European Medicines Agency

London, 15 November 2007
Doc. Ref. EMEA/CHMP/PGxWP/201914/2006

COMMITTEE FOR HUMAN MEDICINAL PRODUCTS

(CHMP)

REFLECTION PAPER ON PHARMACOGENOMIC SAMPLES, TESTING AND DATA HANDLING

DRAFT AGREED BY THE PGWP WORKING PARTY	October 2006
ADOPTION BY CHMP FOR RELEASE FOR CONSULTATION	November 2006
END OF CONSULTATION (DEADLINE FOR COMMENTS)	February 2007
AGREED BY THE PGWP WORKING PARTY	October 2007
ADOPTION BY CHMP	November 2007

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Reflection Paper on PGx samples, testing and data handling

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1. INTRODUCTION

Pharmacogenomics (PGx)[1] offer a potential for better understanding the mechanisms of diseases and optimizing the development and use of medicinal products. PGx information may allow Regulators to optimize the benefit/risk balance evaluation and provide focussed information as guidance to prescribers and patients. In addition, the PGx evaluation may become a valuable tool in risk management and pharmacovigilance strategies.

It is therefore envisaged that in the future PGx testing in clinical trials and large-scale epidemiological studies will be increasingly considered in pre- and post- approval development and assessment of medicinal products.

The objective of this reflection paper is to highlight some key principles to be considered by both the pharmaceutical Industry and the assessors for PGx sampling in clinical trials intended for Regulatory submission. Those principles are underpinned by rapidly evolving science and technologies and therefore this paper will not enter in depth technological details.

2. SCOPE

The potential of PGx analysis is strongly dependant on the reliability of PGx information. Reliability of PGx information again will strongly depend on the overall quality of the test sample, validation of the assay and methods, the reproducibility of data and their association with the clinical phenotype of interest, e.g. therapeutic response.

This paper addressed reflections on some aspects surrounding pre-analytical, analytical and post-analytical steps surrounding PGx samples, testing and data handling, key for the scientific reliability of PGx data submitted for regulatory evaluation.

3. PRE-ANALYTICAL ASPECTS

3.1 Sampling

The availability of nucleic acids (DNA, RNA) of suitable quality is essential for PGx studies where the genetic background of individuals or expression profiles is investigated. Nucleic acids from different tissues or other biological origin are used to address different questions. Nucleic acid quality is assured by the combination of appropriate handling when taking the sample for PGx studies, sample storage, fixation and nucleic acid extraction procedures.

RNA

Because of ubiquitous present RNA degrading enzymes (RNAses) RNA is much less stable compared to DNA. On the other hand often minute amounts of RNA molecules have to be analysed. Therefore special attention is needed to assure RNAse-free collection equipment, efficient RNA extraction and RNA integrity. For expression profiling fast processing of biological materials (e.g. immediate flash frozen storage in liquid nitrogen, fixation, nucleic acid extraction, or other adequate pre-analytical preservation procedure such as preservation of blood in special blood RNA tubes, or preservation of tissue samples in respectively designed RNA preservation media) is recommended since expression patterns may change significantly shortly after bringing cells or organisms into a new environment and since a rapid degradation of the nuclei acid might occur.

[1] RE ICH- E15 EWG definition: The investigation of variations of DNA and RNA characteristics as related to drug response.

The potential effects of temperature on expression patterns may have to be evaluated and the handling process validated.

DNA

Genomic DNA is often collected from easily accessible tissues such as peripheral blood. DNA target structures used for genotyping are much more stable compared to RNA, however continuous degradation of DNA may occur under unsuitable collection (e.g. presence of DNAses) and storage (e.g. long-time room temperature) conditions.

3.2 Storage

Biological samples may contain varying amounts of nucleases with different activities under different conditions, with in general RNA being much less stable compared to DNA. Freeze-thaw cycles may be minimized by splitting the nucleic acid samples into multiple aliquots. Lyophilisation of biological samples has been shown to support long-term storage under moderate temperatures (4°C and -20°C) (Vaughan et al., 2006). Depending on the methodology used, different levels of integrity of nucleic acids may be acceptable. With the appropriate consent, PGx samples may be stored beyond the duration of the clinical trial, potentially for the duration of a clinical development program and beyond. Therefore suitable integrity of the nucleic acids under the chosen storage conditions should be checked and validated at least for the primary target regions and for potential control target regions. Furthermore proper controls shall be performed for the sequence identity of the amplified DNA and the identity of the analysed mRNA.

RNA

Many protocols foresee storage of RNA containing biological samples at -65°C to -80°C temperature at which no significant effects on stability of nucleic acids are expected over time. Storage at -20°C has been described to be associated with continuous degradation of RNA with low kinetics (Kasahara et al., 2006). Biological specimens for PGx analysis may be also stored as extracted nucleic acid samples. Extracted RNA samples are usually stored in RNAse-free water at -65 to -80°C.

DNA

DNA storage usually does not require as stringent conditions as RNA storage. DNA containing materials biological samples may be stored at -20°C or at -80°C. Extracted DNA may be stored at -20°C in water or in a suitable buffer, e.g. TE buffer (Tris/HCl, EDTA). Even at -20 C care must be taken to observe that the DNA samples are not evaporated since dry DNA is often very difficult to resolubilize.

3.3 Fixation

For fixation of tissues and other materials different protocols exist which may be followed after suitable validation of appropriateness for the purpose of the planned studies.

Potential interference of fixation with subsequent nucleic acid detection, identification or quantification procedures should be investigated. Potential interference may occur on different levels: by inhibition of subsequent reactions (e.g. reverse transcription of RNA, amplification of nucleic acid target regions, labelling of nucleic acids, specific hybridization to probes) by fixation reagents or by potential degradation or chemical modification of nucleic acids by the fixation process.

Influence of the fixation process on nucleic acid presence, stability and extractability from fixated tissues is an issue, which should be taken into consideration.

For qualitative analysis of nucleic acids simple fixation protocols, e.g. for DNA dried blood on filter paper or appropriate storage reagents for RNA may be sufficient.

Furthermore, reliability of results is strongly increased by their confirmation on a different platform for which equivalent validation of the fixation process should be performed.

3.4 Nucleic acid extraction and quantification

Several different DNA and RNA extraction reagents and procedures are available.

Compounds of the biological materials other than nucleic acids may be co-purified with the nucleic acids and residual amounts of extraction reagents may still be present in purified nucleic acids. Potential interference with subsequent steps of PGx analysis should be validated for the combinations sample matrix, chosen extraction protocol and PGx analysis method(s).
Validation of consistent extraction efficiency may become an issue when absolute quantitation of nucleic acids is an integral part of the PGx analysis.

RNA

Even after nucleic acid extraction residual amounts of RNAses may still be present sufficient to interfere with subsequent analysis. Overall RNA content, quality (purity) and integrity may be shown by a variety of methods, e.g. by OD260/280 absorbance ratio and by analysis of ribosomal 28S and 18S RNA on a gel.

DNA

It is essential that the extracted DNA is fully in solution since the accuracy and precision of estimates of DNA concentration are critical factors for efficient use of DNA samples in high-throughput genotype and sequence analyses. In the application of a specific DNA quantification method to a particular molecular genetic laboratory protocol must take into account the accuracy and precision of the specific method, as well as the requirements of the experimental workflow with respect to sample volumes and throughput.

4. ANALYTICAL ASPECTS

4.1 Test performance

Different platforms for PGx analysis are characterized by different techniques. Probes consist of oligonucleotides of different lengths manufactured by organic chemistry or of in vitro synthesized cDNAs probes.

The validity of the oligonucleotide probes to be used should be checked using appropriate software. It is of critical importance that the identities of the products of the PCR reactions from genomic DNA are verified. Impurities in the DNA preparations may lead to improper annealing or improper hybridization in specific samples.

For validation of the method used for SNP detection positive and negative control DNA samples should be used. This includes tubes with water and tubes containing e.g. genomic DNA from subjects' known to have different genotypes of the polymorphism being analysed: homozygous for each of the allele and heterozygous for both alleles. In case of rare alleles where appropriate DNA (mostly genomic) standards are unavailable, proper DNA sequencing analysis from both directions has to be done in order to validate the method for SNP detection. These control samples should be analysed at every experiment.
Un-validated SNPs (exploratory genomic biomarkers) need to be tested both in silico and in vitro in order to demonstrate their reliability (e.g. specie-specificity, population frequencies, absence of copy number variation in the segment where the SNP maps, presence/absence of linkage disequilibrium..).

Lack of reproducibility between different platforms is an issue, which may decrease reliability of PGx test results. Non-accurate test results may be generated by different means such as cross-hybridisation

of targets with some sequence homology or by background signal in the absence of any sequence homology or by secondary structures (folding) of target and/or probe sequences preventing specific binding.

Cross-validation of crucial results on either different platforms or formats is a key aspect for the confirmation of the data.

4.2 Quality assurance

Internal quality assurance
Assay standardization. Internationally accepted standard or reference materials for PGx are currently not available. At present, the use of suitable platform- specific reference materials for assay standardization and calibration is recommended. Independent verification of individually determined test results may include highly specific, sensitive and accurate test systems such as validated real time (RT) PCRs.

Assay Controls. Appropriate assay controls are important quality control measures. Controls may include spike-in controls where the hybridization and detection process is controlled by spiking of known amounts of characterized nucleic acids into the extracted nucleic acids to assess the accuracy of the test system. For gene expression studies absolute measurements of gene expression is associated with higher variation compared to relative measurements. Known comparator RNAs, e.g. from constitutively expressed genes may be chosen for normalisation.

External Quality assurance
External proficiency testing programs using well-characterized test samples have already been introduced for the molecular diagnosis of some genetic markers. These programs reveal the degree of inter- and intra-laboratory variation of respective test results and may illustrate the potential need for corrective measures.

Introduction of proficiency-testing program for expression profiling studies is currently under progress. Once such systems are introduced for PGx regular participation in suitable testing programs is considered as an important quality control measure. Systems for the characterisation of potential reference materials for RNA expression analysis systems are just being built up (Shi et al. (2006)). International collaboration addressed to establish analytical and clinical validity should be encouraged.

5. OTHER ASPECTS

5.1 Sample handling systems

Adequate physical storage and an effective labelling and inventory management system are essential. Labelling of samples so that they are efficiently tracked and retrieved can be done with validated electronic data management programs.

Bar coding or other labelling technologies of biological specimens allows automation of the banking system and error-proof operation. A unique barcode ID is given to each sample, generating a system of easily tracked specimens. The characteristics of each sample and related epidemiological and clinical information are linked to the barcode ID in the database system.

Innovative programs for the long-term storage of DNA that combines purification processes proven to produce archival quality DNA, chain of custody documentation through a proprietary Laboratory Information Management System (LIMS), sample security and retrieval efficiency have been developed and are available.

Facilities which meet appropriate quality standards include (non fully exhaustive list):

- GCP-compliant facility for confirmatory studies or established SOPs for exploratory ones
- Interactive, consultative development of customized archiving programs

- LIMS supported, documented chain of custody and sample management processes
- Secure storage systems
- Efficient and flexible retrieval and shipping
- Documented quality control

5.2 Storage duration and associated data handling

The full development of medicinal products takes years and the knowledge in the field of PGx continuously increases. Therefore, for the purposes of PGx and drug development, long term storing of the samples, and the use of appropriate identification codes, must be considered.[2]

An inherent value of the PGx samples is an opportunity for research on genomic factors involved in therapeutic drug response and adverse effects at any time across the development and life cycle management of a medicine. This is only possible via linking the clinical information to the results of the genetic analysis. On a case-by-case basis these longitudinal studies may be appropriate for the regulatory approval of the medicinal products and/or for the post-approval follow-up or monitoring studies.

A basic component in data collection and handling is informed consent that should cover all relevant issues important to the subject as per Good Clinical Practices Guidelines and relevant European legislation.

The consent has to be sufficient to cover the goals of the trial and/or to give opportunity for re-contacting the subject for new informed consent for additional sample and data collection and research. The consent process must strike a reasonable balance between sufficiently describing research purposes and not being overly restrictive so that data and samples become limited in use in light of new scientific knowledge and technology. In special circumstances, if the scope of the proposed research is beyond the original consent obtained, subject re-consent may be considered. Although the majority of the trials and sample collections still are using narrow focused informed consent models, current international trends are indicating an increased shift to broader scope of the consent. This is particularly important considering the large scale of trials and the fact that most current studies are of exploratory nature, the result of which might need confirmation in the future.

Trial subject has irrevocable right at any time to withdrawn his/ her consent for participating at the trial and the provision of samples, including for PGx testing. However, subject's personal decision autonomy to withdraw the informed consent can have practical value in the existence of the sample/data identification code(s) only.

Data obtained/generated prior to the consent withdrawal may continue to be used. The use, and generation, of data subsequent to consent withdrawal will be guided upon the informed consent obtained. The general data security measures have to correspond to the Directive 95/46/EC.

6. GLOSSARY

The following definitions refer to features relevant to PGx assays and variation of PGx data

Accuracy describes the degree of conformity of measured results in one test system with the true (actual) value.

Precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous material under the prescribed conditions. Precision may be considered at three different levels: repeatability, intermediate precision and reproducibility.

[2] ICH E15

Repeatability expresses the precision under the same operating conditions over a short interval of time (intra-assay precision).

Intermediate precision describes the within-laboratory variation of repeat test results with one test system (different days, different analysts, different equipment).

Reproducibility is the inter-laboratory precision, which may be determined as the variation of repeat test results for one test system in different laboratories (intra platform variation) or for different test systems (inter-platform precision).

Analytical specificity is the ability to unequivocally assess the target nucleic acid in the presence of other nucleic acids or other components, which may be expected to be present.

The **linear range** of a quantitative assay describes the concentration range of targets, which is consistent with accurate results.

The **analytical sensitivity** defines the detection limit, which is the lowest amount of nucleic acid, which can be specifically detected by a PGx assay.

Clinical sensitivity is the proportion of individuals with a specified clinical disorder or clinical effect whose test values indicate that the disorder or clinical effect is present (e.g. the mutation associated with the disorder is identified).

Clinical specificity is the proportion of individuals who do not have a specified clinical disorder or effect and whose test results indicate that the disorder or clinical effect is not present.

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