THE COMMON TECHNICAL DOCUMENT FOR THE REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

MODULE 2.4 – NON-CLINICAL OVERVIEW

PARACETAMOL 10 MG/ML SOLUTION FOR INFUSION

VERSION NUMBER: 1.0

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ABBREVIATIONS

5-HT	5-Hydroxytryptamine
AM404	N-arachidonoylphenolamine
ATC	Anatomical Therapeutic Chemical
BDNF	Brain-Derived Neurotrophic Factor
CNS	Central Nervous System
COX	Cyclooxygenase
Eur. Ph	European Pharmacopoeia
GSH	Glutathione
LD	Lethal Dose
NMDA	M-Methyl-D-Aspartate
NO	Nitric Oxide
NSAID	Nonsteroidal Anti-inflammatory Drug
PG	Prostaglandin
PPM	Parts Per Million
PGHs	Prostaglandin H_2 Synthase
SGPT	Serum Glutamate-pyruvate Transaminase
SmPC	Summary of Product Characteristics
$t_{1/2}$	Half-life
TDL_o	Lowest Published Toxic Dose

2.4. Non-Clinical Overview

2.4.1 Overview of the Nonclinical Testing Strategy

The medicinal product is Paracetamol 10 mg/mL solution for infusion which contains the active substance paracetamol. This is a medicinal product subject to medical prescription. The product is intended to intravenous route.

Paracetamol belongs to the pharmacotherapeutic group: Nervous system; Analgesics; Other analgesics and antipyretics; Anilides. ATC code: N02BE01.

Paracetamol (acetaminophen) was introduced into the pharmacological market in 1955 by McNeil Laboratories as a prescribed analgesic and antipyretic drug for children under its trade name Tylenol Children's Elixir (the name tylenol derives from its chemical name – (N-acetyl-p-aminophenol). One year later, 500-mg tablets of paracetamol were available over the counter in Great Britain under the trade name of Panadol, which were produced by Frederick Stearns & Co, the branch of Sterling Drug Inc. Paracetamol became the one of the most frequently sold analgesic and antipyretic medications. There are several hundred preparations in the trade offer, which contain paracetamol alone or in combination with other active substances (Jozwiak-Bebenista and Nowak 2014).

The precise mechanism of the analgesic and antipyretic properties of paracetamol has still to be established; it may involve central and peripheral actions.

Paracetamol provides onset of pain relief within 5 to 10 min after the start of administration. The peak analgesic effect is obtained in 1 h and the duration of this effect is usually 4 to 6 h. paracetamol reduces fever within 30 min after the start of administration with a duration of the antipyretic effect of at least 6 h.

Paracetamol 10 mg/mL solution for infusion is indicated for:

- short-term treatment of moderate pain, especially following surgery,
- short-term treatment of fever,

when administration by intravenous route is clinically justified by an urgent need to treat pain or hyperthermia and/or when other routes of administration are not possible.

2.4.1.1 Regulatory Guidance and Non-Clinical Development Programme

This Marketing Authorisation is based on Article 10(1) of Directive 2001/83/EC (generic medicinal product). Paracetamol is registered in the European Union since 21/12/1959 (EURD). The EURD is not available for paracetamol intravenous formulation.

Paracetamol 10 mg/mL solution for infusion is a generic formulation similar to the reference product Paracetamol B. Braun 10 mg/ml Infusionslösung, manufactured by B. Braun Melsungen AG.

This procedure concerns an application for a generic medicinal product, which has the same qualitative and quantitative composition in active substance as the reference medicinal product, as well as the same pharmaceutical form. Paracetamol 10 mg/mL solution for infusion is a generic medicinal product of an active substance available on the European market for 60 years. For this kind of application, it is not necessary to provide pre-clinical and clinical data relating to the active substance. Therefore, this application is based in clinical summary data for paracetamol, mainly based on the existing marketing authorization dossier of the reference medicinal product and literature data.

No new non-clinical studies were performed in support of this application. Extensive literature data is available, and these are sufficient to evaluate the potential safety issues with paracetamol at dose levels proposed for use in humans.

2.4.1.2 Literature Search Strategy

This Non-clinical overview is based on published scientific literature. Searched were carried out in bibliographic, and factual databases. Specific search criteria were used, adjusted to the specific database terminology, scope, and structure, covering all aspects required for this overview. Primary English language literature was selected initially on the basis of search results including abstracts, and subsequently on the basis of original publications acquired. Where necessary, reference lists of original publications were searched manually for complementary publications. Websites of EMA and FDA were also searched for relevant data.

2.4.1.3 Concordance with Current Research Practice

Due to the long use of paracetamol, some studies cited in this report were performed before the most recent guidelines on Good Laboratory Practice went into force. However, as these

studies were published in peer-reviewed journals there is no reason to believe that a repetition of these studies under current requirements would produce significantly different results.

2.4.2 Pharmacology

Paracetamol belongs to the pharmacotherapeutic group: Nervous system; Analgesics; Other analgesics and antipyretics; Anilides. ATC code: N02BE01.

The precise mechanism of the analgesic and antipyretic properties of paracetamol has still to be established; it may involve central and peripheral actions (Paracetamol B Braun - SmPC 2022).

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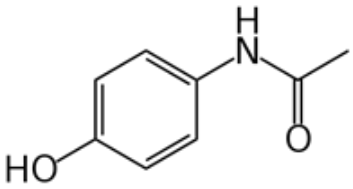
The chemical properties of the active substance paracetamol are presented in Table 1.

Table 1 - Chemical properties of paracetamol.

Parameter	Paracetamol
Chemical Name	N-(4-hydroxyphenyl)acetamide
Synonymous	Acetaminophen
CAS No.	103-90-2
Molecular Formula	$C_8H_9NO_2$
Molecular Weight	151.16 g/mol
Structural Formula	
Solubility	Very slightly soluble in cold water and soluble in boiling water. Freely soluble in alcohol. Soluble in methanol, ethanol, dimethylformamide, ethylene dichloride, acetone, and ethyl acetate. Slightly soluble in ether. Practically insoluble in petroleum ether, pentane, and benzene.
Physical description	Odorless white crystalline solid. White powder. pH (saturated aqueous solution) about 6.

Data retrieved from (PubChem 2023).

A good number of non-clinical studies is available concerning the pharmacological and pharmacodynamic of paracetamol. Literature publications refer to the direct effects of paracetamol treatment in *in vitro* and *in vivo* models.

2.4.2.2 Pharmacodynamics

2.4.2.2.1 Mechanism of Action

The precise mechanism of the analgesic and antipyretic properties of paracetamol has still to be established; it may involve central and peripheral actions (Paracetamol B Braun - SmPC 2022).

Paracetamol is a p-aminophenol derivative with analgesic and antipyretic activities. Although the exact mechanism through which acetaminophen exert its effects has yet to be fully determined, paracetamol may inhibit the nitric oxide (NO) pathway mediated by a variety of neurotransmitter receptors including N-methyl-D-aspartate (NMDA) and substance P, resulting in elevation of the pain threshold. The antipyretic activity may release in the central nervous system (CNS) and prostaglandin-mediated effects on the heat-regulating center in the anterior hypothalamus (PubChem 2023).

Despite the similarities to nonsteroidal anti-inflammatory drugs (NSAIDs), the mode of action of paracetamol has been uncertain, but it is now generally accepted that it inhibits cyclooxygenase (COX)-1 and COX-2 through metabolism by the peroxidase function of these isoenzymes. This results in inhibition of phenoxyl radical formation from a critical tyrosine residue essential for the cyclooxygenase activity of COX-1 and COX-2 and PG synthesis. Paracetamol shows selectivity for inhibition of the synthesis of prostaglandins (PGs) and related factors when low levels of arachidonic acid and peroxides are available but conversely, it has little activity at substantial levels of arachidonic acid and peroxides. The result is that paracetamol does not suppress the severe inflammation of rheumatoid arthritis and acute gout but does inhibit the lesser inflammation resulting from extraction of teeth and is also active in a variety of inflammatory tests in experimental animals. Paracetamol often appears to have COX-2 selectivity. The apparent COX-2 selectivity of action of paracetamol is shown by its poor anti-platelet activity and good gastrointestinal tolerance. Unlike both non-selective NSAIDs and selective COX-2 inhibitors, paracetamol inhibits other peroxidase enzymes including myeloperoxidase. Inhibition of myeloperoxidase involves paracetamol oxidation and concomitant decreased formation of halogenating oxidants (e.g., hypochlorous acid,

hypobromous acid) that may be associated with multiple inflammatory pathologies including atherosclerosis and rheumatic diseases. Paracetamol may, therefore, slow the development of these diseases. Paracetamol, NSAIDs and selective COX-2 inhibitors all have central and peripheral effects. As is the case with the NSAIDs, including the selective COX-2 inhibitors, the analgesic effects of paracetamol are reduced by inhibitors of many endogenous neurotransmitter systems including serotonergic, opioid and cannabinoid systems (Graham, Davies et al. 2013).

Experimental evidence suggests that inhibition of prostaglandin biosynthesis contributes to the pharmacological actions of paracetamol. Three COX isoenzymes are involved in prostaglandin biosynthesis, COX-1, COX-2 and a recently discovered splice-variant of COX-1, COX-3 (Chandrasekharan, Dai et al. 2002). Comparison of canine COX-3 activity with murine COX-1 and -2 demonstrates that this enzyme is selectively inhibited by analgesic/antipyretic drugs such as paracetamol, phenacetin, antipyrine, and dipyrone, and is potently inhibited by some nonsteroidal anti-inflammatory drugs. Thus, inhibition of COX-3 could represent a primary central mechanism by which these drugs decrease pain and possibly fever (Chandrasekharan, Dai et al. 2002).

Paracetamol has a central analgesic effect that is mediated through activation of descending serotonergic pathways. Debate exists about its primary site of action, which may be inhibition of PG synthesis or through an active metabolite influencing cannabinoid receptors. Prostaglandin H_2 synthase (PGHS) is the enzyme responsible for metabolism of arachidonic acid to the unstable PGH_2. The two major forms of this enzyme are the constitutive PGHS-1 and the inducible PGHS-2. PGHS comprises of two sites: a COX site and a peroxidase site. The conversion of arachidonic acid to PGG2 is dependent on a tyrosine-385 radical at the COX site. Formation of a ferryl protoporphyrin IX radical cation from the reducing agent Fe^{3+} at the peroxidase site is essential for conversion of tyrosine-385 to its radical form. Paracetamol acts as a reducing co-substrate on the peroxidase site and lessens availability of the ferryl protoporphyrin IX radical cation. This effect can be reduced in the presence of hydroperoxide-generating lipoxygenase enzymes within the cell (peroxide tone) or by swamping the peroxidase site with substrate such as PGG_2. Peroxide tone and swamping explain lack of peripheral analgesic effect, platelet effect, and anti-inflammatory effect by paracetamol. Alternatively, paracetamol effects may be mediated by an active metabolite (p-aminophenol). P-aminophenol is conjugated with arachidonic acid by fatty acid amide hydrolase to form N-arachidonoylphenolamine. N-arachidonoylphenolamine exerts effect through cannabinoid

receptors. It may also work through PGHS, particularly in areas of the brain with high concentrations of fatty acid amide hydrolase (Anderson 2008).

Novel studies on the mechanism of action of paracetamol regard it as a pro-drug, which due to its active metabolites demonstrates an association with the endocannabinoid system. It has been observed that in mouse brain and spinal cord, paracetamol is subject to deacetylation to p-aminophenol that in turn reacts with arachidonic acid affected by fatty acid amide hydrolase, resulting in the formation of an active metabolite of the drug, the fatty acid amide N-arachidonoylphenolamine (Hogestatt, Jonsson et al. 2005) (Ottani, Leone et al. 2006). N-arachidonoylphenolamine (AM404) does not act directly on cannabinoid receptors, however, it increases activity of endocannabinoid system in an indirect way (Kelley and Thayer 2004). On one hand, this compound is a strong activator of the vanilloid receptor subtype 1, being a ligand of receptors for cannabinoids CB1, and on the other hand, it leads to an increase in the endogenous pool of these compounds as an inhibitor of the endogenous cannabinoid reuptake. According to more recent findings, AM404 acts through T-type calcium-channel inhibition (Cav3.2) (Kerckhove, Mallet et al. 2014).

Some studies have shown the essential role of descending serotonergic pathways and spinal 5-hydroxytryptamine $(5\text{-HT})_{1A}$, 5-HT_{2A}, or 5-HT_3 receptors in the antinociceptive effects of paracetamol, other studies have presented conflicting results, and the particular subtype of spinal 5-HT receptors involved in paracetamol-induced analgesia remains to be clarified. Recent findings suggest that systemic administration of paracetamol may activate descending serotonergic pathways and spinal 5-HT_7 receptors to produce a central antinociceptive and antihyperalgesic effects (Dogrul, Seyrek et al. 2012). Peripheral adenosine A_1 receptors have also been implicated in the antinociceptive effects of locally- and systemically-administered acetaminophen (Liu, Reid et al. 2013).

In conclusion, paracetamol acts at all levels of pain stimulus conduction from the tissue receptors through the spinal cord to the thalamus and the cerebral cortex in which pain sensations are evoked. The mechanism of analgesic action of paracetamol is complex. The following possibilities are still taken into consideration: affecting both peripheral (inhibition of COX activity) and central (COX, descending serotoninergic pathways, L-arginine/NO pathway, cannabinoid system) antinociceptive processes as well as the redox mechanism (Smith 2009).

2.4.2.2.2 Primary Pharmacodynamics

***In vitro* and mouse:** Paracetamol produces analgesia in the mouse writhing test through a central action which is paralleled by a reduction in brain PGE_2 concentrations. In contrast, diclofenac has a peripheral analgesic action in this test. Paracetamol-induced hypothermia is also accompanied by a reduction in brain PGE_2 concentrations in C57/B16 mice. This hypothermic effect of paracetamol was reduced in COX-1 but not in COX-2 gene-deleted mice. These results support the view that analgesia and hypothermia due to paracetamol are mediated by inhibition of a third COX isoenzyme (designated COX-3). In cultured mouse macrophages, COX-2 is induced by treatment with lipopolysaccharide (LPS) or with high concentrations of diclofenac. Diclofenac-induced COX-2 is inhibited with low concentrations of paracetamol, whereas LPS-induced COX-2 is insensitive to paracetamol inhibition. The mechanisms of induction and possibly the functions of these two COX-2 enzymes are also different (Botting and Ayoub 2005).

Mouse: Ayoub et al. compared the antinociceptive action of paracetamol with the non-selective NSAID drug, diclofenac as well as paracetamol antinociception in COX-1 and COX-2 knockout mice. Paracetamol (100-400 mg/kg) inhibited both acetic acid- and iloprost-induced writhing responses. In contrast, diclofenac (10-100 mg/kg) inhibited only acetic acid-induced writhing. Only diclofenac reduced peripheral prostaglandin biosynthesis whereas both drugs reduced central prostaglandin production. PGE_2 concentrations were reduced in different brain regions by administration of paracetamol. COX-1, COX-2 and COX-3 enzyme proteins were expressed in the same brain regions. The effects of paracetamol on writhing responses and on brain PGE_2 levels were reduced in COX-1, but not COX-2, knockout mice. The selective COX-3 inhibitors, aminopyrine and antipyrine also reduced writhing responses and brain PGE_2 biosynthesis. These results suggest that the antinociceptive action of paracetamol may be mediated by inhibition of a tentative COX-3 (Ayoub, Colville-Nash et al. 2006).

Paracetamol reduces the core temperatures of febrile and non-febrile mice alike. Evidence has been adduced that the selectively paracetamol-sensitive PGHS isoform, PGHS-1b (COX-3), mediates these effects. PGHS-1b, however, has no catalytic potency in mice. To resolve this contradiction, paracetamol was injected intravenously into conscious PGHS-1 gene-sufficient (wild-type (WT)) and -deficient (PGHS-$1^{-/-}$) mice 60 min before or after pyrogen-free saline or *E. coli* LPS (10 µg/kg) intravenously. Core temperature was monitored continuously; brain and plasma PGE_2 levels were determined hourly. Paracetamol at <160 mg/kg did not affect core temperature when given before pyrogen-free saline or LPS; at 160 mg/kg, it caused an approx.

2.5°C core temperature fall in 60 min. LPS given after paracetamol (all doses) induced a approx. 1°C fever, not different from that in paracetamol-untreated mice. But this rise was insufficient to overcome the hypothermia of the 160 mg/kg-treated mice; their core temperature culminated 1°C below baseline. LPS given before paracetamol similarly elevated core temperature approx. 1°C. This rise was reduced to baseline in 30 min by 80 mg/kg paracetamol; core temperature rebounded to its febrile level over the next 30 min. At 160 mg/kg, paracetamol reduced core temperature to 4°C below baseline in 60 min, where it remained until the end of the experiment. WT and PGHS-$1^{-/-}$ mice responded similarly to all the treatments. The basal brain and plasma PGE_2 levels of pyrogen-free saline mice and the elevated plasma levels of LPS mice were unchanged by core temperature at 160 mg/kg; but the latter's brain levels were reduced at 1 h, then recovered. Thus, paracetamol could exert an anti-PGHS-2 effect when this enzyme is upregulated in the brain of febrile mice. The hypothermia it induces in non-febrile mice, therefore, is due to another mechanism. PGHS-1b is not involved in either case (Li, Dou et al. 2008).

Rat: The analgesic efficacy of paracetamol and the descending noradrenergic systems using rodent models of inflammatory pain was evaluated. Inflammatory pain models were established by carrageenan injection into rats' paws. The models were defined as acute (4 h after carrageenan injection), subacute (24 h after carrageenan injection), and late (1 week after carrageenan injection) phase. To evaluate intravenous paracetamol treatment, the withdrawal threshold to mechanical stimuli was assessed simultaneously with *in vivo* microdialysis assay of noradrenaline levels in the locus coeruleus. In all phases, intravenous paracetamol had a significant anti-hyperalgesic effect. There was a significant time-dependent increase in the noradrenaline concentration within the locus coeruleus (paracetamol vs. saline treatment) in the subacute pain model, but not in the acute and late phase pain models. In conclusion, descending noradrenergic inhibitory system is involved in the antinociceptive action of paracetamol in the subacute phase of inflammatory pain (Juri, Fujimoto et al. 2021).

Ottani et al. have shown that in rats, using the hot plate test, the analgesic effect of paracetamol is prevented by two antagonists at cannabinoid CB_1 receptors (AM281 and SR141716A) at doses that prevent the analgesic activity of the cannabinoid CB_1 agonist HU210. Their results suggest that paracetamol-induced antinociception involves the cannabinoid system (Ottani, Leone et al. 2006).

2.4.2.2.3 Secondary Pharmacodynamics

***In vitro*:** Godfrey et al. investigated the effect of paracetamol and the analgesic adjuvant caffeine on the activity of NO synthase in mouse spinal cord and cerebellar slices *in vitro*, by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline. Paracetamol (100 µM) had no effect on NO synthase activity in cerebellum, but in the spinal cord both paracetamol (100 µM) and caffeine (30 µM) attenuated glutamate (5 mM)-induced [^{3}H]citrulline production and in combination they abolished it. In conclusion paracetamol inhibits spinal cord NO synthesis and this may be related to its analgesic effects (Godfrey, Bailey et al. 2007).

Antipyretic analgesic drugs (including NSAIDs) inhibit COX-2 and inducible NO synthase, resulting in decreases of the proinflammatory mediators PGE_2 and NO, respectively. Both mediators are regulated by nuclear factor-kappa B, a key transcription factor in inflammation. Few reports have compared the efficacy and potency of anti-inflammatory drugs as NO inhibitors. The effects of 4 popular antipyretic analgesic drugs on NO production induced in hepatocytes and macrophages were assessed in mouse RAW264.7 macrophages treated with bacterial lipopolysaccharide showed the highest efficacy with regard to NO production. Aspirin, loxoprofen, ibuprofen, and acetaminophen dose-dependently suppressed NO induction. Ibuprofen showed the highest potency in suppressing the induced production of NO. In rat hepatocytes, all the drugs inhibited interleukin 1β-induced NO production and ibuprofen and loxoprofen inhibited NO induction effectively. Unexpectedly, the potency of NO suppression of each drug in hepatocytes did not always correlate with that observed in RAW264.7 cells. Microarray analyses of mRNA expression in hepatocytes revealed that the effects of the four antipyretic analgesic drugs modulated the nuclear factor-kappa B signaling pathway in a similar manner to the regulation of the expression of genes associated with inflammation, including the inducible NO synthase gene. However, the affected signal-transducing molecules in the nuclear factor-kappa B pathway were different for each drug. Therefore, antipyretic analgesic drugs may decrease NO production by modulating the nuclear factor-kappa B pathway in different ways, which could confer different efficacies and potencies with regard to their anti-inflammatory effects (Inaba, Yoshigai et al. 2015).

2.4.2.2.4 Pharmacodynamic Interactions

Rat: The effects of analgesic drugs were studied in a model of acute inflammatory pain in carrageenin-injected rats using the vocalization threshold to paw pressure. A combination of

3 different intravenous drugs were used: morphine, diclofenac, and propacetamol, a pro-drug of acetaminophen. The dose-response curves were first obtained for each drug alone. The analgesic potencies of the combinations of morphine and diclofenac (ratios, 1:5.66 and 1:10), morphine and propacetamol (ratio, 1:250), and diclofenac and propacetamol (ratio, 1:65.7) were thereafter evaluated and compared with the effects of the drugs alone. Propacetamol and morphine or diclofenac and propacetamol combinations were additive for all doses tested (Fletcher, Benoist et al. 1997)

The effects of intraperitoneal injections of a combination of 2 COX inhibitors, ibuprofen and paracetamol, with a weak opiate, codeine, on nociception were investigated in Sprague Dawley rats. Administration of paracetamol (11, 44, and 88 mg/kg), ibuprofen (8.75, 35, and 140 mg/kg) or codeine (0.44, 1.75, and 3.5mg/kg) alone caused a dose-dependent inhibition of reperfusion hypernociception. Administration of a combination of 0.44 mg/kg codeine +8.75 mg/kg ibuprofen +11 mg/kg paracetamol, drug doses that did not significantly reduce reperfusion hypernociception when administered individually or in pairs, abolished reperfusion hypernociception, such that the antihypernociceptive efficacy of the combination was approximately 2.5-fold greater than that of the sum of the antihypernociceptive efficacy of the individual drugs. Coordinated motor function, tested using a rotarod, was not impaired at the doses they used. In conclusion, codeine, paracetamol and ibuprofen act synergistically to induce antihypernociception in rats at doses which do not affect motor function (Mitchell, Gelgor et al. 2010).

Oral administration of N-acetylcysteine (163 mg/kg at zero time and 82 mg/kg 30 min later) to adult male Sprague-Dawley rats given an intravenous injection of paracetamol, 150 mg/kg at zero time, increased the formation of paracetamol sulphate and thereby enhanced the elimination of paracetamol Apparently, N-acetylcysteine is an *in vivo* source of inorganic sulphate since availability of the latter is rate-limiting in the formation of paracetamol sulphate. Increased metabolic conversion of paracetamol to its sulphate conjugate results in decreased formation of other metabolites of paracetamol presumably including the reactive metabolite responsible for the hepatotoxic effect of the drug. This may account, at least in part, for the protective effect of N-acetylcysteine against paracetamol-induced hepatotoxicity (Galinsky and Levy 1979).

2.4.2.2.5 Safety Pharmacology

No formal safety pharmacology studies have been performed. However, the human safety of paracetamol is well known (see 2.5 Clinical Overview).

2.4.2.2.6 Discussion and Conclusions

Primary pharmacodynamic studies showed that paracetamol has analgesic and antipyretic properties in mice and rats. Additionally, paracetamol has anti-inflammatory effects *in vitro*.

Pharmacodynamic interactions of paracetamol and morphine, diclofenac, propacetamol, and N-acetylcysteine were observed in rats.

No formal safety pharmacology studies have been performed. However, the human safety of paracetamol is well known.

2.4.3 Pharmacokinetics

The pharmacokinetic properties of paracetamol were extensively investigated in *in vitro* and *in vivo* (mice, rats, hamsters, and guinea pigs) models.

Relevant data from pre-clinical pharmacokinetic studies are shown in the following sections.

2.4.3.1 Pharmacokinetic Properties

2.4.2.2.1 Metabolism and Elimination

The metabolism of paracetamol in various species is presented in Figure 1.

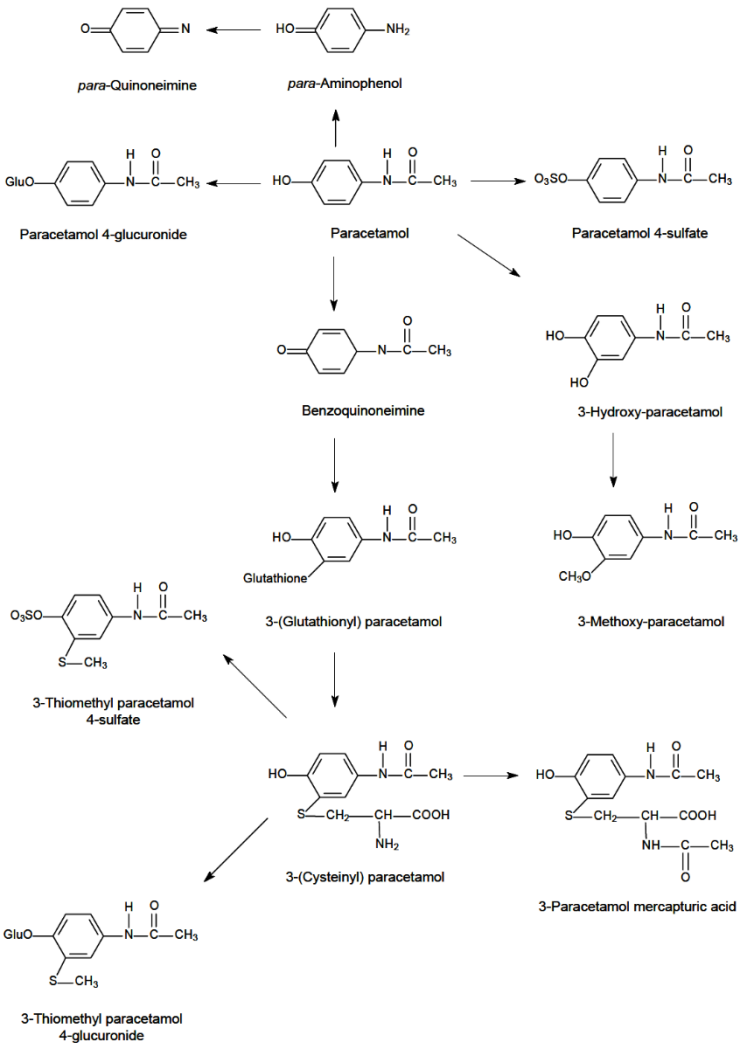


Figure 1- Summary of metabolism of paracetamol based on data for different species (WHO 1999).

***In vitro*, rat and hamster:** In a study in hamsters, para-aminophenol was identified as a urinary metabolite. When [acetyl-^{14}C] paracetamol and [ring-^{14}C] paracetamol were compared in binding studies with renal cortex protein, the amount of covalent binding of radiolabel from [ring-^{14}C] paracetamol was 4 times higher in Fischer rats, which are sensitive to paracetamol nephrotoxicity. This suggests that deacetylation to para-aminophenol is the primary pathway of bioactivation. In contrast, binding of ring- and acetyl-labelled paracetamol to renal protein was similar in non-susceptible Sprague-Dawley rats, suggesting that oxidation may be an important metabolic route. These data indicate that para-aminophenol may be responsible for paracetamol-induced renal necrosis in Fischer 344 rats. Studies *in vitro* with microsomes from Sprague-Dawley rats and *in vivo* with Sprague-Dawley rats that received a 1100 mg/kg body weight intraperitoneal dose of paracetamol in combination with specific inhibitors of deacetylation and oxidation suggested that both oxidative metabolism and deacetylation contribute to the nephrotoxicity of paracetamol (WHO 1999).

Rat: In rats, the percentage of parent compound excreted in the urine ranged between 0.5-3.8% of the dose administered. The percentage of sulphate metabolite ranged between 6.6% (at dose of 600 mg/kg) to 67% (at dose of 37.5 mg/kg), while that of glucuronide conjugate ranged between 12.3% to 17.0%, irrespective of the administered dose. The biliary excretion rate of parent compound plus metabolites remained unchanged (range: 20.2-29.3%) (Hjelle and Klaassen 1984).

2.4.2.2.2 Pharmacokinetic Studies

***In vitro* and rat:** The effect of conjugated-metabolite, paracetamol sulphate, on the pharmacokinetics of its parent drug, paracetamol, was examined in rats following the intravenous bolus administration of paracetamol with paracetamol sulphate, the plasma elimination of paracetamol was delayed, and the distribution volume of paracetamol was increased at the paracetamol sulphate coadministration with 60 mg paracetamol equivalent per kg (eq/kg). The percentages of dose excreted in the urine and bile in 4 h as paracetamol and its conjugated metabolites, paracetamol sulphate and paracetamol glucuronide, were significantly decreased. On the other hand, following the intravenous bolus administration of paracetamol under the steady-state concentration of paracetamol sulphate, the distribution volume and total body clearance of paracetamol were significantly increased. Competitive displacement in serum protein binding of paracetamol by paracetamol sulphate was

ascertained *in vitro* and *in vivo*. A part of the conflict between the bolus and infusion experiment may be explained by the changes in the distribution volume of paracetamol contributed to the paracetamol sulphate concentration-dependent serum protein binding of paracetamol. It was speculated that the pharmacokinetics of paracetamol was partly interacted with paracetamol sulphate by the displacement of serum protein binding (Sawamoto, Kurosaki et al. 1997).

Mouse, rat, hamster and guinea pig: Adult male Sprague-Dawley rats received an intravenous injection of 15, 30, 150 or 300 mg/kg paracetamol, and plasma and urine were assayed during 6-7 h after dosing for paracetamol and its glucuronide and sulfate conjugates. At each dose, the plasma concentrations of paracetamol decreased exponentially with time, but at the two higher doses total clearance and the fraction excreted as sulfate conjugate decreased. Both paracetamol glucuronide and paracetamol sulfate formation appeared to be capacity limited. A correlation has been found between species sensitivity to the hepatotoxicity of paracetamol and the balance between two pathways: (i) formation of glutathione conjugates and the corresponding hydrolysis products (indicative of the 'toxic' pathway) and (ii) metabolism via formation of glucuronide and sulfate esters (the 'detoxification pathway'). The susceptible species (hamsters, mice) excreted 27-42% of the dose as metabolite in the toxication pathway, whereas the more resistant species (rats, rabbits, guinea pigs excreted only 5-7% of the dose via this route. At sufficiently high doses of paracetamol, glutathione is depleted. and the reactive metabolite binds covalently to cell macromolecules. It has also been noted that paracetamol and N-acetyl-para-benzoquinone imine may exert their cytotoxic effects via disruption of Ca^{2+} homeostasis secondary to the depletion of soluble and protein-bound thiols (Galinsky and Levy 1981, WHO 1999).

Rat: In order to investigate the *in vivo* 1st-pass metabolism of paracetamol following the oral and intraduodenal administration in rats, a pharmacokinetic compartment model including absorption process was developed. Using the parameters for the disposition kinetics of paracetamol and its metabolites, sulphate and glucuronide, which were determined in the separate study, the extent of the first-pass metabolism and the contribution of sulphation and glucuronidation to the total first-pass metabolism *in vivo* were quantitatively estimated. As for the results, the 1st-pass metabolism of paracetamol following the oral and intraduodenal administration was mainly attributable to the sulphoconjugation pathway in rats. The sulphation of paracetamol in the intestine and/or in the liver during the 1st-pass was proved to be a saturable process, Then, the sulphation in the 1st-pass metabolism showed the dose- and absorption rate-dependent kinetics (Tone, Kawamata et al. 1990).

Dose-dependent paracetamol pharmacokinetics is thought to be due to saturation of sulphation and glucuronidation, although its glucuronidation has not been thoroughly examined. Because many drug-glucuronides are extensively excreted into bile, the excretion of paracetamol metabolites in bile was examined in urethane-anesthetized rats which received 37.5, 75, 150, 300 or 600 mg/kg of paracetamol intravenously. Disappearance of paracetamol from plasma exhibited clear dose-dependency as determined by prolongation of half-life ($t_{1/2}$) and decreases in total body clearance at 150 mg/kg or higher. Biliary excretion of the various metabolites of paracetamol increased from 20 to 49% as the dosage was increased from 37.5-600 mg/kg. The glucuronide conjugate was the major form of paracetamol in bile at all dosages. Biliary excretion of the glucuronide conjugate increased from 10.5-40.2% of the recovered dose as the amount administered was increased to 600 mg/kg, whereas urinary excretion of the glucuronide conjugate remained relatively constant at approximately 20% of that recovered. Although the fraction of paracetamol excreted as the glucuronide conjugate increased to over 70% of that recovered at the highest dose, a significant decline in the rate constant for glucuronide formation was noted at 300 mg/kg and higher. Likewise, the rate for glutathione conjugation was also lower at 300 mg/kg, whereas the formation of the sulphate conjugate was decreased at lower dosages (75 mg/kg). The results of this study show that glucuronidation is a high-capacity, high-dose saturable pathway of paracetamol biotransformation whose product is preferentially eliminated in bile after high dosages. The shift in paracetamol conjugation from the low-dose saturable sulphation pathway to the high-capacity glucuronidation route is associated with a decrease in paracetamol metabolite excretion in urine and a large increase in its biliary excretion (Hjelle and Klaassen 1984).

2.4.2.2.3 Pharmacokinetic Interactions

Rat: Oral administration of N-acetylcysteine (163 mg/kg at zero time and 82 mg/kg 30 min later) to adult male Sprague-Dawley rats given an intravenous injection of paracetamol, 150 mg/kg at zero time, increased the formation of paracetamol sulphate and thereby enhanced the elimination of paracetamol. Apparently, N-acetylcysteine is an *in vivo* source of inorganic sulphate since availability of the latter is rate-limiting in the formation of paracetamol sulphate. Increased metabolic conversion of paracetamol to its sulphate conjugate results in decreased formation of other metabolites of paracetamol presumably including the reactive metabolite responsible for the hepatotoxic effect of the drug. This may account, at least in part,

for the protective effect of N-acetylcysteine against paracetamol-induced hepatotoxicity (Galinsky and Levy 1979).

2.4.2.2.4 Discussion and Conclusions

The pharmacokinetic properties of paracetamol were investigated *in vitro* and in mice, rats and hamsters.

In rats, the percentage of parent compound excreted in the urine ranged between 0.5-3.8% of the dose administered. Also, the biliary excretion rate of parent compound plus metabolites remained unchanged (range: 20.2-29.3%).

The plasma concentrations of paracetamol decreased exponentially with time in rats, but at higher doses total clearance and the fraction excreted as sulfate conjugate decreased.

In rats, the first-pass metabolism of paracetamol following oral and intraduodenal administration was mainly attributable to the sulphoconjugation pathway.

Also, in rats, disappearance of paracetamol from plasma exhibited a clear dose-dependency as determined by prolongation of $t_{1/2}$ and decreases in total body clearance at 150 mg/kg or higher. Biliary excretion of the various metabolites of paracetamol increased from 20-49% as the dosage was increased from 37.5 to 600 mg/kg-

Pharmacokientic drug interactions between paracetamol and N-acetylcysteine were observed in rats.

2.4.4 Toxicology

The toxicology of paracetamol was investigated *in vitro* and in mice, rats, guinea pigs, dogs and monkeys.

Relevant data from pre-clinical toxicology studies are shown in the following sections.

2.4.4.1 Single Dose Toxicity

Mouse, rat, guinea pig and dog: Lethal dose (LD_{50}) values for paracetamol are presented in Table 2.

Table 2 – Lethal dose $(LD)_{50}$ values of paracetamol (SIRI 2008, HSDB 2023).

Route	Species	LD_{50}	Toxic Effects
Oral	Mouse	338 mg/kg	No details given.
Intraperitoneal	Mouse	367 mg/kg	Analgesia, body temperature decrease.
Subcutaneous	Mouse	310 mg/kg	No details given.
Oral	Rat	1944 mg/kg	No details given.
Intraperitoneal	Rat	1205 mg/kg	Somnolence, tremor.
Oral	Guinea pig	2620 mg/kg	Altered sleep time (including change in righting reflex); somnolence (general depressed activity); tremor.
Oral	Dog	2 g/kg	Altered sleep time (including change in righting reflex); changes in spleen; temperature decrease.
Intravenous	Dog	826 mg/kg	Analgesia.

Mouse: Paracetamol is without known ocular adverse effects, with the exception that in genetically very special mice it can cause irreversible opacification of the anterior portion of the lens when a large dose is given intraperitoneally (HSDB 2023)

The effects of acetaminophen exposures at levels that do not result in significant hepatic injury on mature lung were evaluated in adult male mice. Animals were exposed to paracetamol (140 mg/kg or 280 mg/kg, intraperitoneal). Paracetamol 140 mg/kg did not induce significant hepatic injury; however, this exposure resulted in a significant proinflammatory pulmonary transcriptional response and an influx of immune cells in the bronchoalveolar lavage fluid. These proinflammatory changes were associated with measurable morphometric changes suggesting parenchymal tissue remodeling and alveoli wall thinning at the low paracetamol dose. Exposure to the highest dose of paracetamol (280 mg/kg) given intraperitoneally induces

both hepatic and pulmonary injury in adult mice. In conclusion, acetaminophen exposures that do not cause significant hepatic injury result in acute inflammatory, morphometric and metabolic changes in the mature lung (Dobrinskikh, Al-Juboori et al. 2021).

Rat: Post-mitochondrial supernatants isolated from the livers of mature rats (3-6-month-old) 2 h or more after administration of a single large oral dose of paracetamol (800 mg/kg) showed rapid rates of lipid peroxidation. In similar experiment with old rats (27-30-month-old) the time between administration of paracetamol and the onset of lipid peroxidation was much longer, up to 6 h (HSDB 2023).

Male Wistar rats were fasted 24 h and administered a single dose of paracetamol/water suspension (2 g paracetamol/kg) by gavage. Rats were killed, and liver and blood samples taken at 0, 6, 9, 12, and 24 h post paracetamol administration. Hepatic reduced glutathione levels were lowered within 6 h after paracetamol treatment and remained so until returning to control levels at 12-24 h. Serum glutamate pyruvate transaminase (SGPT) levels were increased from control (n=7) levels of 30-40 mU/mL to 700-3000 mU/mL at 24 h after paracetamol administration. Blood glucose concentrations of paracetamol treated rats (n=13) were 5.85 ± 0.50 mM compared to the control values of 5.28 ± 0.36 mM. Based on trypan blue exclusion, paracetamol-induced necrosis around the central vein was noted at 9-12 h and was much more extensive at 24 h after treatment. A concurrent activation of glycogen phosphorylase in perivenous hepatocytes and an increase in periportal hepatocyte glycogen content was observed at 12 h post treatment (HSDB 2023).

The effects of paracetamol overdose on glycogen metabolism in rat liver have been investigated and related to its cytotoxicity. Paracetamol was administered to male rats by gavage after a 24-h fast and refeeding was not permitted. An early (9-12 h) increase in histochemical demonstrable glycogen phosphorylase an activity in perivenous hepatocytes preceded major loss of membrane integrity as assessed by SGPT activity and uptake of trypan blue during perfusion. These changes occurred only after a decrease in the concentration of reduced glutathione, which is generally observed about 4 h after paracetamol treatment. The activation of glycogen phosphorylase in perivenous hepatocytes occurred concurrently with an increase in glycogen content of periportal hepatocytes, indicating a clear heterogeneity in the response of the two-cell populations to the hepatotoxin (Jepson, Davis et al. 1987).

Cat: Paracetamol was administered to 4 adult cats. A marked degree of cyanosis was seen in these animals within 4 h after administration of single tablet containing 325 mg due to hypoxia

associated with conversion of hemoglobin to methemoglobin. In addition, anemia, hemoglobinuria, and icterus were eventually seen in the cats. Hemolysis of red blood cells was responsible for development of anemia and hemoglobinuria. Icterus was attributed to both lysis of erythrocytes and hepatic necrosis. Facial edema was also observed in 3/4 experimental cats (HSDB 2023).

2.4.4.2 Repeat Dose Toxicity

Mouse: Lowest published toxic dose (TDLo) were determined in mice (SIRI 2008): TDLo oral: 136 g/kg for 13 weeks, continuous. Toxic effects: liver - other changes; kidney, ureter, bladder - changes in bladder weight; nutritional and gross metabolic - weight loss or decreased weight gain; TDLo oral: 336 g/kg for 40 weeks, continuous. Toxic effects: liver - hepatitis (hepatocellular necrosis), diffuse; other changes; changes in liver weight.

Rat: TDLo were determined in rats (SIRI 2008). TDLo oral: 105 g/kg for 35 days, continuous. Toxic effects: liver - other changes; nutritional and gross metabolic - weight loss or decreased weight gain; related to chronic data – death; TDLo oral: 68 g/kg for 13 weeks, continuous. Toxic effects: liver - other changes; kidney, ureter, bladder - changes in bladder weight; nutritional and gross metabolic - weight loss or decreased weight gain; TDLo oral: 6080 mg/kg for 19 days, intermittent. Toxic effects: gastrointestinal - other changes; liver - changes in liver weight; blood - changes in leukocyte white blood cell count; and TDLo intraperitoneal: 1600 mg/kg for 2 days, intermittent. Toxic effects: liver - other changes; biochemical - enzyme inhibition, induction, or change in blood or tissue levels - hepatic microsomal mixed oxidase (dealkylation, hydroxylation, etc.); enzyme inhibition, induction, or change in blood or tissue levels - cytochrome oxidases (including oxidative phosphorylation).

Sub-Chronic and Chronic Toxicity

14-Day Study

Mouse and rat: Rats were fed diets containing 0, 800, 1600, 3100, 6200, or 12500 parts per million (ppm) paracetamol and mice were fed diets containing 0, 250, 500, l000, 2000, or 4000 ppm paracetamol. There were no deaths among any groups during the study; the final mean body weight of male rats that received 12500 ppm was significantly lower than that of the controls. Final mean body weights of male and female mice and female rats that received

paracetamol were similar to those of the controls. Feed consumption by male and female rats that received 12500 ppm paracetamol was lower than that of the controls; feed consumption by all other exposed groups was higher than that of the controls (NTP 1993).

28-Day Study

Rat: A study evaluated the subacute toxicity of paracetamol infusion in albino Wistar rats (male and female) at different dose levels, ranging from 16 to 66 mg/kg body weight. No mortality was seen in any of the treatment groups during the course of study. Various physiological, hematological as well as biochemical parameters were studied and found not to be changed significantly, indicating that paracetamol infusion is non-toxic even at higher dose level in Wistar rats. Overall safety and tolerability profile of paracetamol infusion is proved good and does not appear to carry risk of serious adverse effects (Payasi A 2010).

13-Week Study

Mouse and rat: Rats and mice were fed diets containing 0, 800, 1600, 3200, 6200, 12500, or 25000 ppm paracetamol. Two male and two female rats, and one male and one female mouse that received 25000 ppm, and two male mice that received 12500 ppm died from paracetamol-related toxicity the end of the studies. Final mean body weights of male and female rats and mice that before received 12500 or 25000 ppm were lower than those of the controls. The patterns of feed consumption and reduced body weights that occurred among rats and mice that received diets containing 12500 or 25000 ppm were indicative of poor feed palatability.

Rat: Paracetamol-related lesions were observed in the liver (necrosis, chronic active inflammation, hepatocytomegaly), kidney (tubule cast, tubule necrosis, tubule regeneration), reproductive organs (atrophy of testis, ovary, and uterus), thymus and lymph nodes (lymphoid depletion) of rats that received 25000 ppm, and of the liver (chronic active inflammation, hepatocytomegaly) and testis (atrophy) of male rats receiving 12500 ppm. Compound-related lesions in mice were found in the liver (hepatocytomegaly, focal calcification, pigmentation, necrosis) of males that received 6200,12500, or 25000 ppm and females that received 12500 or 25000 ppm.

2-Year Study

Mouse and rat: Diets containing 0, 600, 3000, or 6000 ppm paracetamol were given continuously to groups of 60 rats and mice of each sex for up to 104 weeks. After 65 weeks of exposure, 10 animals from each group were evaluated for histopathology and for hematology, urinalysis, and clinical chemistry parameters. Survival and mean body weights of rats that received paracetamol were similar to those of the controls throughout the study. The average severity of nephropathy was increased in exposed male and female rats. In males this was associated with an increased incidence of parathyroid hyperplasia (renal hyperparathyroidism). The incidence of focal renal tubule hyperplasia was also increased in exposed male rats. The incidence of mononuclear cell leukemia was increased in exposed female rats and was significantly increased in the 6000 ppm group (9/50; 17/50; 15/50; 24/50). Survival of exposed and control mice was similar throughout the study. Mean body weights of mice that received paracetamol were generally lower than those of the controls throughout the study. Although the incidence of thyroid follicular cell hyperplasia increased with dose among groups of exposed male and female mice, there was no increase in the incidence of follicular cell neoplasms. Renal tubule hyperplasia occurred in one low-dose and two high-dose males and a renal tubule adenoma was present in one low-dose and one high-dose male (NTP 1993).

Dose selection for the 2-year studies was based on reduced body weights and the liver lesions observed in rats and mice at 12500 and 25000 ppm (NTP 1993).

2.4.4.3 Genotoxicity

***In vitro*:** Acetaminophen was not mutagenic in *Salmonella typhimurium* strains TA100, TAI535, TA1537, or TA98 with or without S9. In cytogenetic tests with Chinese hamster ovary cells, acetaminophen induced sister chromatid exchanges and chromosomal aberrations in both the presence and absence of S9 (NTP 1993).

Paracetamol did not induce gene mutation in *Salmonella typhimurium* TA100, TA102, TA1535, TA1537, TA1538 or TA98 or in *E. coli* with or without exogenous metabolic activation, expect in one study in which a metabolic activation system from hamster was used. It induced chromosomal aberrations in Allium cepa. Paracetamol did not induce sex-limited chromatid exchange in Chinese hamster cells and in mouse cells with and without exogenous recessive lethal mutation in Drosophila melanogaster. It gave negative or weakly positive results for the induction of DNA strand breaks in rodent cells *in vitro*. The chemical induced unscheduled

DNA synthesis in mouse hepatocytes; it gave inconsistent results in rat hepatocytes and did not induce this effect in hamster or guinea-pig hepatocytes or in Chinese hamster lung cells *in vitro*. Paracetamol did not induce gene mutation in mammalian cells *in vitro*. It induced sister metabolic activation *in vitro*. It induced micronuclei in a rat kidney cell line *in vitro* but not in primary rat hepatocytes without exogenous metabolic activation. It induced chromosomal aberrations in Chinese hamster and weakly in mouse cells *in vitro*. Paracetamol weakly induced cell transformation in mouse cells. It induced sister chromatid exchange and chromosomal aberration in human lymphocytes without exogenous metabolic activation *in vitro* (WHO 1999).

***In vitro* and *in vivo*:** Paracetamol does not cause gene mutations, either in bacteria or in mammalian cells. There are, however, published data giving clear evidence that paracetamol causes chromosomal damage *in vitro* in mammalian cells at high concentrations and indicating that similar effects occur *in vivo* at high dosages. These studies, which employed doses ranging from the dose resulting in human therapeutic peak plasma levels to highly toxic doses, give convincing evidence that genotoxic effects of paracetamol appear only at dosages inducing pronounced liver and bone marrow toxicity and that the threshold level for genotoxicity is not reached at therapeutic dosage. Reliable studies on the ability of paracetamol to affect germ cell DNA are not available. However, based on the amount of drug likely to reach germ cells and the evidence of thresholds, paracetamol is not expected to cause heritable damage in man. Various old and poorly designed long-term studies of paracetamol in the mouse and rat have given equivocal results. A few of these studies showed increased incidence of liver and bladder tumors at hepatotoxic doses. NTP feeding studies have shown that paracetamol is non-carcinogenic when given at non-hepatotoxic doses up to 300 mg/kg/day to the rat and up to 1000 mg/kg/day to the mouse. Taking into account the knowledge of the hepatotoxicity and metabolism of paracetamol and the existence of thresholds for its genotoxicity, the animal studies do not indicate a carcinogenic potential at non-hepatotoxic dose levels. Based on this updated assessment of the genotoxicity and carcinogenicity of paracetamol, it is concluded that there is no need for regulatory action (Bergman, Muller et al. 1996).

Studies *in vitro* and *in vivo* indicate that the reactive metabolite of paracetamol can bind irreversibly to DNA and cause DNA strand breaks. Paracetamol inhibits both replicative DNA synthesis and DNA repair synthesis in vitro and in experimental animals. Paracetamol does not cause gene mutations, either in bacteria or in mammalian cells. On the other hand, a co-mutagenic effect of paracetamol has been reported. Furthermore, paracetamol increases the

frequency of chromosomal damage in mammalian cell lines, isolated human lymphocytes and experimental animals. Two independent studies have shown an increase in chromosomal damage in lymphocytes of human volunteers after intake of therapeutic doses of paracetamol, whereas a third study was negative. Paracetamol-induced chromosomal damage appears to be caused by an inhibition of ribonucleotide reductase. This indicates that a threshold level for the paracetamol-induced chromosomal damage may exist. Genotoxic effects of paracetamol have, however, been demonstrated both *in vitro* and *in vivo* at or near therapeutic concentrations. The data indicate that the use of paracetamol may contribute to an increase in the total burden of genotoxic damage in man. Thus, there may be a need to evaluate the therapeutic benefit of paracetamol, taking into consideration not only its potential to induce acute and chronic organ damage, but also genotoxic effects (Rannung, Holme et al. 1995).

Paracetamol showed no evidence of induction of point or gene mutations in bacterial and mammalian cell systems or in *in vivo* studies. In reliable, well-controlled test systems, clastogenic effects were only observed in unstable, p53-deficient cell systems or at toxic and/or excessively high concentrations that adversely affect cellular processes (e.g., mitochondrial respiration) and cause cytotoxicity. Across the studies, there was no clear evidence that acetaminophen causes DNA damage in the absence of toxicity. In well-controlled clinical studies, there was no meaningful evidence of chromosomal damage. Based on this weight-of-evidence assessment, acetaminophen overwhelmingly produces negative results (i.e., is not a genotoxic hazard) in reliable, robust high-weight studies (Kirkland, Kovochich et al. 2021).

Mouse and rat: Paracetamol inhibited DNA synthesis in several tissues of mice and rats treated *in vivo*. It induced DNA single strand breaks in mouse liver cells but not in mouse kidney or rat liver or kidney cells *in vivo*. Paracetamol did not induce micronuclei in mice *in vivo* but did induce chromosomal aberrations in mouse bone marrow cells. Paracetamol induced aneuploidy in rat embryo cells *in vivo* (WHO 1999).

Rat: Paracetamol metabolism, cytotoxicity, and genotoxicity were measured in primary cultures of rat hepatocytes Although 3 mM paracetamol caused a slight increase in cellular release of lactate dehydrogenase into the culture medium, cellular glutathione concentration (an index of paracetamol metabolism) was reduced by 50%. Paracetamol at 7 mM was significantly more toxic to these hepatocytes and had a similar but more marked effect on glutathione concentrations. In spite of its cytotoxicity, neither dose of paracetamol stimulated DNA repair synthesis when monitored by the rate of incorporation of [^{3}H] thymidine into DNA following exposure to paracetamol. Thus, although paracetamol has been shown to be both

hepato- and nephrotoxic in several *in vivo* and *in vitro* systems, the reactive toxic metabolite of paracetamol is not genotoxic in rat primary hepatocyte cultures (Milam and Byard 1985).

2.4.4.4 Carcinogenicity

Rat: Under the conditions of the 2-year feed studies there was no evidence of carcinogenic activity of paracetamol in male F344/N rats that received 600, 3000, or 6000 ppm. There was equivocal evidence of carcinogenic activity of paracetamol in female F344/N rats based on increased incidences of mononuclear cell leukemia. There was no evidence of carcinogenic activity of paracetamol in male and female B6C3F_1 mice that received 600, 3000, or 6000 ppm (NTP 1993).

Animal studies do not indicate a carcinogenic potential at non-hepatotoxic dose levels. Based on this updated assessment of the genotoxicity and carcinogenicity of paracetamol, it is concluded that there is no need for regulatory action (Bergman, Muller et al. 1996).

According to a comprehensive review from the California Office of Environmental Health Hazard Assessment paracetamol is not a carcinogenic hazard at any dose level (Sirois 2021).

The mechanistic data related to the steps and timing of cellular events following therapeutic recommended (≤4 g/day) and higher doses of paracetamol that may cause hepatotoxicity and whether these changes indicate that paracetamol is a carcinogenic hazard were evaluated. At therapeutic recommended doses, paracetamol forms limited amounts of N-acetyl-p-benzoquinone-imine without adverse cellular effects. Following overdoses of paracetamol, there is potential for more extensive formation of NAPQI and depletion of glutathione, which may result in mitochondrial dysfunction and DNA damage, but only at doses that result in cell death - thus making it implausible for paracetamol to induce the kind of stable, genetic damage in the nucleus indicative of a genotoxic or carcinogenic hazard in humans. In conclusion, the data demonstrate a lack of a plausible mechanism related to carcinogenicity (Jaeschke, Murray et al. 2021).

2.4.4.5 Reproductive and Developmental Toxicity

Mouse: Paracetamol was tested for its effects on reproduction and fertility in CD-1 mice, following the reproductive assessment by continuous breeding (RACB) protocol Data on body weights, clinical signs, and food and water consumption from a 2-week dose-range-finding

study (Task 1) were used to set, exposure levels for the Task 2 continuous cohabitation phase at 0.25, 0.5, and 1.0% in the diet. Feed consumption was reduced only in females at the top dose level by 10-20%. Measured body weight and feed consumption allowed exposure to be estimate as approximately 370, 770, and 1400 mg/kg/day. During Task 2, 4 animals died: 2, 1, and 1 each in the low, middle, and high dose groups. During Task 2, the number of litters per pair decreased by 3% for the high dose group. No changes were noted in the number of pups per litter, viability, or in adjusted pup weight. The slight reduction in number of litters per pair was judged to be too small to yield a detectable change during the statistically less powerful Task 3 crossover mating, so no crossover test was conducted. For the F_1 evaluation, the last litter in Task 2 from all dose groups was nursed to weaning and reared on the diet consumed by their parents. F_1 pup body weights were reduced at all doses for both sexes by approx. 6-18%. Pup body weight gain to weaning was also reduced for the medium and high dose males (17 and 34%) and for females at all doses (10-28%). All dose groups were reared consuming the same diet provided to their parents. The body weight differences that were seen during nursing were reduced, but still present, at the time of mating. At the F_1 mating, the F_2 pup weight adjusted for litter size was decreased by 11% at the high dose level. No other reproductive end points were affected. After the F_2 pups were delivered and evaluated, the F_I adults from only the control and high dose groups were killed and necropsied. Compared to controls, the high dose males weighed 10% less, while organ weights were not affected. Sperm abnormalities increased from 7% (controls) to 16% at the high dose. High dose females weighed 8% less, while adjusted liver weight was increased by 10%. In summary, the greatest toxicity produced by acetaminophen in the diet of Swiss mice was on the growing neonate. Fertility endpoints (ability to bear normal numbers of normal-weight young) were generally not affected (Lamb 1997).

High doses of paracetamol (400 mg/kg) or hydroxyurea (200 mg/kg) given intraperitoneally daily for 5 days caused reduction in relative testicular weight in mice (B6C3/F1/BOM M). Testicular atrophy of several tubules was seen in the hydroxyurea-treated mice 5 days after the last exposure, whereas paracetamol did not lead to such changes. Exposure to paracetamol caused neither a depletion of glutathione in the testis nor a marked increase in covalent binding. In contrast, significant decreases in the incorporation of thymidine into the testis were observed during the first 3 h following a single treatment with paracetamol (100-400 mg/kg) or hydroxyurea (100-200 mg/kg). In mice treated with paracetamol (400 mg/kg) or hydroxyurea (200 mg/kg) daily for 5 days, flow cytometric analysis revealed large reductions

in one of the tetraploid populations of testicular cells (mostly early pachytene spermatocytes) on days 5 and 10. Changes in the populations of the various spermatid stages occurred later; thus, both compounds appeared to cause a delay in spermiogenesis. Indications of abnormal chromatin structure were seen in an increased frequency of vas deferens sperm on days 27 and 33 after the last exposure, when measured as increased susceptibility towards DNA denaturation in situ. In conclusion, high doses of paracetamol or hydroxyurea inhibit DNA synthesis in the testis. These data indicate that this leads to reduced testicular weight, a reduction in the number of early pachytene spermatocytes, changes in the proportions of the various spermatid stages, and an apparent alteration in sperm chromatin structure (HSDB 2023).

Viberg et al. examined if neonatal paracetamol exposure could affect the development of the brain, manifested as adult behavior and cognitive deficits, as well as changes in the response to paracetamol. Ten-day-old mice were administered a single dose of paracetamol (30 mg/kg body weight) or repeated doses of paracetamol (30 + 30 mg/kg body weight, 4 h apart). Concentrations of paracetamol and brain-derived neurotrophic factor (BDNF) were measured in the neonatal brain, and behavioral testing was done when animals reached adulthood. This study shows that acute neonatal exposure to paracetamol (2 x 30 mg) results in altered locomotor activity on exposure to a novel home cage arena and a failure to acquire spatial learning in adulthood, without affecting thermal nociceptive responding or anxiety-related behavior. However, mice neonatally exposed to paracetamol (2 x 30 mg) fail to exhibit paracetamol-induced antinociceptive and anxiogenic-like behavior in adulthood. Behavioral alterations in adulthood may, in part, be due to paracetamol-induced changes in BDNF levels in key brain regions at a critical time during development. This indicates that exposure to and presence of paracetamol during a critical period of brain development can induce long-lasting effects on cognitive function and alter the adult response to paracetamol in mice (Viberg, Eriksson et al. 2014).

Mice were fed diets containing paracetamol at doses of 0, 0.25, 0.5 and 1% (equivalent to intakes of 0, 360, 720, and 1400 mg/kg body weight per day). The parental generation was exposed for 14 weeks during cohabitation, and the number of litters produced was used as a primary index of reproductive capacity. Feed consumption was reduced in the females at the highest concentration. No changes were noted in the number of pups per litter, pup viability, or adjusted pup weight, but 6/19 pairings at the high dose did not result in a fifth litter, and this accounted for the significantly decreased number of litters per pair in this group. The fifth litter

that was produced by 13 pairs at the high dose contained fewer pups. The last litter produced by the parental generation was maintained on exposure through adulthood. A reduction in body weight gain (range 6-18%) was seen in treated animals of each sex throughout mating. An increase in the percentage of abnormal sperm was observed in males of the F_1 generation at the high dose. In the F_2 generation, a reduction in pup weight adjusted for litter size (11%) was seen at the high dose (Reel, Lawton et al. 1992, WHO 1999).

Groups of 24-26 male B6C3F1/BOM M mice received intraperitoneal injections of 0 or 400 mg/kg body weight paracetamol daily for 5 consecutive days. Treated mice lost weight during the exposure, but there was no difference between control and treated animals throughout the remainder of the post-treatment period. The relative testis weights were reduced by 16-18% by paracetamol 27 and 33 days after treatment, but the only alteration in histological appearance up to 10 days was an approx. 6% reduction in the diameter of the seminiferous tubules 5 days after treatment. DNA synthesis (measured by [^{3}H]thymidine incorporation in the testis) was reduced for several hours immediately after the 5th dose, and this response was seen with concentration as low as 100 mg/kg body weight per day. Flow cytometric analysis of testicular ploidy 26 days after the last dose of 400 mg/kg body weight per day revealed alterations in the cell types indicative of altered transit through the cell cycle, but no effect on the chromatin structure of late-maturing spermatids (Wiger, Hongslo et al. 1995, WHO 1999).

2.4.4.6 Other Toxicity

***In vitro* and *in vivo*:** Paracetamol metabolism, cytotoxicity, and genotoxicity were measured in primary cultures of rat hepatocytes. Although 3 mM paracetamol caused a slight increase in cellular release of lactate dehydrogenase into the culture medium, cellular glutathione concentration (an index of paracetamol metabolism) was reduced by 50%. Paracetamol at 7 mM was significantly more toxic to these hepatocytes and had a similar but more marked effect on glutathione concentrations. In spite of its cytotoxicity, neither dose of paracetamol stimulated DNA repair synthesis when monitored by the rate of incorporation of [^{3}H] thymidine into DNA following exposure to paracetamol. Thus, although paracetamol has been shown to be both hepato- and nephrotoxic in several *in vivo* and *in vitro* systems, the reactive toxic metabolite of paracetamol is not genotoxic in rat primary hepatocyte cultures (Milam and Byard 1985).

Large doses of paracetamol cause centrilobular hepatic necrosis in man and in experimental animals. It has been shown that acetaminophen is metabolically activated by cytochrome P450 enzymes to N-acetyl-p-benzoquinone imine. This species is normally detoxified by glutathione (GSH) but following a toxic dose GSH is depleted, and the metabolite covalently binds to a number of different proteins. Covalent binding occurs only to the cells developing necrosis. It has been shown that these cells also contain nitrated tyrosine residues. Nitrotyrosine is mediated by peroxynitrite, a reactive nitrogen species formed by rapid reaction between nitric oxide and superoxide and is normally detoxified by GSH. Thus, acetaminophen toxicity occurs with increased oxygen/nitrogen stress (Hinson, Reid et al. 2004).

Rat: Gastric irritant effects of paracetamol and some phenolic and non-phenolic analgesics and antipyretics were studied in rats with adjuvant or collagen II-induced arthritis or zymosan-induced paw inflammation and given 1.0 ml hydrochloric acid 0.1 M and/or an intraperitoneal injection of the cholinomimetic, acetyl-β-methyl choline chloride 5.0 mg/kg. Gastric lesions were determined 2 h after oral administration of 100 or 250 mg/kg paracetamol or at therapeutically effective doses of the phenolic or non-phenolic analgesics/antipyretics. The results showed that gastric mucosal injury occurred with all these agents when given to animals that received all treatments so indicating there is an adverse synergy of these three factors, namely: (i) intrinsic disease; (ii) hyperacidity; and (iii) vagal stimulation for rapidly promoting gastric damage, both in the fundic as well as the antral mucosa, for producing gastric damage by paracetamol, as well as the other agents. Removing one of these three predisposing factors effectively blunts/abolishes expression of this paracetamol-induced gastrotoxicity in rats. These three factors, without paracetamol, did not cause significant acute gastropathy (Rainsford and Whitehouse 2006).

Several studies have demonstrated that long-term treatment of rats with paracetamol, even at therapeutic doses, can induce the alteration in the circulatory system. In a study, the effects of acute (1 h) and chronic (30 days) paracetamol treatments on cerebral microvessels in a cortical spreading depression migraine animal model were investigated Based on their results, they suggest that short-term treatment with paracetamol has no effect on cerebral microvessels and that chronic paracetamol treatment can alter cerebral microvasculature, especially when combined with cortical spreading depression activation (Yisarakun, Supornsilpchai et al. 2014).

Monkey: Hepatotoxicity of paracetamol is well known. However, there is a species diversity in the hepatotoxicity. This is attributes to the differences in the rate of formation of the reactive metabolite, NAPQI and the capacity-limited clearance of paracetamol via conjugation. have

shown that a cynomolgus monkey model of paracetamol hepatotoxicity is translatable to humans in terms of toxicokinetic and its toxic nature (Tamai, Iguchi et al. 2017).

2.4.4.7 Local Tolerance

Rat and rabbit: Studies on local tolerance of paracetamol in rats and rabbits showed good tolerability. Absence of delayed contact hypersensitivity has been tested in guinea pigs (Paracetamol B Braun - SmPC 2022).

2.4.4.8 Discussion and Conclusions

The toxicology of paracetamol was investigated *in vitro* and in mice, rats, guinea pigs, dogs and monkeys.

The oral LD_{50} values of paracetamol were 338 mg/kg in mice, 1944 mg/kg in rats, 2620 mg/kg in guinea pigs and 2 g/kg in dogs. The intraperitoneal LD_{50} values of paracetamol were 367 mg/kg in mice and 1205 mg/kg in rats. The subcutaneous LD_{50} value of paracetamol was 310 mg/kg in mice, and the intravenous LD_{50} value of paracetamol was 826 mg/kg in dogs.

In mice, paracetamol 140 mg/kg did not induce significant hepatic injury; however, this exposure resulted in a significant proinflammatory pulmonary transcriptional response and an influx of immune cells in the bronchoalveolar lavage fluid. Cats administered with paracetamol 350 mg/kg orally developed anemia, hemoglobinuria, and icterus.

Various sub-chronic and chronic toxicity studies were conducted. In a 14-day study, paracetamol reduced feed consumption in male and female rats; whereas in a 28-day study, it did not changed physiological, hematological, and biochemical parameters. In a 13-week study, patterns of food consumption and reduced body weights occurred in rats and mice receiving diets of 12500 and 25000 ppm of paracetamol. Also, paracetamol-related lesions were observed in the liver, kidney, reproductive organs, thymus, lymph nodes, and testis of rats and in the liver of male and female mice. In a 2-year study, the average severity of nephropathy was increased in exposed male and female rats. In males, this was associated with an increased incidence of parathyroid hyperplasia.

Paracetamol was not mutagenic in various *in vitro* and *in vivo* tests. Under the conditions of the 2-year feed studies, there was no evidence of carcinogenic activity of paracetamol in male rats.

In reproductive and developmental toxicity studies conducted in mice, the high dose males weighted 10% less compared to controls, while organ weights were not affected. Sperm abnormalities increased from 7% to 16% at the high dose. Fertility endpoints were generally not affected. Paracetamol reduced testicular weight, reduced the number of early pachytene spermatocytes, changed the proportions of the various spermatid and altered sperm chromatin structure.

In mice, exposure to and presence of paracetamol during a critical period of brain development can induce long-lasting effects on cognitive function and alter the adult response to paracetamol.

Also, mice fed with diets containing paracetamol presented an increase in the percentage of abnormal sperm of the F_1 generation at the high dose. In the F_2 generation, a reduction in pup weight adjusted to litter size was seen at the high dose.

Other toxicity studies indicated that paracetamol may have hepatic and nephrotic effects *in vitro* and *in vivo*, as well as gastric effects in rats, and hepatotoxic effects in monkeys.

Studies on local tolerance of paracetamol conducted in rats and rabbits showed good tolerability. Absence of delayed contact hypersensitivity has been tested in guinea pigs.

2.4.4.9 Toxic Potential of Impurities and Residual Solvents of the Pharmaceutical

The active substance paracetamol specifications are justified since are according to the monograph 0049 for paracetamol of the Eur. Ph. and as are per the CEP: R1-CEP 1195-050-Rev 04.

Paracetamol 10 mg/ml solution for infusion specifications are established according to the “Note for Guidance Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and Now Drug Products: Chemicals Substances. Q6A; CPMP/ICH/367/96” and Ph. Eur.

No pharmacological or toxicological effect is expected.

Further information on the impurities of the pharmaceutical product may be found in the quality documentation.

2.4.5 Integrated Overview and Conclusions

This non-clinical overview assesses the pharmacological, pharmacokinetic and toxicological documentation on Paracetamol 10 mg/mL solution for infusion, with paracetamol as the pharmacologically active ingredient. Overall, the pharmacodynamic, pharmacokinetic and toxicological properties of paracetamol are well described in non-clinical studies available in the literature.

Paracetamol belongs to the pharmacotherapeutic group: Nervous system; Analgesics; Other analgesics and antipyretics; Anilides. ATC code: N02BE01.

The precise mechanism of the analgesic and antipyretic properties of paracetamol has still to be established; it may involve central and peripheral actions.

Primary pharmacodynamic studies showed that paracetamol has analgesic and antipyretic properties in mice and rats. Additionally, paracetamol has anti-inflammatory effects *in vitro*.

Pharmacodynamic interactions of paracetamol and morphine, diclofenac, propacetamol, and N-acetylcysteine were observed in rats.

No formal safety pharmacology studies have been performed. However, the human safety of paracetamol is well known.

The pharmacokinetic properties of paracetamol were investigated *in vitro* and in mice, rats and hamsters.

In rats, the percentage of parent compound excreted in the urine ranged between 0.5-3.8% of the dose administered. Also, the biliary excretion rate of parent compound plus metabolites remained unchanged (range: 20.2-29.3%).

The plasma concentrations of paracetamol decreased exponentially with time in rats, but at higher doses, total clearance and the fraction excreted as sulfate conjugate decreased.

In rats, the first-pass metabolism of paracetamol following oral and intraduodenal administration was mainly attributable to the sulphoconjugation pathway.

Also, in rats, disappearance of paracetamol from plasma exhibited a clear dose-dependency as determined by prolongation of $t_{1/2}$ and decreases in total body clearance at 150 mg/kg or

higher. Biliary excretion of the various metabolites of paracetamol increased from 20-49% as the dosage was increased from 37.5 to 600 mg/kg-

Pharmacokientic drug interactions between paracetamol and N-acetylcysteine were observed in rats.

The toxicology of paracetamol was investigated *in vitro* and in mice, rats, guinea pigs, dogs and monkeys.

The oral LD_{50} values of paracetamol were 338 mg/kg in mice, 1944 mg/kg in rats, 2620 mg/kg in guinea pigs and 2 g/kg in dogs. The intraperitoneal LD_{50} values of paracetamol were 367 mg/kg in mice and 1205 mg/kg in rats. The subcutaneous LD_{50} value of paracetamol was 310 mg/kg in mice, and the intravenous LD_{50} value of paracetamol was 826 mg/kg in dogs.

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Various sub-chronic and chronic toxicity studies were conducted. In a 14-day study, paracetamol reduced feed consumption in male and female rats; whereas in a 28-day study, it did not changed physiological, hematological, and biochemical parameters. In a 13-week study, patterns of food consumption and reduced body weights occurred in rats and mice receiving diets of 12500 and 25000 ppm of paracetamol. Also, paracetamol-related lesions were observed in the liver, kidney, reproductive organs, thymus, lymph nodes, and testis of rats and in the liver of male and female mice. In a 2-year study, the average severity of nephropathy was increased in exposed male and female rats. In males, this was associated with an increased incidence of parathyroid hyperplasia.

Paracetamol was not mutagenic in various *in vitro* and *in vivo* tests.

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abnormalities increased from 7% to 16% at the high dose. Fertility endpoints were generally not affected.

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Other toxicity studies indicated that paracetamol may have hepatic and nephrotic effects in vitro and in vivo, as well as gastric effects in rats, and hepatotoxic effects in monkeys.

Studies on local tolerance of paracetamol conducted in rats and rabbits showed good tolerability. Absence of delayed contact hypersensitivity has been tested in guinea pigs.

In conclusion, the data presented in this Non-Clinical Overview concerning Paracetamol 10 mg/mL solution for infusion, confirms the safety, suitability, and efficacy of the product, when used under the prescribed recommended conditions.

2.4.6 Literature References

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