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The presence of drug in control samples during toxicokinetic investigations—A Novartis perspective

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Abstract

During a submission procedure, the validity of a few dietary toxicity studies was questioned because low levels of the drug were detected among control toxicokinetic samples. Although several lines of reasoning suggested that these findings arose from ex vivo contamination, the Regulatory Authority stated that it was not possible to establish a no-effect-level in any of the studies and so the submission was withdrawn. In response, Novartis conducted a thorough review and modification of the procedures involved in the collection and analysis of toxicokinetic samples to minimize such contamination in future studies. Ongoing monitoring of contamination in toxicology studies has subsequently revealed that although it was not possible to completely eliminate the problem, the new procedures together with an increasing awareness of the issue have considerably reduced the incidence of contamination. The process of contamination and its control was also modeled in a feeding study in mice. This provided good evidence that the detection of drug in control samples in the previous studies originated from external sources and not from in vivo exposure.

Keywords: Toxicokinetics; Rodents; Ex vivo contamination; Control sample; Sampling procedure

1. Introduction

In the ICH S3A guideline for assessing systemic exposure in toxicology studies issued in November 1994 (CPMP/ICH/384/95), the primary objective of toxicokinetics is to describe the systemic exposure achieved in animals and its relationship to dose level and the time course of the toxicity study. This exposure might be represented by plasma concentrations or AUCs of parent compound and/or metabolites or in some specific cases by tissue or fluid concentrations. It is specified that "it may not be necessary for toxicokinetic data to be collected in all studies and scientific judgment should dictate when such data may be useful." Note 8 specified that "it is often considered unnecessary to assay samples from control groups. Samples may be collected and then assayed if it is deemed that this may help in the interpretation of the toxicity findings or in the validation of the assay method."

During a submission procedure, the validity of a few dietary toxicity studies was questioned by the assessors because low levels of the drug were detected among control samples. Several lines of reasoning suggested that the levels detected in the control samples arose from ex vivo sources rather than from in vivo exposure of the animals. These conclusions were based on the outcome of Quality Assurance reviews by the Facility and Sponsor prior to submission and again after the files were withdrawn, analyses of the control diets which were negative for test item and the occurrence of a few examples where two animals were housed in the same cage and received the same diet but only one animal had measurable plasma concentrations of the test item. It is widely

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known from the literature that low level contamination of the experimental environment with powdered diet is unavoidable (Sansone and Fox, 1977; Sansone et al., 1977). It was also shown that cross-contamination with dietary dust would not provide sufficient intakes ingested via grooming or by inhalation to give detectable levels (Andrews and Folkerts, 2000). Studies may still be reliable for human safety evaluation even in the absence of valid controls. In such cases, pre-requisites for a reliable assessment are an adequate number of treatment groups with evidence of dose-related and high exposures relative to human exposures in combination with clear evidence for absence of adverse effects. Nevertheless, the Regulatory Authority stated in 2001 that it was not possible to establish a no-effect-level (NOEL) in any of the submitted toxicity studies.

2. How has Novartis dealt with the issue?

In June 2001, two working groups were formed, one in the United States and one in Switzerland, with the goal to find ways to reduce the occurrence of contaminated control specimens. The groups were led by the local Quality Assurance Unit Heads with team members selected from the test article formulation, animal, pathology, and bioanalytical laboratories. Two teams from each site rather than one global team were formed because of the different nature of studies conducted at the two sites, i.e., one site tends to conduct earlier studies of shorter duration and the other is involved primarily with chronic studies. Discussion items and the results of the two groups were continuously exchanged. The teams met at regular intervals and launched several initiatives. The working atmosphere was very open and constructive and avoided any assignment of blame. Management support and the necessary time to consider the issues were also provided.

Since the sources for cross-contamination were not known and as the topic is very complex, the team started by compiling preventive measures currently implemented in each department to avoid contamination including measures which seemed self-evident or trivial. Over the next few weeks, the measures were continuously adapted to reflect any new insights until a final list was agreed. Examples of implemented measures included:

General

- Cleaning procedures for equipment adapted and enforced
- Protective clothing changed when control materials or animals were handled after treated materials or animals
- Separate equipment for control animals (e.g., balances, pipettes, necropsy instruments, and anesthesia boxes)
- Color coding for dosing formulation containers/syringes, glassware, and animal room materials

Test article formulation

- Complete separation of control and test item formulations at all stages (separate room, equipment, and materials)
- Control and test item formulations samples taken for analysis on the same day as toxicokinetic blood collection

Toxicology (rodent/non-rodent laboratories)

- All study activities performed first for controls and then in ascending dose group order
- Control animals exercised separately from test item-treated animals
- Control specimens processed at a separate dedicated workstation from treated specimens
- Dedicated areas within freezers for control specimens

Pathology (for collection of tissue specimens)

- Unique control area identified for isolation of control animal necropsies
- Separate containers for storage and shipment of control specimens to the analytical laboratory

Bioanalytics and pharmacokinetics

- Control specimens processed separately
- Only one control specimen opened at any time during processing
- Separate analysis of control specimens: one blank was placed before and after control specimens in the analysis equipment
- Criteria for the relevance of a contamination were developed

The impact of the revised procedures was then assessed in a feeding study as described below.

2.1. Two-week in feed (powdered diet) methodological study in male mice

The primary objectives of this study were to assess proposed measures to avoid contamination of blood and tissue samples used for toxicokinetic investigations and to identify any potential new sources to minimize these in future studies.

2.2. Design

AFY861 (ethoxy-homolog of the drug substance in the original submission) was given continuously by

Table 1
Study design, animal allocation, and test items dosages

Room	A	B[a]		C[b]	
Group number	1	2	3	4	5
Dose (mg/kg/day)	0	0	600	0	600
Number of animals	40	20	40	20	40

[a] Control and treated animals were housed on separate batteries in the same room.

[b] Control and treated animals were placed on the same battery using the repeated horizontal sequence of one control cage then two treated cages.

dietary administration to two groups of 40 male Crl:CD-1(ICR)BR mice at a dosage of 600 mg/kg/day for 14 days. A further three control groups of 20 or 40 male mice similarly received untreated diet. The animals were housed in three different rooms. One control group (group 1) was housed alone in a separate room. The second control group (group 2) was housed in the same room as a treated group (group 3) but contact between the two groups of animals was minimized as far as possible. Blood and tissue sampling for groups 1, 2, and 3 was strictly in accordance with the newly proposed procedures to avoid contamination. The third control group (group 4) was housed in the same room and on the same cage battery as treated animals (group 5). Blood and tissue sampling for groups 4 and 5 was in accordance with the current standards (see Table 1).

During the study, clinical observations, body weight, and food consumption were regularly performed. On completion of the study, blood samples were collected from the retro-orbital sinus and directly from the vena cava. Samples of fur, liver, and jejunum (contents, mucosa, and residual tissue) were then collected at necropsy. In addition, swab samples for test article residue analyses were taken from the cleaned equipment used in the test article formulation laboratories, animal room, and necropsy area.

2.3. Analysis

2.3.1. Sample preparation of content, mucosa, and residual jejunal tissue

The jejunal tissues (content, mucosa, and residual tissue) were thawed, weighed, and supplemented by the 19-fold volume of pH 7.4 phosphate buffer (0.201 mol/L K_2HPO_4 and 0.049 mol/L KH_2PO_4) to obtain a homogenate corresponding to 50 mg tissue per milliliter homogenate.

2.3.2. Extraction procedure for plasma and tissue homogenate

After addition of internal standard to 50 μL plasma or homogenate, the compounds were extracted from matrix using 5 mL methyl *tert*-butyl ether at basic pH with 1 mL of ammonia solution 0.05 mol/L in a silanized glass tube. After shaking for 30 min and centrifugation for 10 min, the aqueous phase was frozen by dipping the tube in dry ice; the organic layer was transferred into a 5-mL silanized glass tube and evaporated to dryness under nitrogen at 30 °C. The residue was dissolved in 100 μL of a mixture of methanol/0.05 mol/L ammonium acetate (80:20, v/v) and 10 μL was injected onto the analytical column.

2.3.3. Extraction procedure for fur and swab samples

After thawing of the sample, a part of weighed fur or the entire swab and 5 mL of methanol were added in a silanized glass tube. After shaking for 20 min and centrifugation for 10 min, an aliquot of 1 mL was transferred into a 5 mL silanized glass tube with 50 μL internal standard solution and evaporated to dryness under nitrogen at 30 °C. The residue was dissolved in 100 μL of a mixture of methanol/0.05 mol/L ammonium acetate (80:20, v/v) and 10 μL was injected onto the analytical column.

2.3.4. Chromatography and mass spectrometry

The compounds were analyzed on a Zorbax Eclipse XDB-C_{18} 3.5 μm (50 × 2.1 mm) using a Zorbax Eclipse XDB-C_8 3.5 μm (10 × 2.1 mm) pre-column interfaced with a Applied Biosystems API 3000 triple quadruple mass spectrometer. The mobile phase consisting of 0.05 mol/L ammonium acetate (10:90 v/v) and methanol was delivered with an gradient of elution at a flow rate of 250 μL/min. Atmospheric pressure chemical ionization (APCI) was employed as the ionization source. The analyte and its internal standard were detected by use of multiple reaction monitoring (MRM) in the positive ionization mode to detect ion pairs at *m/z* 316/187 (AFY861) and *m/z* 306/174 (internal standard) (see Table 2).

3. Results

There was no contamination in the tissue or blood samples taken from the separately housed control group. Plasma samples obtained from control animals housed in the same room as treated groups were also essentially free of contamination. The tissue samples taken from groups 2 and 4 were all contaminated to minimal or moderate degree. It is likely that airborne food particles from the AFY861-treated feed contaminated the fur and cages of the control animals and the test article was then transferred to other tissues during the necropsy procedure.

AFY861 was detected in all samples from treated groups. A 3-fold difference in the amounts measured in plasma was noted between the two groups which may reflect the difference in the time of blood sampling. Group 3 was sampled approximately 2 h later in the day than group 5, and as rodents generally eat during the night, the additional time prior to blood sampling for group 3 may have allowed a higher absorption of the test item from the last feed (see Table 3).

Table 2
Assay performance

AFY861	Range of concentration	LLOQ (Mean bias %, N) (CV%)	QCS (Mean bias %, N) (CV%)		
Plasma (ng/mL)	2.00–200	2.00 (0%, 10) (8.0%)	4 (+4%, 9) (9.6%)	80 (+7%, 10) (5.6%)	160 (+5%, 10) (4.8%)
Jejunum					
Content (μg/g)	0.100–4.00	0.100 (0%, 10) (7%)	0.3 (−3%, 12) (11.3%)	1.6 (+1%, 12) (7.9%)	3.2 (0%, 12) (7.0%)
Mucosa (μg/g)	0.100–4.00	0.100 (+5%, 7) (4.8%)	0.3 (+5%, 8) (10.5%)	1.6 (+4%, 8) (8.7%)	3.2 (10%, 8) (10.0%)
Residual (μg/g)	0.100–4.00	0.100 (+1%, 8) (7.9%)	0.3 (+1%, 8) (3.0%)	1.6 (3.8%) (+6%, 8)	3.2 (+7%, 8) (6.5%)
Fur (μg/sample)	0.0100–1.00	0.0100 (+1%, 4) (11.9%)	0.02 (+10%, 4) (11.8%)	0.4 (+3%, 4) (1.9%)	0.8 (−0.7%, 4) (1.7%)
Swab (μg/sample)	0.0100–1.00	0.0100 (+1%, 4) (5.0%)	0.02 (+6%, 4) (4.7%)	0.4 (0%, 4) (7.0%)	0.8 (−9.4%, 4) (4.9%)

LLOQ, lower limit of quantification. QCS, quality control sample. CV (%), coefficient of variation (precision) = 100 × standard deviation/mean. Bias (%) = 100 × [(mean measured or back-calculated value − nominal value)/nominal value].

Table 3
Toxicokinetics from the two-week methodological study in mice

Dose (mg/kg/day)	0	0[1]	600[1]	0[2]	600[2]
Number of mice	40	20	40	20	40
Housing	Separate room	Control and treated group in the same room but on different batteries		Control and treated group in the same room and on the same battery	
Mean plasma concentration (ng/mL)	0	0	15 ± 6	0[3]	5 ± 4
Jejunal content					
Concentration range (μg/g)	0	0.105–0.227	2.65–662	0.105–4.57	<50–535
No. of positive samples	0	6	34	20	37
No. of samples < LLOQ	40	14	6	0	3
Jejunal mucosa					
Concentration range (μg/g)	0	0.100	<5–150	0.102–0.746	<5–68
No. of positive samples	0	2	37	13	31
No. of samples < LLOQ	40	18	3	7	9
Fur					
Concentration range (μg/g)	0	0	6–55	0.076–0.747	4–65
No. of positive samples	0	0	40	16	40
No. of samples < LLOQ	40	20	0	4	0

LLOQ, lower limit of quantification: 2 ng/mL in plasma, 0.100 μg/g or higher in tissue, 0.010 μg/g in fur 1, new procedures; 2, procedures in ⩽2000; 3, one of the 0 samples showed a detectable trace of AFY861, though below LLOQ.

Analyses of swab samples taken from cleaned equipment in the test article formulation laboratories, animal room, and necropsy laboratory were generally free of contamination. In the test article laboratory, however, the cleaning procedures for the balance and mortar and pestle were reviewed as relatively large amounts of AFY861 were detected (mean values of 0.874 μg on the balance and 0.525 μg on the mortar and pestle). Swab samples taken from the overalls, shoes, and gloves after working in the animal room contained significant amounts of AFY861 (2.31 μg). These results confirm that it is essential to wear protective clothing at all times and that these items should be discarded when leaving the room to prevent contamination of the surrounding laboratory facility. The air filters in the animal room contained the largest amounts of the test article (up to 2.96 μg) which is consistent with airborne material being a likely source of contamination. Small amounts of AFY861 (0.136 μg) were also found on the paperwork in the animal room (see Table 4).

Table 4
Summary of AFY861 concentrations in swab samples

Area/equipment (after cleaning if appropriate)	Mean concentration in two samples (μg/swab)
Test article formulation	
Spatula	0.0099
Balance—inside	0.874
Worktable	0.167
Mortar and pestle	0.525
Stainless steel mixing bowl	0.0377
Diet containers before issue	0.187
Walls of the laboratory	0.0057
Animal room	
Table (animal room)	0.113
Table (blood sampling room)	0.000
Balance	0.0475
Anaesthesia chamber	0.0065
Centrifuge inside	0.0069
Centrifuge outside	0.000
Blood pot holder	0.000
Blood pot (outside)	0.0177
Needle disposal container	0.000
Treated cage battery: top	0.0067
Treated cage battery: middle	0.000
Treated cage battery: bottom	0.000
Treated cage—inside	0.0143
Treated cage—outside	0.000
Treated cage food container	0.0408
Treated cage drinking bottle lid	0.000
Weighing cup	0.206
Balance	0.077
Chip reader	0.095
Computer keyboard	0.191
Paperwork in animal room[a]	0.136
Clothes after weighing animals[a]	0.0176
Clothes after mortality check[a]	0.0136
Overall[a]	0.486
Shoes[a]	0.213
Gloves[a]	2.31
Hands after blood sampling	0.0085
Face after weighing animals[a]	0.171
Technician's blood after Working in the room	0.000[b]
Air filter[a]	2.96
Necropsy	
Scissors	0.063
Tongs	0.0064
Scalpel	0.000
Anesthesia box	0.055
Necropsy table	0.000
Dissection board	0.000
Chip reader	0.000
Balance	0.000
Balance bowl	0.000

LLOQ: 0.0100 μg/swab.
[a] Not cleaned.
[b] LLOQ: 0.002 μg/mL plasma.

4. Implementation of procedures

Cross-contamination of control specimens is only made visible at the very last stage of analysis when it is too late to take corrective action. Total awareness and commitment is therefore required in all groups, starting with procedures in the test article formulation laboratory through the dosing and handling of animals until the analysis of specimens. The agreed measures were therefore visibly displayed at the work sites in each animal room or laboratory to make personnel aware of the requirements. Full details of the processes were documented in the raw data to achieve complete traceability in case of any contamination and the Quality Assurance Unit was asked to continuously inspect the agreed upon procedures and their documentation. Corresponding standard operating procedures were also updated accordingly. Contract Research Organizations were contacted for comparison with their procedures. If gaps in the procedures were found, the laboratory was requested to comply with the Novartis procedures and to document them in their raw data.

Although it was not possible to completely eliminate the problem, increasing awareness together with implementation of the preventative measures and improved communication on the issue considerably reduced the occurrence of contaminations in control specimens within Novartis. This was confirmed in a review of all studies over the previous few years which indicated that the frequency of contaminations, the number of contaminated specimens and also the levels of contamination had decreased after the introduction of the awareness program. We continue to list all ongoing studies with toxicokinetic specimens and include details of any positive results found in control specimens. This helps to evaluate the relevance of any potential cross-contamination and provides information on whether contaminations occur more often with specific test items.

5. Role of good laboratory practice

Can a study containing positive control specimens still be characterized as a good laboratory practice (GLP) study? According to the OECD guidelines (OECD, 1998), good laboratory practice is "a quality system concerned with the organizational process and the conditions under which studies are planned, performed, monitored, recorded, archived, and reported." GLP neither assures that the scientific design of a study is sound nor that standard operating procedures (SOPs) or analyses are scientifically adequate. It will not decide whether the occurrence of positive control specimens invalidates a study since this is a scientific judgment. In other words, a study can be in compliance with GLP even when positive control specimens are found. The Study Director should, however, identify and fully discuss any issues in the final report.

Can compliance with good laboratory practice prevent contamination of control specimens? GLP provides

a full set of basic principles, such as SOPs for all major standard activities, a detailed study plan for each study, training records for all involved individuals, direct and prompt records for all activities, archives that contain all study information, and a Quality Assurance Unit supervising the GLP compliance of each study. All these requirements serve in the reconstruction of the study at a later date and give accountability for each procedure. Despite such procedures it is not always possible to prevent positive control specimens. Consider the situation when a mix-up of two animals occurs in a rat toxicology study and a control animal unintentionally receives the low dose treatment. In all other respects, procedures were followed. Nevertheless, one control was exposed to the drug as shown after the bio-analyses. Based on the results, the Study Director could identify what happened and address the issue in the final report. This example of an incorrect dosing of a control animal is easy to understand but what about ex vivo contamination? There are many possibilities why this may happen during the handling of animals, blood collection, during processing, storage, shipment of specimens or analyses, and due to the specific substance properties. Usually it is not easy to identify the reasons but examination of the procedures laid down in the GLP documentation might identify weak points. In addition, the support of Quality Assurance personnel is helpful as they oversee a large variety of procedures and may find possible sources of the contamination. One can therefore state that GLPs do not necessarily prevent contamination of control specimens but they may reduce the potential for contamination and help to find reasons for any occurrences during study conduct.

6. General discussion and conclusions

Several lines of reasoning suggested that the small amount of drug detected in several control samples (plasma or tissue) in the studies arose from ex vivo sources rather than from in vivo exposure of the control animals due to mis-dosing or other errors in procedure. In particular, it has been calculated (Andrews and Folkerts, 2000) that one contaminating dust particle of 1–5 μg from the food admixture was sufficient to produce a drug concentration in a control plasma sample of approximately 10 ng/mL. Such a concentration is easily measurable as the limit of quantification for the test item was as low as 2 ng/mL to provide a working range that was adequate to cover the lowest expected concentrations in treated animals while not requiring frequent dilutions at the highest exposure.

The process of contamination and its control was modeled by treatment with AFY861 in mice. This also provided good evidence that the detection of drug in control samples in previous studies originated from external sources and not from an in vivo exposure. Although the new procedures to avoid contamination were effective in substantially reducing the amounts of AFY861 detected in control samples it was not possible to completely avoid contamination in a standard rodent feeding study. The separately housed control animals were free from contamination but this procedure was not implemented as standard in Novartis because the data would not be contemporary with those obtained for the treated groups and may also not be acceptable to regulatory authorities. Many laboratories actually randomly assign the cages from the different treatment groups to each cage battery as was done for groups 4 and 5 in the AFY861 mouse study to minimize the effects of environmental variations such as light intensity within the animal room. Although this is certainly justified scientifically, such a procedure is likely to increase the possibility of contaminated control samples. As a compromise, we would suggest that each group is held on a separate cage battery which is then placed in the same animal room in such a way as to minimize any inter-group differences in environmental conditions.

Contamination of the control samples does not affect the GLP status of the study but it is considered essential that the study director clearly identifies any such issue within the study report and addresses the impact on the study integrity. The main considerations should be the route and duration of administration, the number of animals affected, and the concentrations measured. A review of previous studies at Novartis clearly indicates that the feeding and dermal routes of administration are prone to contamination because it is difficult to restrict environmental exposure to the test item during or after the application procedure. In general, we would not consider a contamination to be significant if less than 10% of the control samples are affected or if the concentrations are below those measured at the lowest dosage. In cases where concentrations are similar or above those measured at the lowest dosage, comparison of the findings in control animals with historical control data or with the results of toxicological investigations with the same test item may identify if the animals were systemically exposed or if the values are due to external contamination. Metabolite analyses may also be helpful if sufficient quantities of the samples and a suitable methodology are available. For animal welfare reasons, every effort should be made to avoid a repetition of the study.

Some of the original studies were repeated as a result of the Regulatory Authority concerns. The new investigations produced the same findings as those obtained previously but without any contamination of tissue or plasma samples from control animals. This confirms the lack of any functional consequences of the

"contaminated" control samples in the earlier studies as would be expected if the source of contamination is ex vivo.

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