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DIHYDROSTREPTOMYCIN and STREPTOMYCIN

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ADDENDUM
Dihydrostreptomycin and Streptomycin residue monographs prepared by the 43rd and 48th meetings of the Committee
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nittee (JECFA, 1994) requested the following information on residues for evaluation in

1. An evaluation report or results of experimental studies on the metabolism of dihydrostreptomycin and streptomycin.
2. Data on residues of streptomycin and dihydrostreptomycin in eggs.
3. Results of studies to determine the relationship between the antimicrobial activity of the residues and their concentration, as measured by specific chemical methods.

Data were submitted at the 48th Meeting (JECFA, 1997) that satisfactorily covered the first two items. The sponsors provided some valuable information to support their analytical methods (Item 3) for the 48th Meeting but were only able to complete the work for the current Meeting. The data package supplied to the 52nd Meeting addresses Item 3 (Norbrook, 1998) and is the source for all the cited data in this monograph.

Two different analytical methods were used to measure residues of dihydrostreptomycin and streptomycin. The first of these methods was a microbiological method (a bioassay), which could not distinguish between the two compounds. The second method was an HPLC assay capable of measuring each compound separately. Data was submitted for the evaluation of the performance characteristics of the individual methods.

A specific requirement of the analytical methods requested by JECFA was that the relationship between the antimicrobial activity of the residues and their concentration, as measured by specific chemical methods should be determined. Such measurements were necessary, both in edible tissues fortified with dihydrostreptomycin and streptomycin, as well as in tissues obtained from animals treated with the compounds. Some of these incurred tissues should have residues above the limit of quantification of the methods. The animal studies, including the sampling and analytical procedures, were performed to GLP. The analytical methods are "in house" and do not appear to have any other accreditation.

SUMMARY OF ANALYTICAL METHODS

Sampling

In all the animal studies, samples weighing at least 200 g were collected. The samples were homogenised and divided into four sub-samples before storage in deep freeze conditions. It should be noted that homogenisation before storage could release enzymes which metabolise the antibiotics.

Bioassay using *Bacillus subtilis*

The standard operating procedure for the bioassay method (Norbrook, 1998; SOM: MRD/DSS/010) and its validation in bovine tissues (Norbrook, 1998; VAL: MRD/DSS/010), ovine tissues (Norbrook, 1998; VAL: MRD/DSS/020) and porcine tissues Norbrook, 1998; VAL:MRD/DSS/030) was provided by the sponsor.

Ten grams of muscle, liver or kidney tissue or 7 g fat were homogenised in phosphate buffer, pH 2.0. A calibration or standard curve in the range 300 - 8000 µg/kg was obtained using blank samples fortified with equal amounts of pure

standards of dihydrostreptomycin and streptomycin dissolved in buffer. After vortex mixing, the mixture was centrifuged to remove the tissue debris and denatured proteins and the supernatant was collected and used for the microbiological assay. Monoethanolamine was added to 5 mL of supernatant to raise the pH. The assay uses *Bacillus subtilis* (probably ATCC 6633 but strain not given for any of the assays) as the test organism and penicillinase may be included to destroy any penicillins present in the extract.

The concentration in the test samples was calculated by interpolation with the standard curve. The assay did not cross-react with penicillins when penicillinase was used. The assay characteristics are summarised in Table 1.

HPLC Assay

The standard operating procedure for an HPLC method (Norbrook, 1998; SOM: CRD/DSS/010) and its validation in bovine tissues (Norbrook, 1998; VAL: CRD/DSS/010), ovine tissues (Norbrook, 1998; VAL: CRD/DSS/020) and porcine tissues (Norbrook, 1998; VAL:CRD/DSS/030) was provided by the sponsor. The sponsors have developed an HPLC method for the measurement of dihydrostreptomycin and streptomycin in muscle, liver and kidney of cattle, sheep and pigs; also for fat of cattle and sheep and for fat combined with skin for pigs. Tissues from untreated animals were used as blanks. Fortified samples for the calibration curves and validation procedures were prepared using 10 g of blank tissues with the addition of two times the amount of dihydrostreptomycin to streptomycin over the range of 400 – 6000 µg/kg for dihydrostreptomycin. Ten gram samples of blank tissue, fortified blank tissue and the test samples were deproteinised with perchloric acid and centrifuged. The liquid extracts were placed onto a small solid phase sulphonic acid resin column, washed with water and the dihydrostreptomycin and streptomycin eluted with phosphate buffer, pH 7.5. Water, perchloric acid and the ion pair concentrate were added to the eluate, this was mixed, filtered and retained for assay. The eluate was analysed by HPLC with post-column derivatisation and fluorescent detection.

The accuracy of the method was measured by using replicates at 400 and 5000 µg/kg spikes. The LOQ was determined as 400 µg/kg for dihydrostreptomycin and 200 µg/kg for streptomycin in all tissues. A recovery correction was not necessary because the standard curve was constructed using fortified material. No comparison of this curve with a pure standards only curve was made. The assay characteristics are summarised in Table 1.

The methods were suitable for measuring concentrations of dihydrostreptomycin and streptomycin in spiked muscle, liver, kidney and fat of cattle, sheep and pigs. The bioassay was suitable for measuring the sum of antimicrobial activity of both drugs in tissues. Although the penicillins did not interfere in the bioassay, other antibiotics may interfere. Thus the bioassay is best suited for the measurement of residues of the two drugs in those animals with a known veterinary treatment history. If the bioassay were to be used in a regulatory control system, any positives would have to be examined by more specific methods, e.g., the HPLC method.

Table 1. Characteristics of the analytical methods for dihydrostreptomycin and streptomycin.

Criteria	Bioassay – Summary	HPLC assay – Summary
QA	In house	In house
Matrices	Muscle, liver, kidney, fat, fat/skin for pig, injection site	Muscle, liver, kidney, fat, fat/skin for pig, injection site
Accuracy at LOQ	See table 2 CVs 0.8 – 11.4%	See table 2 CVs 0 – 13.4%
Recovery	Used fortified standard curve.	Used fortified standard curve
Linearity	$r^2 \geq 0.991$	$r^2 \geq 0.980$
LOD	200 µg/kg in liver 300 µg/kg other tissues.	12 – 153 µg/kg
LOQ	300 µg/kg	streptomycin 200 µg/kg dihydrostreptomycin 400 µg/kg
Specificity from blank	Good	Good at LOQ
Specificity from related compounds.	Poor with certain antibiotics	No cross reaction with penicillin-G, gentamicin and lincomycin

Correlation of the results for the bioassay and HPLC assay.

Fortified Samples.

The initial study reported to the 48th JECFA compared the results when sheep liver and kidney tissues were spiked with dihydrostreptomycin at 500 µg/kg and 1200 µg/kg and were measured using both assays. This experiment confirmed that the results for spiked samples obtained, if based on antimicrobial activity, were equivalent to results obtained from a specific chemical assay. The new studies used fortified blank tissue samples from cattle, sheep and pigs to produce the calibration curves for each assay. Both assays gave a good linear response over a wide range with r^2 values that were acceptable. The accuracy of the methods was checked using samples fortified with dihydrostreptomycin and streptomycin. A summary of the results is shown in Table 2.

Table 2. Assay criteria using fortified cattle, sheep and pig tissues.

Species	Tissue	Method		r^2 Experiment 1[a]	Accuracy (%) and CV (%) at LOQ*		r^2 Experiment 2[b]	Accuracy (%) and CV (%) at LOQ*	
					Accuracy	CV		Accuracy	CV
Cattle	Muscle	Bioassay		>0.992 (7)	96.9	4.7	NA	96.1	3.7
		HPLC	DS	0.981	87.8	0	0.997	95.2	2.6
			S	0.984	92.7	2.5	0.989	110	5.3
	Liver	Bioassay		>0.992 (6)	105	8.0	NA	101	7.0
		HPLC	DS	0.984	88.0	0	0.995	100	12.0
			S	0.996	97.3	6.5	0.984	95.8	5.0
	Kidney	Bioassay		>0.994 (6)	102	5.9	NA	99.8	3.8
		HPLC	DS	0.983	96.6	5.8	0.992	96.4	6.0
			S	0.984	91.5	0	0.983	96.3	3.9
	Fat	Bioassay		>0.996 (7)	105	4.2	NA	101	6.5
		HPLC	DS	0.995	98.9	4.3	0.987	103	9.0
			S	0.989	106	5.4	0.984	104	4.4
Sheep	Muscle	Bioassay		>0.997 (6)	96.0	5.1	NA	99.7	0.8
		HPLC	DS	0.981	98.5	9.1	0.988	88.7	13.4
			S	0.983	88.1	3.5	0.984	109	5.8
	Liver	Bioassay		>0.995 (7)	97.1	5.2	NA	94.1	7.6
		HPLC	DS	0.996	113	6.7	0.997	97.5	3.9
			S	0.994	95.0	5.4	0.988	106	2.5
	Kidney	Bioassay		>0.992 (7)	96.8	5.4	NA	103	4.4
		HPLC	DS	0.984	90.7	4.7	0.992	97.3	2.9
			S	0.984	89.8	10.6	0.990	95.8	1.1
	Fat	Bioassay		>0.991	103	5.1	NA	88.8	2.5
		HPLC	DS	0.981	91.5	3.8	0.991	104	4.4
			S	0.981	108	2.7	0.995	105	1.4

Table 2 (continued). Assay criteria using fortified cattle, sheep and pig tissues.

Pig	Muscle	Bioassay		>0.993 (6)	95.8	4.2	NA	100	4.7
		HPLC	DS	0.981	87.5	5.1	0.981	115	6.6
			S	0.981	85.0	1.2	0.987	113	4.9
	Liver	Bioassay		>0.991 (6)	105	3.5	NA	99.0	11.4
		HPLC	DS	0.987	109	0	0.995	118	7.4
			S	0.981	110	1.6	0.992	113	2.3
	Kidney	Bioassay		>0.996 (6)	103	8.8	NA	101	6.7
		HPLC	DS	0.981	90.6	7.4	0.995	97.9	5.2
			S	0.981	97.9	5.2	0.995	107	3.1
	Fat and	Bioassay		>0.993 (6)	100	3.8	NA	99.6	9.0
	Skin	HPLC	DS	0.981	100	4.0	0.994	94.2	11.6
			S	0.980	109	3.1	0.990	101	3.9

LOQ for HPLC method for Dihydrostreptomycin is 400 µg/kg; for Streptomycin it is 200 µg/kg. For the bioassay the LOQ is 300 µg/kg. [a] and [b] two separate experiments on different days.
Ranges: Dihydrostreptomycin it is 400 - 5000 µg/kg: for Streptomycin it is 200 - 2500 µg/kg. For the bioassay it is 300 - 8000 µg/kg. NA is not applicable.

Incurred Samples

Three new studies were carried out in which animals were treated with proprietary preparations of the two drugs.

1. Cattle were dosed IM with a combination of dihydrostreptomycin sulphate (10 mg/kg BW) and procaine penicillin (8 mg/kg BW). After 2 days the animals were sacrificed and tissues collected for assay.

2. Sheep were dosed IM with streptomycin sulphate (10 mg/kg BW). After 2 days the animals were sacrificed and tissues collected for assay.

3. Pigs were dosed IM with a combination of dihydrostreptomycin sulphate (5 mg/kg BW) and streptomycin sulphate (5 mg/kg BW). After 2 days the animals were sacrificed and tissues collected for assay.

The results for both assays are shown in Table 3. Although the withdrawal time of 2 days is short, there were no measurable residues in any of the muscle and fat tissues. Residues were found in liver, kidney and at the injection site. There was close agreement between the values for both methods.

Table 3. Residues of dihydrostreptomycin and streptomycin (mg/kg) in tissues of treated farm animals.

Cattle	**Muscle**	**Liver**	**Kidney**	**Fat**	**Injection Site**
Bioassay	<0.3	1.13 ± 0.13	6.61 ± 0.72	<0.3	1.70 ± 0.86
HPLC	<0.4	1.49 ± 0.11	5.78 ± 2.28	<0.4	1.71 ± 0.78
Sheep					
Bioassay	<0.3	0.65 ± 0.24	0.91 ± 0.21	<0.3	1.23 ± 0.47
HPLC	<0.2	0.94 ± 0.46	0.89 ± 0.21	<0.2	1.17 ± 0.30
Pigs					
Bioassay	<0.3	1.21 ± 0.44	5.66 ± 1.70	<0.3	1.61 ± 0.30
HPLC	ND	1.04 ± 0.35	5.12 ± 2.26	ND	1.71 ± 0.48

Each value is the mean ± SD for four animals. ND is not detectable for either drug.

APPRAISAL

MRL

The Committee had recommended temporary MRLs for streptomycin and dihyrostreptomycin in muscle, liver, kidney and fat of cattle, sheep, pigs and chickens; also for milk

The Committee considered that the sponsor had provided satisfactory answers to all the requests made at the forty-third meeting and, therefore, decided to delete the temporary status of the MRLs, except for milk. Taking into account the higher limit of quantification of the bioassay method compared with the HPLC method for streptomycin, the Committee recommended MRLs for muscle, liver and fat of 600 µg/kg, and for kidney, 1000 µg/kg in cattle, sheep, pigs and chickens. The temporary MRL for cattle milk is 200 µg/kg.

The ADI is equivalent to 0 – 3000 µg per 60 kg person. The theoretical maximum daily intake for all tissues and milk using the recommended MRLs is 620 µg and when milk is not included, 320 µg .

REFERENCES.

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