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The Challenge of Standard Stability in LC-MS Based Multi-Analyte Approaches for Veterinary Drugs (英文原文)

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Abstract: The short- and long-term stability of multi-component mixtures and intermediate mixtures of analytical standards of veterinary drugs, which can potentially occur in food and feed chains, was examined by an isochronous measurement approach. Short-term stability testing of calibrants included storage for 1, 2, 4, and 7 days at –20 ℃ (as a baseline) 4 ℃, and 23 ℃ (with and without exposure to sunlight), respectively. Long-term stability testing conditions of intermediate mixes were –20 ℃, 4 ℃, 23 ℃ (with and without exposure to sunlight), and control temperature at –80 ℃ while the testing period was 2, 4, 8, and 12 weeks, respectively. Results indicated that calibration standards should ideally be stored at 4 ℃ for only 1~2 days, without the presence of acid. Neutral storage conditions were acceptable even at room temperature. Storage of intermediate mixtures containing ß-lactams and cephalosporins for longer than 1 month under 4 ℃ and room temperature resulted in a loss of almost 90%. When it comes to the intermediate mixtures with penicillin V and G, acceptable storage conditions were 2 weeks at –20 ℃, without the presence of acid. Other classes of veterinary drugs were less critical as considers long-term stability. Overall, storage conditions at –20 ℃ were considered optimal for long-term storage of intermediate mixes of veterinary drug standards.

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1. INTRODUCTION

As veterinary drugs are an extremely heterogeneous class of compounds concerning their polarity, solubility and stability, special attention must be paid when developing a new analytical multi-analyte method[1]. Most veterinary drug standards are commercially available as solids, and thus need to be accurately weighed and dissolved. For the latter step it is necessary to take into account that the applied solvents are appropriate and do not cause degradation of the standard. The issue of standard stability is one of the prerequisites related to validation, as pointed out by the SANTE guideline[2] or Commission Decision 2002/657/EC[3]. In the case of the development of a method targeting various sub-classes of compounds dissolved in several different solvents it is necessary to find a way to facilitate manual work. The solution for this can be to group all compounds dissolved in the same solvent in an intermediate mixture[4-5]. From these mixtures, a combined stock solution is prepared that serves as base for preparing serial dilutions of external calibration standards. This approach requires the determination of long-term stability of stock solutions and intermediate mixes as well of the short- term stability of the calibration standards. However, to our best knowledge, the only paper examining the stability of such a large number of veterinary drugs is Desmarchelier et al. [6]. In this work, the stability of more than 150 residues of different classes of veterinary drugs which can potentially occur also in food and feed was investigated. The stability of > 120 veterinary drug standard solutions in multi-component mixtures (1 week) and intermediate solutions (12 weeks) was tested. The stability results were expected to provide important information on the proper management of the calibrants employed in respective LC-MS/MS multi-analyte methods.

2. MATERIALS AND METHODS

2.1 Chemicals

LC gradient-grade acetonitrile and methanol as well as MS-grade glacial acetic acid (p.a.) and ammonium acetate were purchased from Sigma-Aldrich (Vienna, Austria). Reference standards were purchased from Sigma-Aldrich (Vienna, Austria), Dr. Ehrenstorfer (Augsburg, Germany), European Union Reference Laboratory (Berlin, Germany) or were obtained as gifts from various research groups. Reference standards (>CL), divided into classes (XVIII) according to their chemical properties, analyzed in this work were:

(1) anthelmintic: fenbendazole, fenbendazole sulfone, triclabendazole sulfone, triclabendazole sulfoxide, triclabendazole, albendazole, albendazole sulfone, albendazole sulfoxide, albendazole-2-aminosulfone, rafoxanide, closantel, oxyclozanide, clorsulon, cambendazole, oxibendazole, praziquantel, niclosamide, levamizole, flubendazole, mebendazole, mebendazole amine, morantel, nitroxynil, pyrantel pamoate, thiabendazole

(2) antiprotozoal: ronidazole, dimetridazole, ornidazole, carnidazole, ipronidazole

(3) macrocyclic lactones: doramectin, eprinomectin, moxidectin

(4) sulfonamides: sulfasalazine, sulfaethoxypyridazin, dapsone, sulfacetamide, sulfaguanidin, sulfadiazine,sulfathiazole, sulfapyridin, sulfamerazine, sulfamoxole, sulfisoxazolel, sulfameter, sulfamethazine, sulfamethizole, sulfamethoxazoleol, sulfaclozine, sulfaphenazole, phtalylsulfathiazole, trimethoprim (antifolate antibacterial agent-acts synergistically with sulfonamides), sulfachloropyridazine, sulfadimethoxine, sulfadimidine, sulfadoxine, sulfamethoxypyridazine, sulfamonomethoxine

(5) aminoglycosides: streptomycin, apramycin, dihidrostreptomycin, kanamycin, neomycin B, sisomycin

(6) macrolides: spiramycin, oleandomycin, josamycin, lincomycin, clindamycin, erythromycin A, roxithromycin tulathromycin, tilmicosin, tylosin

(7) tetracyclines: demeclocycline, meclocycline, methacycline, minocycline, chlortetracycline, oxytetracycline, doxycycline

(8) quinolones: marbofloxacin, norfloxacin, ofloxacincin, ciprofloxacin, danofloxacin, enrofloxacin, orbifloxacin, difloxacin, nalidixic acid, flumequine, oxolinic acid, perfloxacin, fleroxacin, lomefloxacin, sarafloxacin, pipemidic acid, cinoxacin

(9) nitroimidazoles: metronidazole

(10) polymixin: colistin

(11) pleuromutilin: valnemulin, tiamulin

(12) β-lactams: amoxicillin, ampicillin, dicloxacillin, oxacillin, tazobactam, piperacillin, ticarcillin, sulbactam, clavulanic acid

(13) penicillins: penicillin G, penicillin V, cloxacillin, aspoxicilin

(14) cephalosporins: cefadroxil, ceftizoxime, ceftriaxone, cefuroxime, desfuroylceftiofur, cephapirin, cefalonium, cefazolin, cefoperazone, ceftiofur, cefacetrile, cefquinome, cephalexin

(15) coccidiostats: clazuril, diclazuril, nicarbacin, clopidol, halofuginone, ethopabat, robenidyn, decoquinate, monensin, salinomycin, lasalocid, maduramicin, nequinate, amprolium, dinitrocarbanilide

(16) amphenicols: thiamphenicol, florfenicol, chloramphenicol

(17) NSAIDs: ketoprofen, naproxen, meloxicam, flunixin, carprofen, diclofenac, ibuprofen, mefenamic acid, tolfenamic acid, firocoxib, celecoxib

(18) corticosteroids: dexamethasone, flumethasone, methylprednisolone, betamethasone, prednisolone, triamcinolone.

2.2 Preparation of standard stock solutions

Stock standard solutions were prepared with respect to analyte solubility in a particular solvent as described by Desmarchelier et al. [6]. The solid substance was weighed (minimum weight of 1 mg) and the liquid level was adjusted with appropriate solvents to obtain a targeted concentration of 1 000 μg/mL. Solutions were sonicated until complete dissolution. Respecting the solubility of compounds, six different solvents were used: water, methanol, methanol + water (1∶1), methanol + dimethyl sulfoxide (DMSO) (1∶1), water + acetonitrile (1∶1), 1 mM sodium hydroxide in methanol. Overall, six intermediate mixtures were prepared (every 10 μg/mL) in 10 mL volumetric flasks, except water mixture in 15 mL falcon tube. Intermediate mixtures were prepared by combining individual stock solutions dissolved in the same solvent and then completed with the corresponding solvent. All solutions were stored at –20 ℃. A detailed overview related to the preparation of the 174 individual stock solutions and intermediate mixtures is given in the Supplementary Materials.

2.3 Calibration

External neat-solvent calibration was performed by serial dilutions of the final working solution with acetonitrile/water (1∶1): 1∶1, 1∶3: 1∶10, 1∶30, 1∶100. To check the linearity of the response, linear 1/x weighted calibration curves were constructed for the neat solvent standards. The construction of calibration curves and peak integration were performed using MultiQuant 2.0.2 software (Sciex, Foster City, CA, USA).

2.4 Method

The analytical procedure for this experiment was described by Malachová et al. [7], the existing method was transferred, and new MRM transitions were added following compound optimization. Briefly, a QTrap 5500 MS/MS system (Sciex, Foster City, CA, USA) equipped with a Turbo V electrospray ionization (ESI) source was coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 ℃ on a Gemini C18-column, 150×4.6 mm i.d., 5 μm particle size, equipped with a C18 security guard cartridge, 4×3 mm i.d. (both Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode with a flow rate of 1 000 μL/min. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10∶89∶1 (v/v/v; eluent A) and 97∶2∶1 (v/v/v; eluent B), respectively. For further purification of reverse osmosis water, a Pure-lab Ultra system (ELGA Lab Water, Celle, Germany) was used. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 50% within 3 min. Further linear increase of B to 100% within 9 min was followed by a hold time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The injection volume was 5 μL. ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarity in two separate chromatographic runs. The settings of the ESI source settings were as follows: source temperature 550 ℃, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of nitrogen), ionspray voltage −4 500 V and +5 500 V, respectively, collision gas (nitrogen)-medium. The column temperature was set at 25 ℃. The target cycle time was 1000 ms, the MS pause time was 3 ms, and the detection window width was 40 and 52 s in the positive and negative ESI mode, respectively. According to the SANTE validation guidelines[2], two MRM transitions per analyte are acquired for confidence.

3. STABILITY STUDY

Isochronous measurements were performed to examine the stability of veterinary drug standards. The measurements involved storing standards over different periods at different temperatures, in a way that allowed all measurements to be performed simultaneously[8]. The isochronous measurement approach foresees that in the beginning all samples are stored at temperatures considered to prevent any degradation (–80 ℃ and –20 ℃ in this experiment)

and then transferred to different temperatures for different time periods tested.

3.1 Short term stability study

A multi-component mix was prepared by mixing 30 μL of each intermediate mixture (180 μL overall) and then completed until 1 mL with extraction solvent (acetonitrile/water/acetic acid 79∶20∶1, v/v/v): dilution solvent (acetonitrile/water/acetic acid 20/79/1, v/v/v), 1∶1. After this, 50 μL of the multi-component mix was aliquoted in each vial (overall 13 vials) and diluted with 450 μL of solvent to obtain a 30 ng/mL concentration.

Replicates of the multi-component mix were prepared without the presence of an acid, in acetonitrile: water (1∶1, v/v) and in acidified conditions with acetonitrile/water/acetic acid (79∶20∶1). Short-term stability testing included storing standards for 1, 2, 4, and 7 days at –20 ℃ as a baseline, at +4 ℃ (refrigerator) and at room temperature (both with and without exposure to sunlight). The day after the last time point all standards deriving from the different storing regimes, as well as the control vial (–20 ℃), were brought to room temperature and measured in a randomized sequence to avoid the occurrence of any trend induced by the measurement (Table 1).

Table 1 Short term stability–1 multi-component mix (30 ng/mL) per point

Conditions:	1 day	2 days	4 days	7 days
–20 ℃	Control vial (1.)			
+4 ℃	2.	5.	8.	11.
+23 ℃, light	3.	6.	9.	12.
+23 ℃, dark	4.	7.	10.	13.

3.2 Long term stability study

Long-term stability was tested by preparing six intermediate mixes in the respective solvents. Altogether, there were 17 sets (six vials). Testing conditions were –20 ℃, refrigerator (4 ℃) and room temperature (both with and without exposure to sunlight). The control temperature was –80 ℃ while the testing period was 2, 4, 8, and 12 weeks. The control set (last remaining set at –80 ℃) was taken out on the last day and each mix/point (10 μg/mL) was put together and diluted, with and without the addition of acid to a obtain final analyte concentration of 200 ng/mL. This was carried out in a way that from all six vials in individual set (17 sets in total) 20 μL was transferred to a new vial, mixed (120 μL), and then filled with 880 μL of solvent to bring the total volume to 1 mL. The day after the last time point all standards deriving from the different storing regimes, as well as the control vial (–80 ℃), were brought to room temperature and measured in a randomized sequence to avoid the occurrence of any trend induced by the measurement (Table 2).

Table 2 Long term stability–1 intermediate mixture set per point

Conditions:	12 weeks	8 weeks	4 weeks	2 weeks	
80 ℃					Control set (1.)
–20 ℃	17.	13.	9.	5.	
4 ℃	16.	12.	8.	4.	
23 ℃, light	15.	11.	7.	3.	
23 ℃, dark	14.	10.	6.	2.	

4. DATA EVALUATION

The construction of calibration curves and peak integration were performed using MultiQuant 2.0.2 software (Sciex, Foster City, CA, USA). Further data evaluation was carried out in Microsoft Excel 2013. The results are presented as recovery, obtained by dividing the area of the standard stored at the tested temperatures and the peak area of the standard kept at the baseline temperature (–20 ℃ for short-term and –80 ℃ for long-term stability) (Formula 1)).

$$R(\%) = \frac{\text{area(tested set, or vial)}}{\text{area(control set or vial)}} \times 100 \quad (1)$$

5. RESULTS AND DISCUSSION

5.1 Short term stability study

In order to assess stability of calibrants under typical conditions in an LC autosampler, a short-term stability trial was conducted.

As expected, storage at +4 ℃ for a period of 1~2 days proved to be the optimal conditions for storage of multi-mix standards. Considering the tested solvents, acidified conditions proved to be significantly worse for the storage of a final working solution of particular veterinary drugs classes compared to pure solvent without acid addition. Penicillins, polyether ionophores, and quinolones in particular (Figure 1, for exact values see Supplementary material) showed an absolute preference for an acid-free solvent. Data for the period of “4 days” for acid-free conditions are missing due to instrument operation problems. Beta lactams have shown satisfactory stability within multi-components mixes even in the presence of methanol which is contrary to that re-

ported by other authors[4-6]. Moreover, all compounds remained reasonably stable in neutral conditions even at room temperature, without any significant influence of light exposure (see Supplementary material). This indicates that stability under neutral storage conditions is acceptable in cases where larger batch samples (requiring an analysis time of a few days) are supposed to be tested.

5.2 Long term stability study

The long-term stability study confirmed that acidic conditions were worse compared to an acid-free solvent for several classes of compounds such as penicillins, cephalosporins, macrolides, polyether ionophores (Figure 2, for exact values see Supplementary material). Penicillins can only be stored for 2 weeks in acid-free conditions at –20 ℃, which contradicts the findings of Berendsen et al. [9], who reported the stability of Penicillin V in the presence of methanol for at least 2 months. Storage at higher temperatures or for prolonged periods did not meet the set criteria for penicillins. Any temperature other than –20 ℃ led to almost complete loss (90%) of the analytes belonging to ß-lactams or cephalosporins after 1 month of storage, which confirms the results of Okerman et al. [10]. In contrast, most of the other compounds showed acceptable stability at –20 ℃ and even +4 ℃ for a prolonged period. For example, most compounds from the classes including sulfonamides, coccidiostats, glucocorticoids, and NSAIDs (Supplementary material) were observed to maintain the desired stability even at room temperature and in the presence of light. These results advise that, whenever possible, longer storage of penicillins, cephalosporins, and ß-lactams should use a freezing temperature of –20 ℃ or, even better, of –80 ℃, as it was also recommended by Desmarchelier et al. [6]

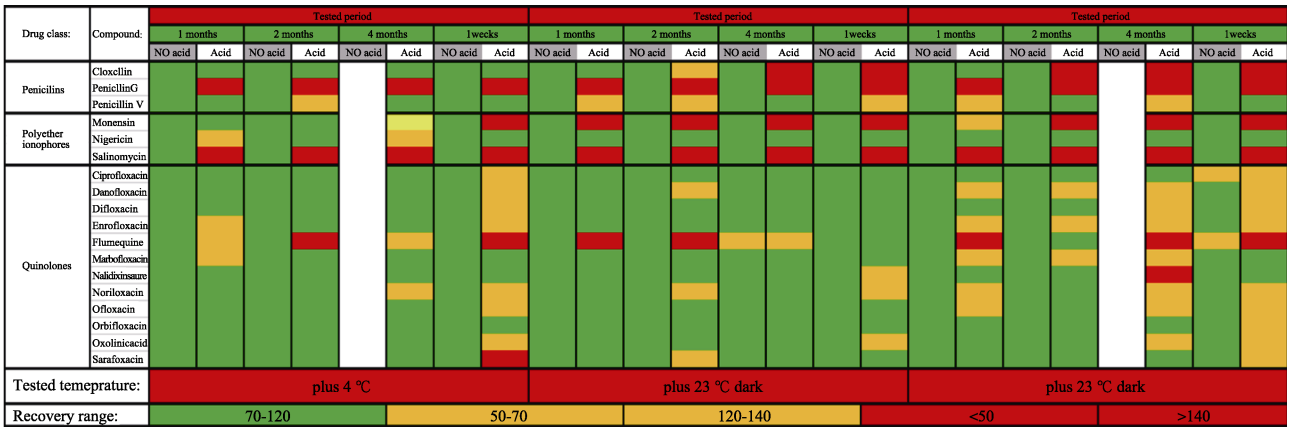

Fig.1 Results of short-term stability study for selected analytes, green color represents the recovery range of 70%~120%, yellow color between 50%~70% and 120%~140%, red color below 50% and above 140% of short-term stability study for selected analytes (Supplementary material), white columns indicate a lack of data due to system operation problem

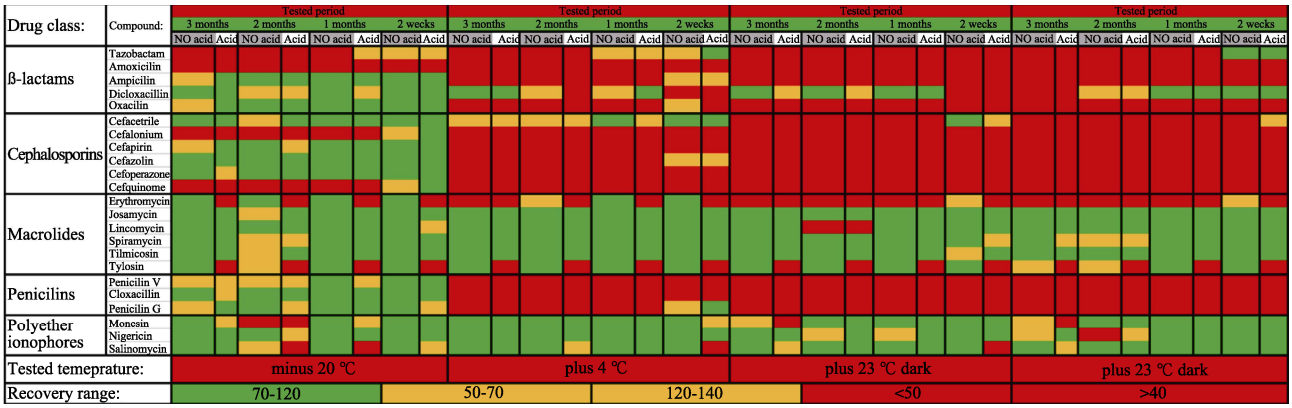

Fig.2 Results of long-term stability study for selected analytes (Supplementary material), green color represents the recovery range of 70%~120%, yellow color between 50%~70% and 120%~140%, red color below 50% and above 140%

6. CONCLUSIONS

To establish and ensure good laboratory practice the stability of intermediate mixes and multi-component mixes of analytical standards of veterinary drugs was tested. The results show consistent stability when

multi-component mixes are stored for up to one week at a temperature of +4 ℃ under neutral storage conditions. However, the stability at room temperature for the typical duration of an analytical sequence is sufficient. When intermediate solutions of veterinary drugs are stored for a period of 1 month or longer, it would be best to apply a temperature of –20 ℃, or lower. In case of storage longer than one month even at a temperature of –20 ℃, the use of freshly prepared intermediate solutions containing classes of veterinary drugs such as penicillins, cephalosporins, and ß-lactams is recommended.

REFERENCES

See in its Chinese version P98-P99.

·信息窗·

金色名片：我国唯一的油脂博物馆在武汉揭牌开馆啦！

2021 年 10 月 1 日，在这举国同庆、共襄盛世的美好日子里，油脂博物馆在武汉举行了盛大的揭牌开馆仪式。这是中国唯一的一座油脂博物馆。中国粮油学会、湖北省粮食局、江南大学等单位，全国油脂行业王瑞元等专家，益海嘉里、中粮集团等 50 余家油脂企业代表汇聚一堂，见证了“油幕新开满庭芳”的一幕。

该馆位于武汉轻工大学金银湖校区，建馆面积 1800 余平方米。馆陈是对中国油脂发展历程的全方位展示，共分为中国古代油脂、近代油脂工业发展、油脂行业名人、油脂学会沿革、油脂名校名企、油脂标准、油脂科普、国家油脂安全等展厅，每个展厅都配置了丰富的藏品展出和相关油脂文化的介绍。开馆恰逢武汉轻工大学建校 70 周年，之后将向社会各界人士免费开放。

国以民为本，民以食为天。油脂，从古至今一直都是人们赖以生存的生活必需品，中国是最早种植油料作物、制取和使用油脂的国家之一。为了让油脂文明活起来，书写油脂人的故事，展示油脂工业的磅礴，以及油脂文化中蕴含的中国智慧、中国精神和中国力量，延续民族的精神血脉，武汉轻工大学在国家粮食和物资储备局、湖北省人民政府、中国粮油学会、众多油脂企业和校友的鼎力支持下，筹备六年，建馆方成。

因粮而生，因油而强。武汉轻工大学建校于 1951 年，是全国最早培养粮食行业专门人才的学校。长期以来，学校坚持立足粮油食品行业，形成了以粮油食品学科为特色，食品营养与人类健康领域相关学科优势明显的多科性大学格局。现有一批以何东平教授等为代表的粮油行业全国性知名专家学者，有大宗粮油精加工省部共建教育部重点实验室、国家粮食局粮油资源综合开发工程技术研究中心和国家粮油标准研究验证测试中心等平台。学校先后被省委、省政府评为“科技服务湖北先进单位”“湖北省农业科技成果转化优秀组织单位”“首批湖北省技术转移示范机构”和“服务湖北经济社会发展先进高校”。

作为全国唯一的油脂博物馆，该馆将作为中国油脂界的“金色名片”，力求打造成为中国油脂文化的展示基地、油脂技术人才的教育与培养基地、油脂科技研发与创新成果展示基地、油脂科学的国际交流与合作基地和爱国主义教育基地。馆小乾坤大，油脂博物馆中蕴含的丰富馆藏馆展和文化知识，必将为我国博物馆事业增添浓墨重彩的一笔。

（撰稿：王丽英；信息提供：何东平 2021 年 10 月 1 日）