21 February 2014
EMA/CVMP/350579/2013
Committee for Medicinal Products for Veterinary Use

European public MRL assessment report (EPMAR)

Chloroform (all mammalian food producing species)

On 10 January 2014 the European Commission adopted a Regulation[1] establishing maximum residue limits for chloroform for all mammalian food producing species, valid throughout the European Union. These maximum residue limits were based on the favourable opinion and the assessment report adopted by the Committee for Medicinal Products for Veterinary Use.

Chloroform was previously included in Table 2 of the Annex to Regulation 37/2010 and, as such, was prohibited for use in veterinary medicinal products for food producing species. MERIAL submitted the application for the modification of the maximum residue limit status for chloroform to the European Medicines Agency, on 1 October 2012.

Chloroform is intended for use as an excipient in vaccines.

Based on the original and complementary data in the dossier, the Committee for Medicinal Products for Veterinary Use recommended, on 13 June 2013, removal of chloroform from Table 2 of the Annex to Regulation No. (EU) 37/2010 and inclusion of the substance in Table 1 of the Regulation.

Subsequently the Commission recommended, on 15 October 2013, inclusion of chloroform in Table 1 of the Annex to Regulation No. (EU) 37/2010. This recommendation was confirmed on 5 November 2013 by the Standing Committee on Veterinary Medicinal Products and adopted by the European Commission on 10 January 2014.

[1] Commission Implementing Regulation (EU) No 19/2014, O.J.L 8/18, of 11.01.2014

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Chloroform (all mammalian food producing species)

Summary of the scientific discussion for the establishment of MRLs

Substance name:	Chloroform
Therapeutic class:	For use as an excipient
Procedure number:	EU/12/203/MER
Applicant:	MERIAL S.A.S.
Target species:	All ruminants, porcine
Intended therapeutic indication:	Not applicable (for use as an excipient)
Route(s) of administration:	Subcutaneous/intramuscular

1. Introduction

Chloroform is a potent central nervous system (CNS) and cardiovascular depressant with anaesthetic, narcotic and other CNS effects. In the past chloroform was used as a general anaesthetic in human and veterinary medicine.

Chloroform was assessed by the Committee for Medicinal Products for Veterinary Use (CVMP) for the purpose of establishing maximum residue limits in 1996 (EMEA/MRL/118/96-FINAL) and concluded at that time that residues of chloroform posed a potential risk to the consumer. The Committee recommended inclusion of the substance in Annex IV of Council Regulation (EEC) No 2377/90.

Currently chloroform is included in Table 2 (Prohibited substances) of the Annex to Commission Regulation (EU) No 37/2010 of 22 December 2009, in in accordance with the following table:

Pharmaco-logically active substance	**MRLs**
Chloroform	MRL cannot be established

MERIAL S.A.S. submitted an application for the modification of the existing MRL status for chloroform to the European Medicines Agency, on 1 October 2012, in order to allow the use of chloroform as a solvent, preservative and stabiliser in vaccines for all ruminants and porcine species. The current application relates to the use of chloroform as a solvent, and a preservative and stabiliser in vaccines.

2. Scientific risk assessment

2.1. Safety assessment

The previous assessment carried out by the CVMP concluded that residues of chloroform posed a potential risk for the health of the consumer as a result of the substance's carcinogenic potential in mice and rats, equivocal data on mutagenicity, data on embryotoxicity and foetotoxicity, and insufficient data on pharmacodynamics and depletion of residues. The CVMP therefore recommended inclusion of chloroform in Annex IV of Regulation (EEC) No 2377/90 (now Table 2 of Regulation (EC) No 37/2010).

Since the CVMP's 1996 evaluation considerable additional data have been generated on the toxicity of chloroform. An updated MRL dossier has now been provided citing primarily published literature data.

2.1.1. Overview of pharmacological properties

Pharmacodynamic properties including mode of action

The pharmacological profile of chloroform in humans has been established during its long history of use as an anaesthetic agent. Exposure to chloroform at 24 to 73 g/m^3 air was used to induce medical anaesthesia, which was maintained via a continuous exposure at 12 to 48 g/m^3 air (WHO, 2004).

Following such a high dose acute inhalation exposure, the primary target organs are the CNS, the liver and the kidneys. CNS related effects include CNS depression, excitement, nausea and vomiting followed by ataxia, dizziness, drowsiness, cardiac arrhythmias, hypotension, convulsions, coma, respiratory failure and possible sudden death. Individuals exposed to high levels of chloroform may also develop hepatic dysfunction and/or necrosis several days later, as measured by increased concentrations of serum bilirubin and transaminases. Renal dysfunction and renal tubular necrosis have also been reported following the high doses of chloroform required for anaesthesia (HPA, 2007).

Chloroform depresses CNS function and cardiac muscle. It has also been used as an antitussive (CVMP, 1996).

No data were provided on the pharmacodynamic effects of chloroform underlying the pharmacological observations reported above.

Pharmacokinetic properties

Chloroform is absorbed, distributed, metabolised and excreted relatively rapidly in all species studied. Oral absorption is high in humans with around 50% (average) absorbed following a single oral dose with a peak chloroform concentration in blood after 1.5 hours. Chloroform is lipophilic and distributes preferentially into fat. The metabolism of chloroform has been thoroughly investigated. There are both oxidative (major) and reductive (minor) pathways of metabolism. Oxidative metabolism, which is mainly dependent on CYP2E1, generates carbon dioxide as the main metabolite, as well as the toxic metabolite, phosgene, and hydrochloric acid. Phosgene causes local toxicity wherever there is a high concentration of the metabolic enzyme CYP2E1 in all species studied. Literature data show no large differences between rat, mouse and human in terms of the activity of CYP2E1. Once the CYP2E1 enzymes have been depleted, phosgene can be formed in man via CYP2A6 activity. However, this pathway makes a very minor contribution to the overall level of phosgene produced and can be considered as negligible. Phosgene is a highly unstable reactive intermediate in the metabolic process that ultimately produces carbon dioxide and chloride ions, or a bound conjugate of phosgene. The half-life of phosgene in water is approximately 0.03 seconds. Phosgene does not last long enough to be measured directly and is instead measured by cysteine derivitization (*in-vitro*) and as a single permanently bound glutathione-phosgene reaction product (*in-vivo*). The production of the stable and safe glutathione-phosgene reaction product is a protective mechanism of the body. This conjugate does not break down to form free phosgene. It is only if glutathione levels are saturated that phosgene can react irreversibly with macromolecules, resulting in toxicity. Phosgene reacts immediately with water, glutathione or proteins and, therefore, is never found as free drug in any tissue and cannot travel throughout the body. It does not last long enough to be present in edible tissues and cannot survive in the aqueous and acidic environment of the human stomach.

2.1.2. Calculation of pharmacological ADI, if relevant

Chloroform is well known for its use as an anaesthetic in the past in humans and in animals. No discussion of the pharmacodynamics underlying this pharmacological effect has been provided, other than information on the range of concentrations that need to be inhaled to both induce and maintain general anaesthesia. This is because there is a dearth of data on the mode of action which leads to this pharmacological effect. However, it is considered that depression of the CNS occurs at high acute doses, whereas the current assessment is based primarily on chronic low dose exposure. As detailed in the CVMP guideline on the approach to establish a pharmacological ADI (EMEA/CVMP/SWP/355689/2006) a pharmacological ADI needs to be established when pharmacological effects can be expected at doses in the same range or lower than toxicological effects. The endpoint of concern (i.e. anaesthesia in humans) occurs at doses that are considerably higher than the NOELs and ADI derived from the toxicological data provided. Therefore, the establishment of pharmacological ADI is not considered relevant for consumer safety.

2.1.3. Overview of toxicology

The majority of the data have been provided in the form of regulatory reviews, conducted by several authoritative bodies, such as the US EPA, WHO and Health Canada/Environment Canada. The reviews neither indicate whether any specific guidelines have been followed for the individual studies commented upon, nor whether the studies were conducted to GLP. Studies using several strains of mice and rats are available, demonstrating a range of sensitivities to chloroform. The studies that were conducted using the oral route of administration were considered since this is the route by which humans would be exposed to any residues left in products derived from animals treated with vaccines that include chloroform as an excipient.

Acute toxicity

Male mice of various strains have been shown to be the most acutely sensitive species to the hepatic and renal adverse effects of chloroform, with oral LD_{50} values ranging from 36 to 460 mg/kg bw. There do not appear to be any doses tested, down to 10 mg/kg bw, that did not induce some kind of cell proliferation or enzyme disturbances in either the liver, the kidney, or both. An increase in renal cell proliferation was seen in male Osborne-Mendel rats given gavage doses of 10 mg/kg bodyweight. F344 rats do appear to be more resilient to the effects of chloroform, since a NOEL of 30 mg/kg bw, administered once, has been established for hepatic damage in this strain. However, lesions in nasal passages were seen at 34 mg/kg bw/day administered for 4 to 5 days. It has been established that this is a site where CYP2E1 is abundant.

Subacute studies also show adverse effects at all doses investigated, down to 10 mg/kg bw. B6C3F1 female mice received 0, 3, 10, 34, 90, 238 and 477 mg/kg bw/day of chloroform by gavage in corn oil for 5 days per week for 3 weeks. Hepatocyte centrilobular proliferation was recorded at 34 mg/kg bw/day, leading to a NOEL of 10 mg/kg bw/day. NOELs have not been established for humans, although studies have generally been conducted using higher doses, so it is not clear where the threshold might be.

Repeated dose toxicity

The lowest dose in sub-chronic testing that produced no 'adverse' effect was 10 mg/kg bw/day for 3 weeks. However, this study in mice was only 3 weeks in duration and the (all female) animals were not dosed at the weekends. These results contrast with those of another study, in which a single dose of 10 mg/kg bw produced an increase in renal cell proliferation in male Osborne-Mendel rats. This indicates that males could be more sensitive than females to renal toxicity of chloroform.

The most universally observed toxic effect of chloroform is damage to the liver. The severity of the effects per unit dose administered depends on the species, the sex, the vehicle and the method by which the chloroform is administered. The lowest dose at which liver damage has been observed is 15 mg/kg bw/day administered to beagle dogs in a toothpaste base over a period of 7.5 years. Somewhat higher doses are required to produce hepatotoxic effects in other species. Although duration of exposure varied in these studies, NOAELs ranged between 10 and 125 mg/kg bw/day.

Reproductive toxicity, including developmental toxicity

Three published studies into reproductive toxicity of chloroform were provided.

In the first study, conducted in CD1 mice, doses received were 6.6, 16, and 41 mg/kg bw in the low, mid and high dose groups, respectively. It was demonstrated that there was no effect on either fertility or reproduction in two generations of mice that were administered chloroform at a dose of 16 mg/kg bw/day or lower. There were adverse effects noted in the epydidymes of males from the second generation, and liver damage in the females at the highest dose of 41 mg/kg bw/day. The NOEL for reproduction and fertility can be considered to be 16 mg/kg bw/day.

The second study investigated the teratogenicity of chloroform in two species, rat and rabbit. Doses of 0, 20, 50, or 126 mg/kg bw/day in the rat and 0, 20, 35, or 50 mg/kg bw/day in the rabbit were given on days 6 to 15 and 6 to 18 of gestation, respectively. Foetotoxicity was noted at the highest dose levels, but there was no teratogenicity noted at any dose tested. The NOEL for foetotoxicity is determined as 35 mg/kg bw/day (rabbit data). The third study, in which chloroform was administered at levels of 100, 200 and 400 mg/kg bw/day by gavage to Sprague-Dawley rats from day 6 to day 15 of gestation, was supportive of the lack of teratogenicity. Effects observed on fertility or in the foetus occurred only at dose levels that were toxic to multiple systems, or maternally toxic, respectively.

Genotoxicity

The question of whether chloroform or a metabolite is mutagenic has been tested extensively across different systems (yeast, bacteria and mammalian systems). Among the 110 tests performed to assess the potential mutagenic action of chloroform, more than 64% gave negative results whereas less than 13% gave positive results. The other 23% gave weak positive or equivocal results.

The CVMP convened an independent expert group to consider the genotoxicity of chloroform. The group concluded that the weight of evidence indicates that chloroform is not a directly DNA reactive genotoxin. Although positive results were seen in some genotoxicity assays, these were mainly observed at high doses where cytotoxicity occurs and genetic damage may be an indirect effect. While a small number of studies did report positive results at non cytotoxic doses/concentrations, taking the evidence as a whole, the contribution of a genotoxic mode of action to the carcinogenic risk was concluded to be negligible.

These conclusions were accepted by the Committee and are in line with those of a number of other authoritative bodies:

- WHO (2004) concluded that the weight of evidence indicated that neither chloroform nor its metabolites appear to interact directly with DNA or possess significant genotoxic potential;
- the US EPA (2001) concluded that the weight of evidence indicated that even though a role for mutagenicity cannot be excluded with certainty, chloroform is not a strong mutagen and that neither chloroform nor its metabolites readily bind to DNA;

- the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC) concluded that the weight of evidence indicates that chloroform should be classified as non-genotoxic;
- an expert panel sponsored by the US EPA and ILSI (2000) concluded that the weight of evidence supports a non-genotoxic classification for chloroform;
- the ECHA Committee for Risk Assessment (2011) concluded that the body of evidence does not support the classification of chloroform as a mutagen according to Classification, Labelling and Packaging (CLP) and Dangerous Substance Directive (DSD) criteria.

Carcinogenicity

Several references have focused on the potential carcinogenic effect of chloroform in mice, transgenic mice and rats. Liver and kidney were the only two organs revealing tumours following chloroform exposure. Differences were observed in the incidence of these tumours depending on the species, the sex, the route of administration and the dose.

The mode of action for chloroform carcinogenesis has been well described in the literature. Phosgene, a cytotoxic metabolite of chloroform, is the compound that induces liver and kidney cell death via destruction of the mitochondria within the cells. Once a proportion of the mitochondria have been disabled, the cell dies. Once a certain threshold proportion of the cells have died, remaining cells undergo regenerative proliferation. During chronic dosing of chloroform this constant proliferation can lead to hyperplasia followed by tumourigenesis as a result of mutations in the parent cell(s). The CVMP's independent expert group on the genotoxicity of chloroform agreed that the data showed that the main driving force for tumorigenesis in rodents is a cytotoxicity driven mechanism of action and that a threshold exisits below which no tumours would be induced.

Although the actual threshold for the adverse events has not been elaborated, the fact that there is a threshold has been adequately demonstrated, and also agreed by several international scientific bodies. The dose at which these events occur is dependent on several variables, including species, strain, sex, vehicle in which the chloroform is delivered, whether the dose is a bolus (as with gavage) or a sustained low level (as in drinking water), and level of mutation conferring hyperplasia in the proliferating cells. The proposed dose response relationship for chloroform tumourigenesis by the cytotoxicity-regenerative hyperplasia mode of action is considered to be non-linear, as it is dependent on biochemical and histopathological events that are non-linear. An oral NOEL of 17 mg/kg bw/day for the most sensitive mice has been established for (renal) carcinogenicity.

Studies of other effects including immunotoxicity and neurotoxicity

There is no evidence from any acute or repeat dose study to suggest that chloroform is immunotoxic.

Chloroform has anaesthetic properties and clearly has major CNS effects. However, the data provided do not indicate that neurotoxicity is an issue with this substance.

2.1.4. Calculation of the toxicological ADI or alternative limit

Summary of NOELs and all relevant studies

Species	Study type and duration	NOEL	Comments
Rats (male; Osborne-Mendel)	Single dose	not established	LOEL = 10 mg/kg bw
Mice	Repeat dose (sub-chronic)	10 mg/kg bw/d	

Species	Study type and duration	NOEL	Comments
Dogs	Repeat dose (chronic)	not established	LOAEL = 15 mg/kg bw/d
Mice	Reproduction/fertility	16 mg/kg bw/d	
Rabbits	Foetotoxicity	35 mg/kg bw/d	
Mice	Carcinogenicity	17 mg/kg bw/d	

The overall point of departure for the calculation of the ADI is therefore 10 mg/kg bw/day from the single dose study as this was the lowest dose tested which produced an (adverse) effect. While it is more usual that a chronic study would result in a lower dose producing adverse effects, in this case the effect seen in the single dose study, namely increased proliferation of the renal epithelial cells, is considered as an important step in the development of tumours. This was therefore considered to be a relevant effect upon which to base the toxicological ADI.

A safety factor of 1000, based on a 10 x 10 extrapolation for intra- and inter-species variability, an additional 5-fold factor for extrapolation from LOEL to NOEL, and a further 2-fold factor to account for the potential seriousness of the effect is considered appropriate for the derivation of the ADI, resulting in a toxicological ADI of 10 µg/kg, which is equivalent to 600 µg for a 60 kg adult.

Coincidentally this ADI is the same as the Permitted Daily Exposure calculated for residual solvents.

2.1.5. Overview of microbiological properties of residues

While chloroform is used as an antibacterial preservative in vaccines, this preservative effect results from the fact that the substance is toxic to bacterial cells, as it is to mammalian cells. In light of the rapid degradation of chloroform and of the substance's volatility, this cytotoxicity is not expected to be a relevant effect of residues in food of animal origin following administration of chloroform-containing vaccines. The proposed use of chloroform is not expected to affect the human gut flora or microorganisms used in food processing.

2.1.6. Calculation of microbiological ADI

The proposed use of chloroform is not expected to affect the human gut flora and consequently a microbiological ADI is not considered necessary.

2.1.7. Observations in humans

The toxicity of chloroform to humans is highly variable, but it can be highly acutely toxic when relatively high levels are ingested or inhaled. No epidemiological studies have been provided that may indicate the effects of long term low dose exposure, but the evidence suggests that as long as proliferative hyperplasia is not triggered, then most changes to the target organs are at least partly reversible. It is interesting to note that the acute inhalation case studies generally involved individuals who were relatively naïve to the exposure to chloroform, perhaps indicating that a 'tolerance' to the acute adverse effects could build up after long term exposure, although this has not been demonstrated in long-term animal studies.

2.1.8. Findings of EU or international scientific bodies

A number of bodies have examined chloroform:

- WHO (1998) used a safety factor of 1000, based on 10 x 10 for inter- and intra-species differences, and another 10 fold factor for extrapolation from LOAEL to NOAEL (15 mg/kg) and

concluded that the ADI should be 13 µg/kg. The LOAEL of 15 mg/kg bw/day induced fatty changes in the livers of dogs;

- The International Programme on Chemical Safety (IPCS) (2000) used a safety factor of 1000 combined with a dose of 10 mg/kg bw/day identified in a 3-week oral gavage mouse study in which hepatic degenerative changes were observed to give a Tolerable Daily Intake (TDI) of 10 µg/kg;
- US Environmental Protection Agency (EPA, 2001) also used 1000 as the safety factor, based on a BMDL of 1 mg/kg bw/day to give an 'Oral reference dose' of 10 µg/kg bw/day. This was based on liver toxicity in the dog;
- WHO (2004) used a PBPK model to reduce the uncertainty factor to 25 and to normalise for human metabolic activity, and then calculated a TDI of 15 µg/kg. This was based on the LOAEL of 15 mg/kg bw/day, seen to induce fatty changes in the livers of dogs;
- EMA CHMP (1996) and EMA CVMP (2000), in their ICH (ICH Q3C) and VICH (VICH Topic GL18) guidelines on residual solvents used a safety factor of 1200, with a point of departure of 60 mg/kg kg/day (based on kidney tumours in mice) to give a Permitted Daily Exposure (PDE) for residual solvents (in pharmaceuticals for human or veterinary use) of 600µg per person (based on 50 kg adult);
- The European Chemicals Agency (ECHA, 2011) adopted an opinion proposing harmonised classification and labelling for chloroform. This did not require the establishment of an ADI.

2.1.9. Overall conclusions on the ADI

In the absence of relevant pharmacodynamics data it has not been possible to derive a pharmacological ADI. However, as the pharmacological effect of concern (i.e. anaesthesia in humans) occurs at doses that are considerably higher than NOELs from toxicological studies, the establishment of pharmacological ADI is not considered relevant for consumer safety. As the proposed use of chloroform is not expected to affect the human gut flora a microbiological ADI is not considered necessary. A toxicological ADI of 10 µg/kg, which is equivalent to 600 µg/day for a 60 kg adult, is established using a LOEL of 10 mg/kg bw, seen in a single dose study in rats, with an uncertainty factor of 1000 (consisting of a factor of 10 for interspecies differences, a factor of 10 for intraspecies differences, a factor of 5 to extrapolate from a LOEL to a NOEL and an additional factor of 2 to account of the potential seriousness of the effect).

Coincidentally this ADI is the same as the PDE (*permitted daily* exposure) calculated for residual solvents.

2.2. Residues assessment

No new studies on chloroform ADME or residues depletion in food producing animals were presented.

2.2.1. Pharmacokinetics in target species

No *in vivo* or *in vitro* data on pharmacokinetics in target species were provided. However, a physiologically based pharmacokinetic (PBPK) model, described in the residue depletion section below, has been extrapolated from validated rat, dog and human models.

The organ toxicity of chloroform is considered to result from the generation of phosgene by CYP2E1 mediated metabolism. No data have been provided that demonstrate that the level of CYP2E1 activity

is conserved across species or that CYP2E1 represents the sole pathway for the production of phosgene in the target species. It is therefore not certain that metabolism in the target species would lead to production of phosgene in the same organs and at the same rate as seen in laboratory species. However, it is clear that isoforms of CYP2E1 are conserved across the relevant species in the organs identified as targets for toxicity; it is therefore likely that the same patterns of metabolic transformation and toxicity would be apparent if high enough doses were administered to these species.

2.2.2. Residue depletion studies

No *in vivo* residues depletion studies have been submitted. The absence of these studies has been justified on the basis of the high volatility of chloroform and low tissue residue concentrations expected. Omission of hot and cold residue depletion studies is considered acceptable in this case as chloroform is a simple lipophilic C-1 molecule which is distributed mainly via diffusion. The data presented were generated using a physiologically based pharmacokinetic (PBPK) model based on physiologic data (experimentally measured/extrapolated data) for the target species cattle, pigs and sheep. The model is based on the model developed for rats, dogs and validated for humans. As well as providing estimates of residue levels in muscle, fat, liver and kidney, the PBPK model was extended to predict residues in injection site tissues.

The anticipated dose is 10 or 20 mg chloroform per animal. If this amount of chloroform were to remain at the injection site and be ingested by the consumer, exposure to residues would be far above the CVMP's ADI of 600 µg/person/day. Therefore, the decline of chloroform residues at injection sites within the first day after administration is of great interest for the assessment of consumer safety. As the subcutaneous injection site is, compared to muscle after intramuscular administration, a relatively poorly perfused tissue and as, additionally, chloroform has high affinity to fat, the subcutaneous tissue may be considered as the worst case in terms of residues concentrations.

The model was not extended to milk. Instead, an estimate of residues in milk was performed using data for a liver to which 10% of residues in fat was added, which is considered to represent a worst-case approach.

Description of the model:

A physiologically based pharmacokinetic (PBPK) model to study the absorption, distribution, metabolism and excretion of chloroform in laboratory animals based on the actual physiology of the species was extended from a model originally developed for rats and humans, and applied to cattle, sheep and pigs. The model includes compartments for liver, kidney, fat, muscle, slowly and richly perfused tissues, and compartments to account for metabolism in liver and kidney. A muscle compartment and a sub-compartment of slowly perfused tissues are included to allow for intramuscular and subcutaneous injection respectively (Figure 1). The injection sites for intramuscular (IM) and subcutaneous (SC) route of administration were modelled as a tissue with a defined volume, partition coefficients and blood flow taking the worst-case approach.

For the IM injection compartment, the blood flow and partition coefficients were based on muscle, which is consistent with the recommendations in the CVMP guideline on injection site residues (EMEA/CVMP/542/2003), which states that "For substances where there is an MRL for muscle, the injection site is usually treated as muscle tissue". The IM injection site was set to a mass of 500 g of muscle for cow, pig and sheep in line with the CVMP guideline on injection site residues, VICH GL46 on metabolism studies to determine the quantity and identify the nature of residues (EMA/CVMP/VICH/463072/2009) and VICH GL48 on marker residue depletion studies to establish

product withdrawal periods (EMA/CVMP/VICH/463199/2009). The injected amount of chloroform was then able to partition into systemic circulation based on the same principle as for other tissues, i.e. venous equilibration.

The SC injection compartment was considered as a portion of the slowly perfused compartment (representing skin and subcutaneous tissue in this case). The blood flow was thus based on slowly perfused tissue (the SC injection is into fat). The fat/blood partition coefficient of 37.7 was used in the PBPK model because this represented the most conservative approach. Indeed, chloroform partitions into fat 38 times more than blood but will not be retained in the tissue for any longer than it takes for the blood flow to fat to take it away. In the model, fat is responsible for the slower phase of clearance from a bolus dose. Thus, using the higher tissue/blood partition (i.e. fat/blood) slows the release of chloroform into systemic circulation in the model resulting in much higher concentrations in tissue compartments (modelled at 12 hours) after injection. Moreover, this approach covered the theoretical possibility that the injection is inadvertently made directly into fat tissue. The injected amount of chloroform was then able to partition into the systemic circulation based on the same principle as other tissues, i.e. venous equilibration although at a slower rate.

While data from several publications comparing PBPK model predictions with measured data show that assessment of injection site residues based on a PBPK model is more complicated than for other tissues, the model described above for the injection site is considered to be plausible (based on appropriate compartment sizes and tissue composition), although it relies on the assumption that absorption from the injection site is slow, leading to high injection site residues and low tissue residues.

On the basis of studies conducted with other small chlorinated molecules it is expected that chloroform will partition into milk as it would do into any other tissue. While the time to maximum milk concentration would be influenced by the blood flow, the maximum concentration in milk will only be affected by the partition coefficient. An in vitro partition coefficient of 16.5 for human milk/air was previously determined for chloroform; this value is very similar to the human liver/air partition coefficient measured for chloroform. The model was not used to directly predict residue levels in milk; rather, levels in milk were estimated based on levels predicted in liver and fat. Liver was chosen (rather than muscle) as this resulted in a higher (more conservative) estimate of residue levels. Additionally and considering data obtained from different sources, cattle milk does not contain more than 5% of fat and sheep milk not more than 8%. An additional safety/uncertainty margin has been incorporated into the estimate of residues in milk by assuming that cattle and sheep milk contain 10% fat. Consequently, milk is considered to contain residues equivalent to that in liver (no metabolism) plus 10% of residues in fat.

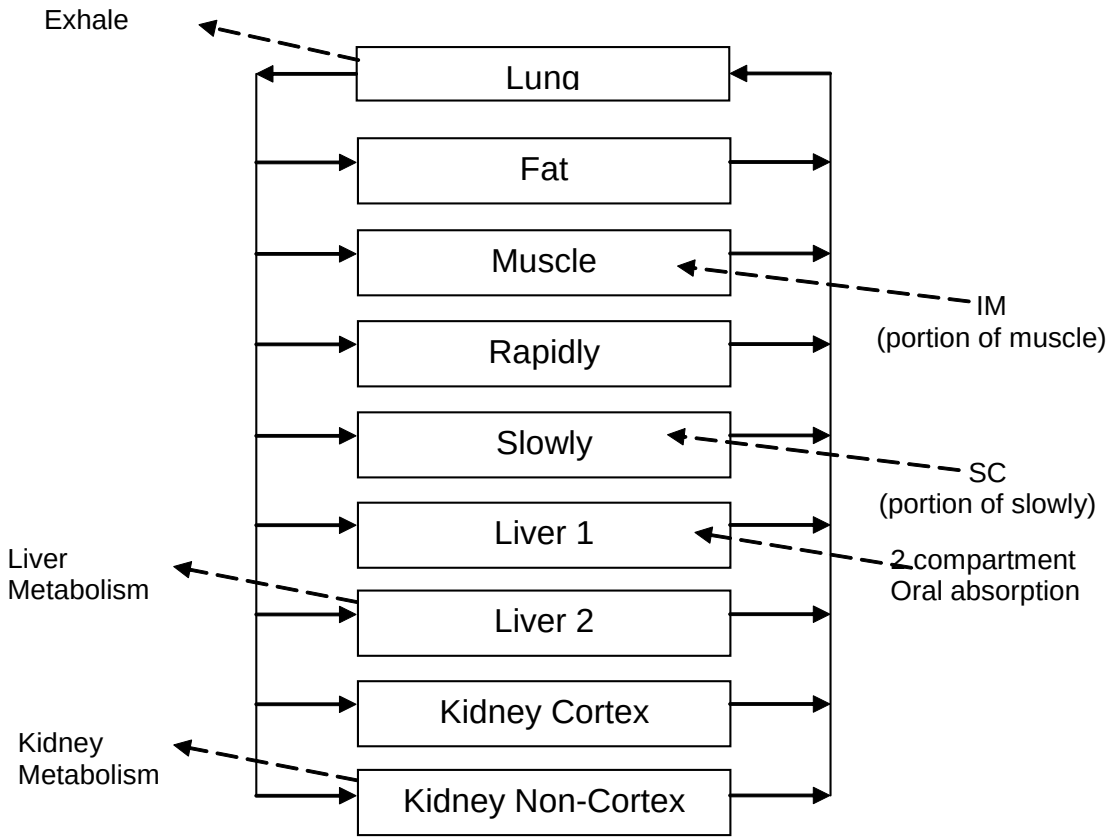

Figure 1 (above): Schematic showing the compartments in the PBPK model extended to include intramuscular and subcutaneous injection in cattle, sheep and pigs. The parameters, pharmacokinetic values and metabolic capacity for the laboratory and domestic species used in the model are given in tables 1 and 2.

Table 1: Physiological values used in the chloroform PBPK model for livestock

	Rat	Human	Cattle	Pig	Sheep
Body Weight (kg)	0.475	70	240	40	40
Tissue Volume (fraction of body weight)					
Fat	0.124	0.2124	0.2	0.2142	0.168
Kidney	0.0073	0.0044	0.0026	0.004	0.003
Liver	0.0366	0.0257	0.03	0.0294	0.015
Muscle	0.404	0.4	0.45	0.4	0.277
Rapidly perfused	0.0621	0.0709	0.0621	0.0621	0.0621
Slowly perfused	0.19	0.0368	0.0368	0.0368	0.0368
Blood	0.074	0.079	0.08	0.06	0.057
Fractional tissue subvolumes (kg)					
Liver periportal (fraction of liver volume)	0.58	0.58	0.58	0.58	0.58
Liver centrilobular (fraction of liver volume	0.42	0.42	0.42	0.42	0.42
Kidney cortical (fraction of kidney volume)	0.7	0.7	0.7	0.7	0.7
Kidney non-critical (fraction of kidney volume)	0.3	0.3	0.3	0.3	0.3
Flows (L/h/kg$^{0.74}$)					
Alveolar ventilation	24.2	19.4	19.4	19.4	19.4
Cardiac output	14.4	13.45	26.27	22.1	21.29
Tissue Blood Flow (fraction of cardiac output)					
Fat	0.07	0.052	0.02	0.07	0.10
Kidney	0.141	0.175	0.11	0.14	0.1673
Liver	0.183	0.227	0.35	0.305	0.183
Muscle	0.278	0.191	0.4	0.252	0.1374
Slowly perfused	0.058	0.086	0.058	0.058	0.058
Rapidly perfused			1-sum remaining tissues		

Table 2: Chemical specific parameters used in the chloroform PBPK model for livestock

	Rat	Human	Cow	Pig	Sheep
Partition coefficients					
Blood/air	20.8	7.43	7.43	7.43	7.43
Fat/air	203	280	280	280	280
Kidney/air	11	11	11	11	11
Liver/air	21.1	17	17	17	17
Rapidly perfused/air	21.1	17	17	17	17
Slowly perfused/air	13.9	12	12	12	12
Fat/blood	-	37.7	37.7	37.7	37.7
Metabolic constants					
VmaxC for liver (mg/h/kg BW$^{0.74}$)	6.44	15.7	6.44	6.44	6.44
KM for liver (mg/L)	0.543	0.448	0.543	0.543	0.543
VmaxC for liver (mg/h/kg BW$^{0.74}$)	0.067	0.089	0.067	0.067	0.067
KM for kidney (mg/L)	0.543	0.448	0.543	0.543	0.543

No data were available in the literature on the metabolic capacity for cattle, pigs or sheep towards chloroform. As such, the rat metabolic rate constants for liver and kidney were retained for the livestock model with the maximum velocity scaled allometrically (BW$^{0.74}$) (most conservative approach). Additionally, the model was run without any capacity of metabolism to generate worst case chloroform tissue concentrations by removing a pathway for chloroform elimination (worst case approach). The model was validated by confirming the terminal half life (1.5 hours) of chloroform in humans after a single oral dose of 500 mg in a gelatin capsule, measured to 6 hours. The results produced were consistent with previously published results. However, since the current model includes inter-species, dose and route to route extrapolations, the CVMP convened an independent expert group to provide advice on the level of confidence that can be had in in the model's predictions. The expert group noted that the reliability of the model predictions will depend upon the reliability of the input parameter values used but considered that the model includes a number of conservative and worst case assumptions and that where there is some uncertainty in the parameters used, this is unlikely to

be sufficient to change the overall conclusions resulting from the data generated using the model. The expert group noted that while it considered the model acceptable, the specific physicochemical and pharmacokinetic characteristics of chloroform are such that this conclusion cannot be readily extrapolated to use of the model for substances other than chloroform. The CVMP accepted the expert group's conclusions.

Model predictions

The blood and tissue time course predictions of chloroform disposition after a 20 mg bolus dose administered to cattle and pigs, or 10 mg to sheep given via the intramuscular and subcutaneous routes were modelled. Results are presented in Tables 3 and 4.

Table 3: PBPK model predicted tissue concentration and depletion (µg/kg) up to 48 hours after 20 mg (cattle and pig) or 10 mg (sheep) intramuscular injection of chloroform

		With Metabolism					Without Metabolism				
	Time (hr)	Fat	Muscle	Liver	Kidney	Injection Site	Fat	Muscle	Liver	Kidney	Injection Site
Cattle	2	23.7	11.8	8.93	7.05	53.4	44.5	38.5	46.2	29.6	80.1
	4	25.3	1.19	0.92	0.72	1.23	57.8	12.6	15.2	9.71	12.6
	8	24.0	0.079	0.084	0.067	0.079	60.6	1.68	2.10	1.35	1.68
	12	22.5	0.065	0.072	0.058	0.065	58.0	0.55	0.75	0.49	0.55
	24	18.7	0.054	0.060	0.048	0.054	49.20	0.36	0.51	0.33	0.36
	48	12.9	0.037	0.041	0.033	0.037	35.36	0.26	0.37	0.24	0.26
Pig	2	392	32.6	18.9	15.7	87.7	719	104	105	67.6	159
	4	360	3.85	3.53	2.97	3.92	733	21.4	25.8	16.6	21.5
	8	284	2.36	2.44	2.06	2.36	619	10.4	14.5	9.37	10.4
	12	224	1.86	1.93	1.62	1.86	516	8.63	12.0	7.78	8.63
	24	110	0.91	0.94	0.79	0.91	300	5.01	6.99	4.52	5.01
	48	26.3	0.22	0.23	0.19	0.22	101	1.69	2.36	1.52	1.69
Sheep	2	347	25.59	12.99	11.66	160	551	52.8	46.9	29.9	188
	4	302	4.69	3.93	3.56	5.60	520	13.6	16.6	10.7	14.5
	8	209	2.66	2.52	2.29	2.66	396	8.13	11.2	7.22	8.13
	12	144	1.84	1.74	1.58	1.84	300	6.16	8.47	5.47	6.16
	24	47.9	0.61	0.58	0.52	0.61	131	2.68	3.69	2.39	2.68
	48	5.24	0.07	0.06	0.06	0.07	24.9	0.51	0.70	0.45	0.51

Table 4: PBPK model predicted tissue concentration and depletion (µg/kg) up to 48 hours after 20 mg (cattle and pig) or 10 mg (sheep) subcutaneous injection of chloroform

	Time (hr)	With Metabolism					Without Metabolism				
		Fat	Muscle	Liver	Kidney	Injection Site	Fat	Muscle	Liver	Kidney	Injection Site
Cattle	2	7.24	10.4	11.4	9.12	25559	12.3	20.3	29.8	19.3	25618
	4	13.6	7.64	8.04	6.42	16352	27.5	19.6	26.9	17.4	16490
	8	20.0	3.23	3.38	2.70	6696	46.8	10.3	13.8	8.90	6876
	12	21.4	1.36	1.43	1.14	2743	53.9	4.73	6.33	4.08	2877
	24	19.7	0.15	0.16	0.13	190	51.7	0.70	0.96	0.62	219
	48	13.7	0.04	0.044	0.036	1.88	37.6	0.28	0.39	0.25	7.97
Pig	2	159	48.3	48.3	40.6	22265	269	91.4	127	81.7	22644
	4	250	29.6	28.9	24.3	12465	470	65.2	86.7	55.9	13085
	8	286	11.0	10.8	9.08	3933	592	29.2	39.1	25.2	4492
	12	253	4.78	4.78	4.03	1264	560	15.8	21.5	13.9	1641
	24	132	1.19	1.23	1.03	67.9	350	6.16	8.58	5.54	209
	48	31.9	0.27	0.28	0.23	7.62	120	2.03	2.84	1.83	54.8
Sheep	2	161	28.6	26.2	23.7	10456	240	45.7	62.0	40.0	10633
	4	233	17.8	15.8	14.3	5517	375	31.8	41.5	26.7	5779
	8	232	7.04	6.37	5.78	1570	411	15.4	20.5	13.2	1806
	12	181	3.44	3.18	2.88	474	349	9.21	12.5	8.05	646
	24	64.1	0.85	0.81	0.73	33.60	163	3.45	4.75	3.07	106
	48	7.17	0.093	0.088	0.080	2.85	32.07	0.67	0.92	0.60	18.95

The main target tissues were predicted to be fat and in particular subcutaneous injection site tissue, modelled with and without metabolism. As can be seen in tables 3 and 4 (above), residues are predicted to deplete rapidly from all edible tissues.

2.2.3. Monitoring or exposure data

No relevant data other than that described elsewhere in this report and in previously published CVMP Summary Reports/EPMARs are available.

2.2.4. Analytical method for monitoring of residues

No analytical method has been proposed. The absence of an analytical method can be considered acceptable for a 'no MRL required' recommendation.

2.2.5. Findings of EU or international scientific bodies

No relevant information was available.

3. Risk management considerations

3.1. Potential effects on the microorganisms used for industrial food processing, if relevant

While chloroform is used as an antibacterial preservative in vaccines, this preservative effect results from the fact that the substance is toxic to bacterial cells, as it is to mammalian cells. In light of the rapid degradation and of chloroform and of the substance's volatility this cytotoxicity is not expected to be a relevant effect of residues in food of animal origin following administration of chloroform-containing vaccines. The proposed use of chloroform is not expected to affect microorganisms used in food processing.

3.2. Other relevant risk management considerations for the establishment of maximum residue limits, if relevant

Chapter 2.1.5 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organisation for Animal Health (OIE), on Foot and mouth disease vaccines previously stated that the two commonly used preservatives in Foot and mouth disease vaccines are chloroform and thiomersal but reference to specific preservatives has been removed from the current version. It has been shown that thiomersal causes dissociation of capsid proteins within the FMD viron particles which reduces immunogenicity and therefore it is not recommended as a stabilizer or preservative in FMD vaccines. The lack of availability of alternatives to chloroform was therefore considered as a relevant risk management consideration.

In its evaluation the CVMP also noted that the feasibility of control of residues in tissues and commodities of animal origin is minimal. This is because the volatility of the substance is such that substantial losses would occur during sample preparation, storage and analysis. This limits both the possibility and the potential value of establishing numerical MRL values.

3.3. Elaboration of MRLs

Chloroform is currently included in Table 2 (Prohibited substance) of the Annex to Regulation 37/2010, in line with the 1996 CVMP Opinion, which highlighted a potential risk for the health of the consumer based on the substance's observed carcinogenic potential in rodents, embryo and foetotoxicity, and insufficient data on mutagenic potential, pharmacodynamics and residue depletion. Since the 1996 evaluation a considerable body of additional data has been generated particularly in relation to the genotoxic and carcinogenic potential of the substance, with the result that a toxicological ADI can now be set. In addition, the applicant has provided acceptable data on residue depletion, generated using a PBPK model. Having evaluated the available data, it is considered that the controlled use of chloroform would not represent a hazard to human health and that consequently, the continued inclusion of chloroform in Table 2 (Prohibited substance) of the Annex to Regulation 37/2010 is not warranted. In reflecting on the need for numerical MRL values the CVMP considered the criteria for inclusion of substances into Annex II of Council Regulation 2377/90 (now Table 1 of Regulation 37/2010) laid down in Volume 8 of The rules governing medicinal products in the European Union, which states:

"Substances complying with the following criteria will be assessed on their own merits to see whether they could be entered into Annex II:

- *Poor or absent absorption from the gastro-intestinal tract or from sites of local application (e.g. skin or eyes)*
- *The substance is rapidly and extensively detoxified or excreted"*

These criteria were updated in the CVMP Note for guidance on the risk analysis approach for residues of veterinary medicinal products in food of animal origin (EMEA/CVMP/187/00-FINAL) to include also the following:

"*Substances complying with the following criteria may be entered into Annex II:*

- *Use in a small number of individual animals, infrequent or non-regular treatments*
- *"The animal is unlikely to be sent for slaughter immediately after treatment".*

Chloroform is rapidly and extensively excreted, the foreseen use of the substance as an excipient in vaccines is expected to result in non-regular treatment of animals, and administration of vaccines containing chloroform is expected to be followed by a significant time interval before sending animals for slaughter.

Furthermore, since the modelling of the worst-case scenarios has indicated that levels considerably lower than the ADI would be expected to be retained in edible tissues (see below) and commodities after treatments with products containing chloroform at the proposed doses, since the substance is rapidly and extensively excreted, since it is anticipated that administration of products containing chloroform will be infrequent or non-regular, since it is not expected that animals will be sent for slaughter immediately after administration of products containing chloroform, the CVMP considers that a 'no MRL required' entry into Table 1 of Regulation 37/2010 would be appropriate.

Need for restrictions of use

While a 'No MRL required' status is considered appropriate, it is clearly necessary for the protection of human health that the use of the substance should be limited in order to ensure that consumer exposure to residues will remain well below the ADI. In the absence of numerical MRL values this can be achieved by limiting the accepted uses of the substance and by limiting the amount of the substance that can be administered. This is in line with Article 14(7) of Regulation (EC) 470/2009. A dose limit (concentrations not exceeding 1% w/v and total doses not exceeding 20 mg per animal) and a restriction to use as an excipient in vaccines only is therefore recommended for inclusion in the 'other provisions' section of Table 1 of Regulation 37/2010.

Calculation of theoretical daily intake of residues

The data generated by the PBPK model (with and without metabolism) were used to estimate the maximum residues to which consumers are likely to be exposed. The calculations were based on an injection, using the subcutaneous or intramuscular routes, of 2 ml of vaccine (containing 20 mg of chloroform, cattle and pig) or 1 ml (containing 10 mg of chloroform, sheep) and slaughter of the animals 12 hours after administration. Twelve hours is considered to be the shortest period after which slaughter would be possible.

Intramuscular injection site concentrations were predicted to be equal to those in normal muscle tissue. Following subcutaneous injection elevated residue levels were predicted in subcutaneous fat. For this reason the food basket used to estimate the potential consumer exposure to residues following subcutaneous administration of chloroform to the animal included 50 g of injection site fat (normally muscle is considered as the injection site tissue). Use of fat as the injection site tissue is accepted for the purpose of a worst-case calculation.

It is considered that the model data generated using the assumption of no metabolism of chloroform in the target species would represent the worst case scenario. Due to the highly unstable nature of the toxic metabolite, phosgene, this substance would not be bioavailable to human consumers of animals treated with a product containing chloroform at the levels modelled, as outlined in section 2.1.1 above.

Table 5: Food basket approach based on residues at 12 hours generated with the highest blood/air partition coefficient, without metabolism and with the injection site (µg), and including milk in all cases

Without metabolism; With the injection site (for IM administration the injection site is considered to be muscle while for SC administration it is considered to be fat)						
	Cattle		Swine		Sheep	
	IM	SC	IM	SC	IM	SC
Fat 50g	2.90	143.85	25.8	82.05	15.0	32.3
Muscle 300g	0.165	1.419	2.589	4.74	1.848	2.763
Liver 100g	0.075	0.633	1.20	2.15	0.847	1.25
Kidney 50g	0.025	0.204	0.389	0.695	0.274	0.403
Milk 1500g						
- 10% fat	8.70	8.085	(8.085)	(8.085)	45.0	52.35
- liver no metabolism	1.125	9.495	(9.495)	(9.495)	12.705	18.75
Total µg	12.99	163.69	29.98	89.64	75.67	107.82
(with cattle milk)			(47.56)	(107.22)		
% ADI 600 µg	2.17	27.28	5.0	14.94	12.61	17.97
(with cattle milk)			(7.9)	(17.9)		

As demonstrated by the figures in table 5, predicted residues at 12 hours after administration of a product containing 10 mg chloroform in sheep, or 20 mg in pigs and cattle would lead to a worst case consumer exposure of approximately 30% of the ADI. This is considered to be a very conservative estimate of residue exposure, as it assumes that animals will be slaughtered 12 hours after slaughter while it does not take account of the fact that, due to the volatility of the substance, chloroform levels will continue to decrease following slaughter/production of milk.

3.4. Considerations on possible extrapolation of MRLs, if applicable

In line with Article 5 of Regulation (EC) No 470/2009 the CVMP considered the possibility of extrapolating its recommendation on maximum residue limits for chloroform in cattle, sheep and pigs to other food producing species and commodities. Taking into account the current scientific knowledge the recommendations on extrapolation are justified as follows:

Animal species/ food commodities	Extrapolation possible (YES/NO)	Justification
All mammalian food producing species	Yes	Residue depletion data generated in the PBPK model indicate that for cattle, sheep and pigs residue levels in food of animal origin will lead to a worst case consumer exposure well below the ADI. It is considered that similar residue behaviour can be expected in other mammalian species.
Avian species/poultry	No	The applicability of the PBPK model used is not known for these species.
Fish	No	The applicability of the PBPK model used is not known for these species.

Animal species/ food commodities	Extrapolation possible (YES/NO)	Justification
Honey	No	Honey specific data are required in order to allow adequate evaluation of the risk to consumers. In addition the restricted use proposed is not relevant to honey bees.

3.5. Conclusions and recommendation for the establishment of maximum residue limits

Having considered that:

- relevant data have been made available in relation to the toxicology of chloroform;
- a toxicological ADI of 10 µg/kg (600 µg/person) has been established as the overall ADI;
- chloroform is rapidly metabolised and eliminated in all species for which data exist;
- while *in vivo* residue depletion data in the target species were not available, an adequately evaluated PBPK model predicted that in the worst case scenarios, the maximum consumer exposure to residues following the intended use would remain well below the ADI, following both intramuscular and subcutaneous administration of chloroform containing products to the target animals;
- the high volatility of chloroform seriously restricts the feasibility of residue controls;
- restricting the use of chloroform is justified in order to ensure that consumer exposure to residues will remain at safe levels even in the absence of numerical MRLs;

the Committee recommends the removal of chloroform from Table 2 of the Annex to Regulation No. (EU) 37/2010 and its inclusion in Table 1 of the regulation, in accordance with the following table:

Pharmacologically active substance	Marker residue	Animal species	MRL	Target tissues	Other provisions	Therapeutic classification
Chloroform	NOT APPLICABLE	All mammalian food producing species.	No MRL required	NOT APPLICABLE	Only to be used as an excipient in vaccines and only at concentrations not exceeding 1% w/v and total doses not exceeding 20 mg per animal	NO ENTRY

4. Background information on the procedure

Submission of the dossier	01.10.2012
Steps taken for assessment of the substance	
Application validated:	10.10.2012
Clock started:	11.10.2012
Meeting of the ad hoc expert group on the genotoxicity of chloroform	04.02.2013
List of questions adopted:	07.02.2013
Consolidated response to list of questions submitted:	19.04.2013
Clock restarted:	20.04.2013
Meeting of the ad hoc expert group on the PBPK model	13.05.2013
CVMP Opinion adopted:	13.06.2013