Towards an EBF recommendation on validation of Dried Blood Spots assays

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Presented at: EBF Open Symposium, Barcelona

2 December 2010

Acknowledgment

- The EBF DBS team
  - John Smeraglia (Pfizer)
  - Leonarda Brunet (Abbott)
  - Philip Timmerman (Janssen Research and Development)
  - Steve White (GlaxoSmithKline)
  - Silke Luedtke (Boehringer Ingelheim Pharma)
  - Sussane Globig (Actelion Ltd)
- The broader EBF community

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Introductory statements

- Dried Blood Spot analysis > micro sampling
- The recommendations only reflect on (potential) differences and special considerations needed for method validation and sample analysis for dried blood spots (DBS) compared to ‘liquid assays’.
- The recommendations are primarily given for “spot & punch” techniques. Some requirements for online DBS analysis may vary.
- All input was gathered from the EBF community (through surveys and iterations thereof, targeted follow up questions) and peer discussion, and summarized by the EBF-DBS sub team

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The prework

DBS Survey within EBF community in preparation of in depth discussions and June 2010 DBS meeting in Brussels:

2 simple questions:

1. Do DBS methods require special validation criteria?
   If yes, which validation criteria need to be included, deleted or adapted
2. Do studies analyzed with validated DBS methods need special acceptance criteria?
   If yes, which acceptation criteria need to be altered?

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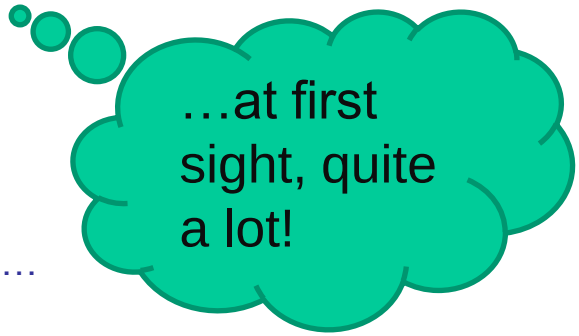
Initial outcome – 1/2

Method Validation: what needs to **added**

- ‘Spot specific’ validation parameters:
  - The effect of spotted volume on precision and accuracy
  - Spot-to-spot accuracy and precision
  - Distribution of the spot on filter paper
    - Impact of filter paper type, spot size and homogeneity, carry over, temperature during spotting and drying, hematocrit, plasma protein binding
- Added or enhanced stability considerations:
  - Stability of analyte during drying conditions
  - Effect of drying time
  - Additional focus on whole blood stability
- “Card Specific” validation parameters
  - Storage conditions temperatures, humidity, packaging, light…
- Additional topics
  - Process of “dilution integrity” to be modified e.g. by diluting the extract of the “dilution QC” disc with pooled extract obtained from blank blood spots
  - Recovery and MF



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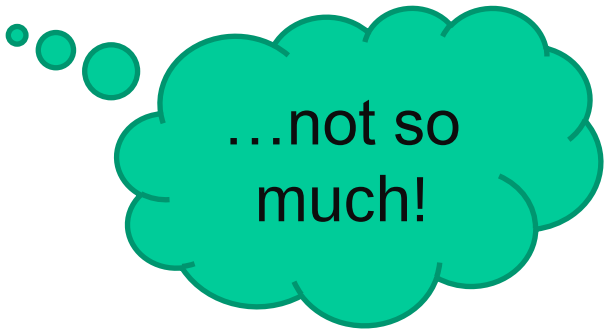
Initial outcome – 1/2

Method Validation: what can be **deleted**

- Basically all stability assessments (short and long term stability) related to storage of liquid samples
  - F/T
  - Long term, short term of plasma



Full slide deck: visit

http://www.europeanbioanalysisforum.eu

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Towards recommendations
Areas discussed

- Definition of a sample
  - Inter *vs.* intra card spotting
- Physical properties of blood
  - Anticoagulant
  - Hematocrit
  - Venous *vs.* capillary (finger prick) blood sampling
- Spiked vs incurred samples
- Spot size
- Sample homogeneity
  - prior to spotting
  - post spotting: Multiple punches from the same spot

- Preparation of Cal/QC samples
- Carry over
- Considerations for addition of internal standards
- Extraction Recovery
- Stability aspects
- Card storage pre & post spotting
- Sample dilutions
- Matrix Effects
- Impact of filter paper types

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Definition of a sample - 1/2

- Observations:
  - Typically, individual study samples are spiked in multiple replicates on one card. It can occur that aliquots of individual study samples are spotted onto more than one card.
  - Typically, individual calibration standards and QC samples (Cal/QC) may be spiked in singles or duplicates on one card.
  - Moreover, preparation of Cal/QC samples *during validation* may differ from the process used *during study sample analysis* with respect to replicates per card.
  - As a consequence, inter-card variability may impact accuracy and precision differences between study samples and Cal/QC samples.

- Challenge:
  - Do we need to consider intra-card versus inter-card variability as part of validation?

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Definition of a sample - 2/2

- Recommendation:
  - When using cards from the same type/manufacturer, inter-card variability does not need to be investigated as a discrete method validation parameter, because:
    - Using current commercially available cards (i.e. 4 spots cards), Cal/QC samples will be spread over multiple cards as per design, covering for inter-card variability.
    - In the event of failing validation, failed run investigation may focus on inter-card variability. For that reason, it should be considered to enable maximal visualization of inter-card variability as part of validation batch design.
  - As a consequence : **1 spot = 1 sample**
    - Any additional spot, originating from the same sample pool, and spotted on the same card or on a different card from the same type/manufacturer can be considered an identical replicate sample (to be used e.g. for re-analysis, metabolite quantification or ISR)

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Physical properties of blood

- Observations:
  - When comparing plasma/serum/urine and blood (with tissue like properties), blood has significantly different physical properties which may impact sample to sample variability and assay robustness.
  - Parameters to consider are:
    - The use of anticoagulants
    - Impact of spot size
    - Impact of hematocrit on spot formation
    - Venous *vs.* capillary (finger prick) blood sampling

In the next slides, above aspects are discussed as part of individual validation parameters or for background consideration

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Physical properties of blood: Anticoagulant

- Considerations of the anticoagulant:
  - Principles of anticoagulant may apply as for plasma
  - Because the process of collection may differ from plasma samples, with a potential impact on clotting, special sampling and spotting techniques may need to be developed and evaluated.
  - Being mindful of micro volumes sampled undue sample dilution caused by addition of anticoagulants needs to be managed during validation and throughout sampling and analysis
    - same considerations apply for additional additives like stabilizing agents,..
  - The added value of anticoagulants needs consideration.
  - Validation experiments and preparation of Cal/QC samples need to reflect above considerations.

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Physical properties of blood: Hematocrit

- Considerations of the hematocrit:
  - Hematocrit may impact spot formation and homogeneity
  - The impact of hematocrit values on the spot size and homogeneity should be understood and documented during validation.
    - Clinically relevant variations of hematocrit values should be evaluated during validation (i.e. from 35 – 55 %).
    - Special populations with physiological conditions affecting the hematocrit beyond normal values (patients with conditions/on medication impacting their hematocrit,… i.e. renal impairment) may require additional validation as they occur.

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Physical properties of blood: Others

- Venous *vs.* capillary (finger prick) blood sampling
  - The difference between venous *vs.* capillary blood is not an analytical property that needs investigation beyond the considerations mentioned under the paragraph “hematocrit”
  - It is acknowledged that analysis of venous *vs.* capillary blood samples may impact the PK parameters. Evaluation of these differences is considered to be the responsibility of the PK teams

- Other considerations
  - The impact of hemolysis should be understood, i.e. on the spot formation or on sample stability.
  - The impact of other blood parameters (i.e. lipemic samples, other?) needs to be understood and validated as appropriate.

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Spiked *vs.* Incurred samples 1/2

- Observation:
  - DBS samples typically originate from micro sampling procedures, providing additional challenges during sampling.

- Challenge:
  - Which considerations (*other than ISR*) apply during validation with respect to incurred *vs.* spiked samples?

Spiked versus Incurred samples 2/2

- Recommendation:
  - As for 'liquid samples' validation, the percentage and type of non-matrix solvents should be limited to prevent differences in analytical behavior from spiked to incurred samples.
  - Critical to quality parameters are:
    - Differences in distribution of the compound on the filter paper.
    - Differences in hemolysis prior to applying to the filter paper
    - Differences in drying time.
  - Incubation/equilibration dynamics of analyte of fortified (spiked) samples *vs*. incurred samples

- ISR
  - ISR is applicable as proposed in the relevant papers (AAPS, EBF)
    - Depending on validation data, ISR can be managed from 2nd spot or from 2nd punch out of same spot.

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Spot size: general

- Recommendation:
  - The validation of the spot size, *in millimeters punched*, from a DBS card (and originating from a preset sample volume), is an integral part of the validation of a DBS method (i.e. accuracy and precision, LLOQ-ULOQ, s/n,…).
  - Additional validation parameters related to the spot size are:
    - Drying time.
    - Drying temperature (and definition thereof i.e. ‘ambient’) and relative humidity (RH).
    - Room temperatures and RH value boundaries may need to be investigated as part of validation.

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Spot size: ‘GSP’

- Adherence to ‘Good Spotting Procedures’ (GSP)
  - Examples of non-GSP are:
    - Incorrect volume (if applicable)
    - Application of spot in fractions (*vs.* one fluent movement)
    - Incidental (partly) double spotting
    - Influence of the capillary touching the filter paper during spotting
    - Wrong manipulation of the filter paper prior to spotting or after drying (contamination)

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Spot size: boundary testing

- Challenge:
  - Is boundary testing needed as part of validation of the spot size?
- Recommendation:
  - If spotting on card is done using accurate pipettes (and documented in the study), no boundary testing is required as part of method validation.
  - If spotting on card is done using non-accurate tools (e.g. capillaries), boundary testing of spotted volume should be considered during validation. (e.g. +/- 50 % of target volume).
  - Successful boundary testing removes the need for accurate pipetting in the animal lab or clinic.
  - Acceptance criteria for boundary testing should be preset and aligned with acceptance criteria applied in regulated bioanalysis.
  - Target spot size for Cal/QC samples = Study samples.

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Sample homogeneity prior to spotting

- Challenge:
  - Micro sampling techniques applied in DBS can impact sample homogeneity.
  - How can homogeneity be safeguarded during sampling and spotting?
- Recommendation:
  - Special considerations need to be taken to safeguard sample homogeneity.
  - Some examples of parameters which can negatively impact sample homogeneity of spiked samples are, but not limited to:
    - Time between spiking the analyte in blood and application of spiked sample on card (e.g. stability aspects and equilibration after spiking)
    - Vigorous shaking of blood prior to spiking on card (e.g. hemolysis)
    - Application of multiple sample aliquots from 1 capillary versus from multiple capillaries

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Multiple punches from the same spot

- Observation:
  - Analyzing multiple punches from the same spot can be needed for a number of reasons:
    - Increase sensitivity
    - Enhance accuracy and precision
    - reanalysis
    - ISR
    - Metabolite
    - Biomarker analysis
- Recommendation:
  - If on-card sample homogeneity is assessed during validation, multiple punches from the same spot can be analyzed during study sample analysis.

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Preparation of Cal/QC samples

- Challenge:
  - Do other dynamics apply when preparing Cal/QC samples for DBS?
- Recommendation:
  - Preparation of Cal/QC samples for DBS needs to adhere to the same principles compared to 'liquid assays'
  - In addition:
    - blood used to prepare Cal/QC samples should be fresh (i.e. harvested on the day of use). If not routinely feasible, the effect of age (and storage) of the blood needs to be assessed (i.e. experiments or from literature) and documented.
    - The use of batch prepared Cal/QC samples is acceptable provided they are used within the documented storage stability period.
    - Although equally true for liquid assays, due to micro sampling, additional considerations are applicable when fortifying blood with analytes (i.e. the percentage of non-aqueous to prevent solvent effects creating differences between spiked *vs.* incurred samples (cfr. slide 14).

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Carry Over

- Recommendation:
  - Both for validation experiments and study sample analysis, and in addition to post preparative and instrument carry over considerations (i.e. autosampler, LC-columns, etc.), following additional carry over considerations need to be properly assessed:
    - Physical carry over from card to card .
    - Spot-to-spot carry over originating from the punching device.
  - All confirmed sources of carry over should be eliminated (i.e. by inclusion of blank cards and/or punches in the run).

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Considerations for Internal Standards

- Challenge:
  - It is important to acknowledge that currently used procedures of adding the internal standards (IS) for DBS analysis (typically in solution in the solvent in which the DBS filter punch is extracted) doesn't cover all necessary aspect of analytical compensation intended by an IS (e.g. recovery).
- Recommendation:
  - Additional scientific validation may be needed to investigate potential differences in behavior of the analyte (i.e. dissolving from the DBS filter punch) versus the IS (added in solution).
  - Other techniques of introducing the IS earlier in the analytical process should be considered and evaluated as appropriate.

Note :

IS are close structural analogues of the analyte being quantified (preferably stable isotope labeled analogues). They are added to Cal, QC and study samples in equal concentrations to compensate for fluctuations in the analyte response during sample preparation and analysis.

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Extraction Recovery

- Recommendation:
  - Being mindful of the limitations of the analytical compensation offered by the IS with respect to extraction recovery, recovery needs to be evaluated and documented more thoroughly for DBS assays.
  - The impact of card storage on extraction recovery from the punched disks needs to be considered and documented (i.e. as part of long term stability). At method development and validation, consideration should be given to anticipate potential changes in extraction behavior of the analytes from old *vs.* recent dried blood.

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Stability aspects 1/3

- From the FDA guidance:
  - *Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis.*
- Challenge:
  - How does this apply to DBS?

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Stability aspects 2/3

- Recommendation - general:
  - Evaluation of stability aspects mentioned in the current regulatory guidances, but which are scientifically or procedurally irrelevant for DBS should be discontinued (i.e. freeze/thaw)
  - Evaluation of pre & post spotting stability unique to DBS should be considered as part of the validation – *see next slide*
  - In addition to investigation of on-card stability, appropriate stability experiments should be conducted on liquid whole blood to be able to provide proper guidance to the collection and handling of blood samples prior to spiking. The blood used for these experiments should be freshly harvested.

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Stability aspects 3/3

- Recommendation - pre & post spotting:
  - Pre spotting
    - Storage of pre spotted cards under extreme environmental conditions (i.e. temperature/RH) can negatively affect their performance .
    - As a consequence, cards should be stored under dry (desiccant) conditions prior to use.
  - Post spotting
    - ‘Extreme‘ storage conditions (during drying, at the sampling site, during shipment and at the bioanalytical site) should be understood.
      - Examples are, but not limited to: high RH, low or high temperature, sudden temperature/RH changes impacting condensation, extreme and long exposure to daylight.
    - Boundary testing as part of the stability experiments during validation should be considered.
    - Appropriate measures should be taken to prevent extreme storage conditions, i.e. desiccants, protect from light /extreme temperatures.

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Sample Dilutions

- Challenge:
  - do we need to consider other dynamics when diluting samples compared to liquid assays?
- Recommendation:
  - Principles of dilutions used for liquid samples apply for DBS.
  - Scientific validation of dilutions should be documented during method validation.
  - Alternative to dilutions with blank DBS extract, other approaches can be evaluated and subsequently validated.
  - It should be understood that, when applying alternative approaches, the potential effect on other validation parameters (i.e. extract sample stability, matrix effect, selectivity, etc…) should be validated as part of the dilution testing.

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Matrix Effects

- Recommendation:
  - Similar to liquid assays, the guiding principles to document and/or prevent of matrix effects apply for DBS assays.
  - Although an integral part of the validation, awareness of the additional impact of the filter paper on matrix effects should be understood.

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Filter paper consideration

- Recommendation:
  - A method is validated per *filter paper type/manufacturer*.
  - Any changes in *filter paper type and/or manufacturer* calls for a partial validation. Parameters belonging to the partial validation initiated by the change *in filter paper type and/or manufacturer* need to be pre-defined.
  - Examples of parameters included in this partial validation are:
    - Accuracy and precision
    - Extraction recovery
    - Matrix effects
    - Drying conditions (i.e. drying time, temperature,)
    - Dilution – depending on dilution technique used
    - Stability of analytes on card

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Conclusion

- EBF intensively discussed the validation requirements needed to generate robust and reproducible concentration date from DBS experiments
- EBF carefully evaluated which recommendations are proposed to the scientific community
- EBF intents to move forward and publish these recommendations
- EBF intents to further support the use of DBS in preclinical and clinical development

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