ERNDIM - Quantitative Schemes
Quantitative Organic Acids

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Annual Report ERNDIM-EQAS 2014

1. Purpose

The purpose of the ERNDIM External Quality Assurance Scheme for Quantitative Organic Acids is the monitoring of the analytical performance of the quantitative analysis of organic acids in urine. For detailed information see www.erndim.org / www.ERNDIMQA.nl

2. Participants

110 Datasets were submitted by laboratories from 33 countries of which 7 did not allow calculation of the annual report due to too few results.11 Labs did not submit any results.

Although the number of participants in this scheme is steadily increasing (6% more participants each year since 2008), only 52% of labs that take part in the qualitative scheme also participate in the quantitative scheme. Labs that do participate to the quantitative scheme typically submit data for only 65% of analytes. Several critical analytes – pathognomonic for specific inborn errors of metabolism – are reported by less than 60% of participating labs, e.g.: 3-hydroxy-3-methylglutaric acid, 4-hydroxybutyric acid, D,L-Glyceric acid, hexanoylglycine, mevalonic acid, tiglylglycine and vanillactic acid. This indicates that half of the laboratories that provide diagnostic testing using organic acids in urine do not feel the need at all for quantitative analysis of organic acids, and that for many critical analytes, less than one third of diagnostic laboratories are able to provide quantitative data.

The need for quantification remains a matter of debate within the Scientific Advisory Board. While diagnosis of many inborn errors of metabolism can be reliably achieved through qualitative profile recognition of large biochemical anomalies, the Scientific Advisor ERNDIM EQA Scheme for Quantitative Organic Acids strongly recommends the uniform implementation of quantitative organic acid assays. These can be most informative in detecting subtle increases of significant organic acids such as ethylmalonic acid in SCAD-deficiency, 3-methylglutaric acid in the 3-methylglutaconic acidurias and 3-hydroxyisovaleric acid in biotinidase deficiency. Another important area of quantitative analysis is that of treatment monitoring.

3. Design

The Scheme has been designed, planned and coordinated by Prof. Geert Martens as scientific advisor and Dr. Cas Weykamp as scheme organiser (subcontractor on behalf of SKML), both appointed by and according to the procedures of the ERNDIM Trust Board. The design includes samples and reports which are connected to provide information with a balance between short-term and long term-reports and between detailed and aggregated information.

Samples

The scheme consisted of 8 lyophilised urine samples, all prepared from the same basic human urine but with various amounts of added analyte. The samples were identical two by two: the pairs, along with the added amounts of analyte and their source are in Table 1 below. The type and level of the analytes were discussed in the Scientific Advisory Board and agreed by the Trust Board. As before, the concentrations varied between the physiological range and the typical pathological range. The latter may be quite high, e.g. for methylmalonic acid, and pyroglutamic acid. Samples have been tested for stability an homogeneity according to ISO 13528.

As compared to the 2013 scheme, the 2014 scheme

- included the same compounds except that N-acetylaspartate was left out.
- Contained 2-fold lower concentrations of various analytes (e.g. 3-hydroxy-3-methylglutaric acid, hexanoylglycine, methylmalonic acid, 3-hydroxyisovaleric acid and mevalonic acid, indicated in bold in Table 1) to better approximate the clinical decision limits

Table 1: Pairs, added amounts (in micromol/L) of organic acids and their source

Analyte	Source	Added to Pair 182 -185	Added to Pair 184-188	Added to Pair 183 -186	Added to Pair 181-187
D-2-OH-glutarate	Aldrich H8378	87,0	0,0	396,2	161,0
3-Methylglutarate	Aldrich M47604	45,1	0,0	148,2	88,4
3-OH-3 methylglutarate	Aldrich H4392	72,0	36,0	0,0	239,8
3-OH-Isobutyrate	Sigma 36105	97,0	0,0	410,2	180,0
3-OH-Isovalerate	Brunet	69,9	35,0	0,0	167,7
4-OH-Butyrate	Sigma H3635	34,8	0,0	338,3	71,7
Adipate	Sigma A26357	0,0	472,0	143,0	73,2
D,L-Glycerate	BioConnect Lip0000373 / TCI G0232	280,0	140,1	0,0	931,1
Ethylmalonate	Aldrich 102687	32,2	17,0	0,0	152,3
Fumarate	Sigma F2752	169,0	50,4	24,4	0,0
Glutarate	Sigma G3407	0,0	307,5	92,0	46,7
Glycolate	Sigma G8284	501,9	236,4	160,2	0,0
Hexanoylglycine	VUmc, Ten Brink	0,0	36,2	21,7	11,0
2-Ketoglutarate	Sigma K2000	614,0	84,6	66,6	0,0
Malic acid	Sigma M9138	0,0	443,2	88,6	48,1
Methylmalonate	Aldrich M5,405.8	999,6	249,5	50,1	0,0
Mevalonate	Sigma M4667	0,0	502,6	100,1	50,7
Pyroglutamate	Aldrich 83160	113,1	0,0	1000,1	299,8
Sebacate	Aldrich 84809	50,0	25,2	0,0	150,2
Suberate	Aldrich S5200	451,0	135,0	67,6	0,0
Tiglylglycine	VUmc, Ten Brink	0,0	286,8	87,3	19,5
Vanillacetate	TCI H0538	25,1	0,0	83,4	49,4

Reports

All data-transfer, the submission of data as well as request and viewing of reports proceeded via the interactive website www.erndimqa.nl which can also be reached through the ERNDIM website (www.erndim.org). The results of your laboratory are confidential and only accessible to you (with your name and password). The anonymised mean results of all labs are accessible to all participants. Statistics of the respective reports are explained in the general information section of the website.

The website supplies short-term and long-term reports. Short-term reports are associated with the eight individual specimens, for which a deadline has previously been established . Two weeks after the respective deadlines participants can request their reports and thus can update the information on their analytical performance. Although technically not required, a delay time of 14 days has been arbitrarily chosen to enable the scientific advisor to inspect the results and add his comment to the report. In contrast to the rapidly available short-term reports the annual long-term report is based on the designed connection between samples – as described above - which enables to report a range of analytical parameters (accuracy, precision, linearity, recovery and inter-laboratory dispersion) once an annual cycle has been completed.

Another characteristic of the website is the variety of result presentations which allows laboratories to make an individual choice for detailed and/or aggregated reports. The most detailed report which can be requested from the website is the “Analyte in Detail” which shows results of a specific analyte in a specific sample (184 such Analyte-in-Detail-reports could be consulted in the 2014 cycle). A more condensed report is the “Cycle Review” which summarizes the performance of all analytes in a specific sample (8 such Cycle-Review-Reports were available in 2014). The highest degree of aggregation is the Annual Report which summarizes the performance of all analytes of all 8 samples. Depending on the information one wants to obtain one can choose to inspect only the annual report (e.g. laboratory managers) or study all 184 detailed reports (person in charge of the workplace, technicians).
Inevitably, every sign of inadequate performance arising from the Annual Report will be followed up by inspecting the relevant Analyte-in-detail reports.

4. Discussion of Results in the Annual Report 2014

Subsequently we present accuracy, recovery, precision, linearity, interlab CV and cross sectional relations. It may be helpful to print your results of the annual report from the Interactive Website before reading the following comments and keep in mind that we only discuss the results of all labs in general: it is up to you to inspect and interpret the results of your laboratory and - where needed – to investigate the cause of unsatisfactory results and to make plans for improving your performance..
Whenever serious problems are encountered, contact may be made with your National Representative or eventually with the Scientific Advisor.

4.1 Accuracy

A first approach to describe accuracy is to compare the mean outcome in the eight samples in your lab with the mean in all labs. This is shown in the column "Your Lab" and "All labs" under the heading "Accuracy". E.g. it can be seen that the mean of all labs for 2-OH-glutaric Acid is 152.

4.2 Recovery

A second approach to describe accuracy is the percentage recovery of added analyte. In this approach it is assumed that the recovery of the weighed quantities is the target value. The correlation between weighed quantities as added to the samples

(on the x-axis) and the measured quantities (on the y-axis) have been calculated. The slope of the correlation multiplied with 100% is the recovery of the added amounts. The column “Recovery” shows your recovery of the respective organic acids in comparison to the median recovery of all laboratories.

The median recovery was acceptable (80% < recovery < 120%)) for 18/22 analytes. Analytes showing low median recoveries in the 2014 scheme typically also did so in previous years, e.g. 4-OH-butyric acid (average median recovery for the years 2008-2014: 67%), 3-OH-isobutyric acid (68%), D,L-glyceric acid (76%) and 3-OH-3-methylglutaric acid (79%), with no trend towards improvement. The low recovery 4-OH-butyric acid is possibly due to lactone formation, either during the production of the samples or during the extraction / derivatisation. Also 2-OH-glutaric acid (median recovery for the years 2008-2014: 94%), and mevalonic acid (median recovery for the years 2008-2014: 84%) are prone to lactone formation which should always be kept in mind when interpreting the recovery data.

Conclusions from aggregated data are generalisations which should render the participants of the QC-programs (and even more the end-users of the data) cautious about utilizing data from other labs without asking about proof of reliability. We strongly recommend that you revise the calibrations of analytes that show a clearly lower recovery in your lab as compared to the median of all labs. One pragmatic option for improved harmonization across diagnostic labs, is to use the residual samples of the previous ERNDIM EQA Scheme for Quantitative Organic Acids as calibrators, taking either added amounts (Table 1) or the median value reported by all labs (Annual Report, www.erndimqa.nl) as indicator of trueness/accuracy. The difficulties we face are certainly a challenge for developing improved methods.

4.2.1 Precision (intra-lab CV)

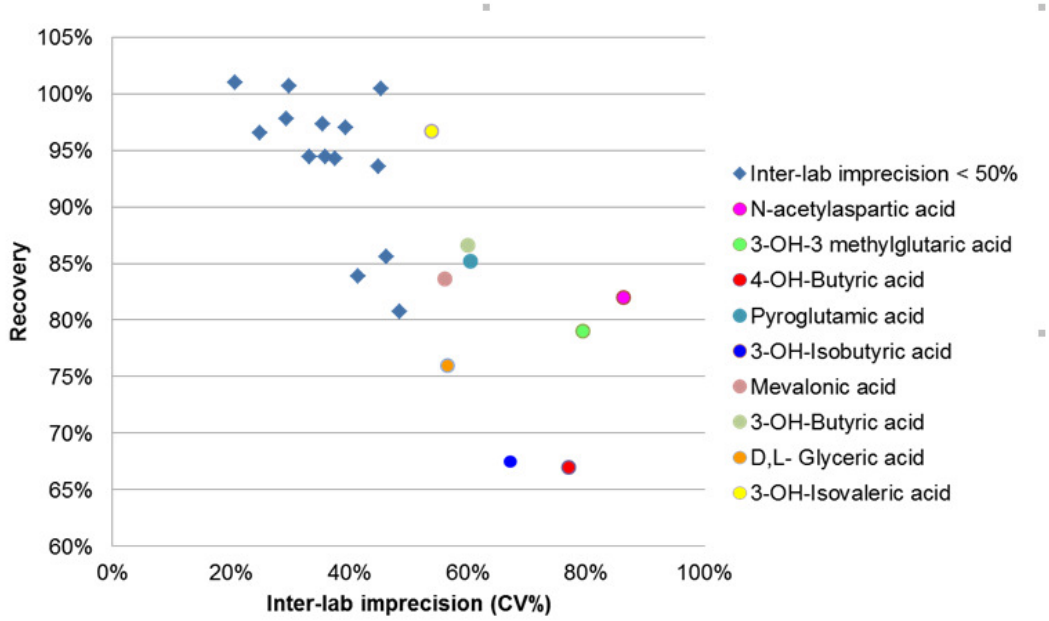
Reproducibility is an important parameter for quality in the laboratory. Your Intra-Laboratory coefficient of variation (CV) is calculated from the 4 pairs of identical samples in the scheme design which can be regarded as technical duplicates, and compared to the median CV on all duplicate results for a given analyte, submitted by the total group of participating laboratories. These calculated precisions thus provide a rough indication of the reproducibility of your laboratory as compared to the total group of participating laboratories, and are shown in column “Precision”.

Median precision was excellent for many compounds with intra-lab CV ≤ 15% e.g. for glutaric acid (9.9%). Higher imprecisions for several hydroxyacids may have been the consequence of non-optimal extraction efficacies. In line with the results of previous years, a number of problematic compounds show poor precision with intra-laboratory CV of > 25% e.g. 4-hydroxybutyric acid, 3-hydroxyisovaleric acid and hexanoylglycine. Rigorous standardization of the extraction parameters, i.e. pH of the sample, exact volumes of extraction solvents and carefully controlled timings of various steps (evaporation of solvents, oximations,…) may be a way to improve this aspect of performance.

4.2.2 Interlab CV

For comparison of outcome for one patient in different hospitals and for use of shared reference values it is relevant to have a high degree of harmonization between results of various laboratories. Part of the scheme design is to monitor this by calculating the Inter-laboratory CV. This, along with the number of laboratories which submitted results, is shown in the column “Data All labs” in the Annual report. It can be seen that most laboratories submitted results for methylmalonic acid (104) whereas only 38 participated for vanillactic acid.

The Inter-lab CV ranges from 21.8 % for glutaric acid to 62.4% for 3-OH-3-methylglutaric acid. As expected, the Inter-lab CV is typically 2 to 3-fold higher than the corresponding Intra-lab CV but for a number of organic acids this discrepancy is more than 3-fold. Key examples, with relevance for disease monitoring and/or diagnosis are 3-hydroxy-3-methylglutaric acid, 3-hydroxyisobutyric acid, D,L-glyceric acid, pyroglutamic acid and previously also N-acetylaspartic acid. For a number of compounds, this discrepancy was persistently observed also in previous schemes from 2008 on. Compounds with a high inter-laboratory CV (>50%) typically show lower median recoveries (< 80%), as indicated in the Figure below. This is another reason to emphasize the need for harmonization of methods between the different laboratories.



4.2.3 *Linearity*

Linearity over the whole relevant analytical range is another important parameter for analytical quality. The regression has been calculated taking the concentration of the addition as independent (x) variable and the measured concentrations as the dependent (=y). The regression coefficient r of the individual and the median of all labs are shown in the column “Linearity” of the annual report. It can be seen that the coefficients of regression range from 0.918 for mevalonic acid to 0.997 for methylmalonic acid. Overall reported linearity is excellent for all compounds, suggesting that the major source of inter-laboratory variations reside at the level of sample extraction/derivatisation rather than at the level of instrument calibration of mass spectrometers.

4.2.4 *Cross Sectional Relations*

The various parameters as described above often have an interrelation: often more than one parameter directs towards good or bad analytical control. This pattern is not clearly seen in the organic acids scheme.

4.3 *Your performance: red and green flags*

ERNDIM has implemented a system to judge performance of individual laboratories. Red flags in the annual report of an individual laboratory indicate poor performance for accuracy, precision, linearity, and/or recovery. Organic acids with satisfactory performance for at least three of the four parameters (thus no or only one red flag or no result) are marked in green.

Thus a green mark indicates satisfactory performance for analysis of that particular organic acid while a grey mark together with two or more red flags indicates that your laboratory has failed to attain satisfactory performance for this analyte.
Criteria for red flags can be found in the general information on the website (general information; interactive website, explanation annual report).

4.4 Poor Performance Policy

A wide dispersion in the overall performance of individual laboratories is evident. Table 2 shows the percentage of red flags observed. 28% of the laboratories have no red flag at all and thus have attained excellent overall performance. In contrast, at the other extreme there are also 2% of laboratories with more than 25% red flags. Following intensive discussion within the ERNDIM Trust Board and Scientific Advisory Board (SAB) and taking into account feedback from participants we have been able to agree on a harmonised scoring system for the various branches of the quantitative schemes as described in our Newsletter of Spring 2009. In parallel to this the SAB has agreed levels of adequate performance for all the schemes and these will be re-evaluated annually. The scoring systems have been carefully evaluated by members of the SAB and have been applied to assess performance in our schemes from 2007 onwards. The ERNDIM Board has decided that the Scientific Advisor will judge the performance of the individual laboratories based on these levels of satisfactory performance and issue a letter of failure with advice to achieve satisfactory performance to those laboratories which do not achieve satisfactory performance. The letter is intended to instigate dialogue between the EQA scheme organiser/advisor and the participating laboratory in order to solve any particular analytical problems, eventually resulting in an improved quality of performance of labs

Table 2. Percentage Red Flags

% Red Flags seen in Annual Report	Percentage Labs In this Category	Cumulative Percentage Of Labs
>25%	2%	2%
20 – 25%	6%	8%
15 – 20%	12%	20%
10 – 15%	3%	23%
5 – 10%	24%	47%
0 – 5%	25%	72%
0%	28%	100%

4.5 Certificates

As for other schemes the performance as it is indicated by the red/green flags in the individual laboratories annual report is summarised in the new style of annual participation certificate. The certificate lists the total number of organic acids in the scheme, the number for which results have been submitted and the number for which satisfactory performance has been achieved. It is important to bear in mind that the certificate has to be backed up by the individual annual report in the case of internal or external auditing.

5 Conclusions & Summary

The high overall interlab CV demonstrates clearly the major problem in the analysis of organic acids: lack of standardization. Precision with a mean CV of 18.3% is much better indicating that reproducibility within the labs is acceptable. Linearity is no major problem and recovery is also quite acceptable. In this respect it should be noted that extra samples can be purchased from the scheme organizer, which may be used as calibrators, given that the weighed additions and the median calculated values are known. These samples are prepared by mixing equal amounts of the four levels of

one of the previous years. Over the years it has become clear that these 'mixed' samples are ideally suited to serve as internal quality assurance samples.
We invite you to review your data carefully and especially study your recoveries. These may give an indication of deviant calibration.

6 Preview Scheme 2015

The analytes in the 2015 scheme will be the same as in 2014.

7 Questions, Comments and Suggestions

If you have any questions, comments or suggestions, please address to the scientific advisor of the scheme Dr. Geert Martens (Geert.Martens@uzbrussel.be) and/or the scheme organiser Dr. Cas Weykamp (c.w.weykamp@skbwinterswijk.nl).
Alternatively you may approach your local National Representative, a list of which is available from ERNDIM.