.9
Tulathromycin	20	403.9	18	158.1
Aivlosin	40	1042.7	42	108.9
Pirlimycin	25	411.2	19	112.0
Aminoglycosides				
Apramycin	25	540.3	20	378.2
Dihydrostreptomycin	35	584.3	30	263.2
Gentamycin C1	20	478.3	15	322.2
Gentamycin C1A	20	450.3	17	322.2
Gentamycin C2 or C2A*	20	464.3	17	322.2

Gentamycin C2 or C2A*	25	485.2	25	163.1
Kanamycin	30	615.3	35	161.1
Paromomycin	30	616.3	30	163.1
Spectinomycin*	30	351.2	25	100.0
Streptomycin	50	582.3	35	263.2

* analysed as a hydrate salt.

Table 2. Stability of antibiotics in solution stored at 4°C.

Compound	Relative response compared to t=0 (%)					Final concluded stability
	t=1w	t=2w	t=1m	t=2m	t=3m	
Tetracyclines						
Oxytetracycline	91	101	**45**	**85**	**74**	2 weeks
Tetracycline	**86**	93	**69**	**56**	**45**	< 1 week
Chlortetracycline	104	109	92	**85**	104	≥ 2 months
Doxycycline	107	119	94	102	114	≥ 3 months
Penicillins						
Amoxicillin	**31**	**11**				< 1 week
Ampicillin	**36**	**15**				< 1 week
Penicillin G	**49**	**27**				< 1 week
Penicillin V	**30**	**9**				< 1 week
Cloxacillin	**38**	**14**				< 1 week
Dicloxacillin	**29**	**9**				< 1 week
Nafcillin	**54**	**30**				< 1 week
Oxacillin	**42**	**19**				< 1 week
	Relative response compared to t=0 (%)					
	t=1m	t=3m	t=6m	t=12m		
Sulfonamides						
Sulfadiazine	99	101	97	**98**		≥ 12 months
Sulfathiazole	100	101	102	**99**		≥ 12 months
Sulfapyridine	100	101	98	**101**		≥ 12 months
Sulfamerazole	100	100	98	**100**		≥ 12 months
Sulfamoxole	100	96	100	**95**		≥ 12 months
Sulfadimidine	100	101	100	**102**		≥ 12 months
Sulfamethizole	101	100	98	**101**		≥ 12 months
Dapsone	102	102	99	**102**		≥ 12 months
Sulfamethoxypyridazine	101	102	100	**101**		≥ 12 months
Sulfamonomethoxine	101	103	99	**101**		≥ 12 months
Sulfachloropyridazine	101	104	101	**102**		≥ 12 months
Sulfadoxine	99	101	99	**101**		≥ 12 months
Sulfamethoxazole	102	102	98	**101**		≥ 12 months
Sulfisoxizole	100	100	97	**99**		≥ 12 months
Sulfadimethoxine	98	99	94	**99**		≥ 12 months
Sulfaquinoxaline	100	101	100	**100**		≥ 12 months
Quinolones						
Marbofloxacin			**97**			≥ 6 months
Norfloxacin			**100**			≥ 6 months
Ciprofloxacin			**98**			≥ 6 months
Enrofloxacin			**102**			≥ 6 months
Danofloxacin			**106**			≥ 6 months
Sarafloxacin			**100**			≥ 6 months
Difloxacin			**101**			≥ 6 months
Oxolinic acid			**102**			≥ 6 months
Nalidixic acid			**101**			≥ 6 months
Fumequine			**103**			≥ 6 months
Aminoglycosides						
Apramycin	97	95	100	**98**		≥ 12 months

Dihydrostreptomycin	96	98	100	**99**	≥ 12 months
Gentamycin C1	99	98	99	**100**	≥ 12 months
Gentamycin C1A	97	97	99	**99**	≥ 12 months
Gentamycin C2 or C2A*	99	99	100	**98**	≥ 12 months
Gentamycin C2 or C2A*	98	98	100	**98**	≥ 12 months
Kanamycin	100	99	99	**99**	≥ 12 months
Neomycin	101	100	100	**99**	≥ 12 months
Paromomycin	99	93	99	**98**	≥ 12 months
Spectinomycin	102	98	97	**99**	≥ 12 months
Streptomycin	96	99	92	**94**	≥ 12 months
Macrolides					
Tilmicosin			96	**97**	≥ 12 months
Tiamulin			114	**105**	≥ 12 months
Erythromycin			103	**112**	≥ 12 months
Tylosin A			98	**98**	≥ 12 months
Valnemulin			108	**105**	≥ 12 months
Josamycin			109	**104**	≥ 12 months
Lincomycin			99	**98**	≥ 12 months
Spiramycin I			102	**109**	≥ 12 months
Tulathromycin			110	**100**	≥ 12 months
Aivlosin			116	**110**	≥ 12 months
Pirlimycin			92	**96**	≥ 12 months

w = weeks, m=months

The results below 90 % are indicated in bold.

* Gentamycin C2 and C2A cannot be distinguished with the applied methodology.

Table 3. Stability of antibiotics in solution stored at -18°C.

Compound	Relative response compared to t=0 (%)					Final concluded stability
	t=1w	t=2w	t=1m	t=2m	t=3m	
Tetracyclines						
Oxytetracycline	101	111	96	107	116	≥ 3 months
Tetracycline	109	103	104	95	**87**	2 months
Chlortetracycline	99	117	106	97	100	≥ 3 months
Doxycycline	115	111	111	104	109	≥ 3 months
Penicillins						
Amoxicillin	95	110	92	91	91	≥ 3 months
Ampicillin	95	109	93	93	**89**	2 months
Penicillin G	95	107	96	98	94	≥ 3 months
Penicillin V	90	110	90	95	**88**	2 months
Cloxacillin	95	108	96	98	96	≥ 3 months
Dicloxacillin	95	109	97	98	94	≥ 3 months
Nafcillin	95	106	98	100	96	≥ 3 months
Oxacillin	92	107	94	98	94	≥ 3 months

w = weeks, m=months

The results below 90 % is indicated in bold.

Table 5. Stability of penicillins in muscle stored at -18 °C after buffering at pH=6.

Compound group	Compound	Species	t (days)	Level [VC%] (µg/kg)		Stable?
				Day 0	Day t	
Penicillins	Ampicillin	Porcine	76	4.5 [15]	5.0 [6.0]	Yes
	Cloxacillin	Porcine	76	149 [9.1]	148 [3.0]	Yes