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The comparability of different neuron-specific enolase immunoassays and its impact on external quality assessment system

Štern P.[1,2], Bartoš V.[3], Uhrová J.[1], Springer D.[1], Vaníčková Z.[1], Tichý V.[4], Průša R.[5], Zima T.[1]

[1]*Institute of Clinical Biochemistry and Laboratory Diagnostics, General University Hospital and First Faculty of Medicine, Charles University, Prague*
[2]*Department of Clinical Biochemistry, Institute for Postgraduate Medical Education, Prague*
[3]*Department of Nuclear Medicine, University Hospital and Policlinic, Ostrava*
[4]*Department of Clinical Biochemistry and Haematology, Centromed, Prague*
[5]*Institute of Clinical Chemistry and Pathobiochemistry, Faculty Hospital Motol, Prague*

SUMMARY
Objective: Long-term external quality assessments suggest that the individual results of different immunoassays are often not comparable. Our goal was to assess the possible sources of these differences.
Methods: The paper is based on the results of analyses using seven different immunoassays: DELFIA (PerkinElmer), Elecsys 2010 (Roche), Kryptor (B.R.A.H.M.S.), the enzyme-linked immunosorbent assay DRG and three methods based on immunoradiometric assays (DiaSorin, Immunotech and Schering-CIS).
Results: The following parameters were evaluated: precision profile of individual methods, linearity on dilution, modified recovery and comparability of immunoassays.
The analytical results for certain low concentration specimens correlate well while others do not (up to five – fold difference), especially in the case of controls prepared synthetically. Therefore, the current non-standardized preparation of controls seems to be questionable. In the cut-off range, the difference in the results of native samples does not exceed its double value. The variation in values higher than 100 µg/l obtained with different assays is under 40 %. All the evaluated immunoassays are efficient and highly comparable (The correlation coefficients are up to 0.994).
Conclusion: Our results confirm the expected matrix interference occurring especially in the range of normal and cut-off NSE concentrations. Another source of discrepancies can be put down to different antibody affinity to αγ- and γγ-enolase isoenzymes.
Key words: neuron-specific enolase, NSE, precision, recovery, comparability, EQA.

SOUHRN
Štern P., Bartoš V., Uhrová J., Springer D., Vaníčková Z., Tichý V., Průša R., Zima T.: Porovnatelnost rozdílných imunoanalytických metod ke stanovení neuron-specifické enolázy a jejich vliv na systém externího hodnocení kvality
Cíl práce: Externí hodnocení kvality z dlouhodobého pohledu ukazují, že výsledky analýz stejného vzorku různými imunoanalytickými metodami často nejsou porovnatelné. Našim cílem bylo hledat příčiny těchto diferencí.
Materiál a metody: Práce vychází z výsledků získaných sedmi rozdílnými imunoanalytickými metodami: DELFIA (PerkinElmer), Elecsys 2010 (Roche), Kryptor (B.R.A.H.M.S.), enzymové imunoanalýzy na imunosorbentu DRG a tří imunoradiometrických metod (DiaSorin, Immunotech and Schering-CIS).
Výsledky: Hodnotili jsme následující parametry: profil přesnosti jednotlivých metod, linearitu po ředění, výtěžnost (modifikovanou) a porovnatelnost imunoanalytických metod. Výsledky analýz u některých vzorků s nízkými koncentracemi NSE korelovaly dobře, zatímco jiné nesouhlasily (diference byla až pětinásobná), zvláště v případě uměle připravovaných kontrolních materiálů. Proto se jeví současná příprava kontrolních materiálů, prováděná bez standardizace, jako sporná. V oblasti hraničních hodnot nepřesahují rozdíly v analytických výsledcích biologických vzorků dvojnásobek. Kolísání hodnot výsledků vyšších než 100 µg/l bylo mezi rozdílnými metodami do 40 %. Všechny hodnocené imunoanalytické metody jsou vhodné a velmi dobře porovnatelné (Korelační koeficienty do 0,994).
Závěr: Naše výsledky potvrzují očekávané interference matric, které se projevují zvláště v oblasti normálních a hraničních koncentrací NSE. Dalším zdrojem diskrepancí může být rozdílná afinita protilátek vůči izoenzymům αγ- a γγ-enolázy.
Klíčová slova: neuron-specifická enoláza, NSE, přesnost, výtěžnost, porovnatelnost, EQA.

Introduction

The glycolytic enzyme, neuron-specific enolase (NSE), 2-phospho-D-glycerate hydrolase EC 4.2.1.11, is an isoform of the enzyme enolase that is found in neurons and cells of endocrine origin, as well as in platelets and erythrocytes. The structure of NSE is sometimes described as a γγ homo-dimer [1, 2, 3, 4, kit insert HyTest – Turku, Finland], and other times as a mixture of dimers, γγ and αγ [5, 6, 7, 8, kit inserts: B.R.A.H.M.S., CIS (Schering-CIS BioInternational), Roche], or only the γ-subunit is mentioned [2]. Despite the fact that the complete sequence of amino-acids is claimed to include 433 residues, the data concerning the monomer and dimer sizes also differ. The indicated MW (molecular weight) is 39,000 [1], 45,000 [9], 47,000 [2, 3, 4, 10] and that of the dimer 77,000 [9, kit insert HyTest], 78,000 [11], 80,000 [kit insert Roche], 95,000 [kit inserts: B.R.A.H.M.S., CIS], 96,000 [3].

NSE is synthesized in the neural and lung tissue of the foetus and in neuro-endocrine tissue in healthy adults. Increased production is observed in non-malignant lung and liver disease (up to 20µg/l). The possibility of false positivity must be emphasized in the case of stored blood or haemolysis (the concentration of NSE is higher in erythrocytes). NSE is significantly increased in neuroblastoma, medulloblastoma, retinoblastoma, tumours of diffuse neuroendocrine system (SCLC – small cell lung carcinoma, carcinoid, pheochromocytoma, gastrinoma, medullar carcinoma of the thyroid gland), in adenocarcinoma of the kidney and prostate, seminoma, and melanoma. The highest levels have been described in well-differentiated ganglioneuroblastoma and in ganglioneuroma. The most frequent indication for NSE determination is the monitoring of patients with neuroblastoma and SCLC [14, 19]. NSE is not suitable for the basic diagnosis or screening of pulmonary carcinoma [14, 19]. Serum NSE levels are also increased in patients with head trauma, where NSE values can be used for the prognosis of brain damage. NSE is also elevated in patients with stroke. NSE values higher than 33 µg/l in patients following successful cardio-pulmonary resuscitation for cardiac arrest predict a poor prognosis.

In view of the interlaboratory comparability of analytical results, NSE determinations rank among those TM (tumour marker) assays, which repeatedly show a poor interlaboratory comparability when checked within the TM EQA (external quality assessment) survey performed by DGKL (Joint German Society for Clinical Chemistry and Laboratory Medicine), as well as SEKK (Czech System of External Quality Control). The aim of this study was to call attention to the possible causes of these discrepancies, as well as to find out whether NSE analysis is really so doubtful.

Specific antibodies are essential reagents in immunoassays. The differences between immunoassays can be attributed to different properties. We can assume that the reason of discrepancies between individual immunoassays can be elucidated by different antibody properties. Unfortunately, we have not found any studies systematically dealing with the antibodies to NSE. Four of the seven companies (B.R.A.H.M.S., CIS, DiaSorin and PerkinElmer) whose kits we compared for NSE assessment did not provide us with information on the antibodies used.

Polyclonal antibodies are no longer used to determine NSE levels as their diagnostic sensitivity is lower than that of monoclonal antibodies, however, the specificity of both polyclonal and monoclonal antibodies is comparable [12].

NSIQ031 [kit inserts: Hybritech, Beckman Coulter], 18E5 [5, kit insert Roche – murine biotinylated], E21 [13, kit insert DRG], 9601 [kit insert Medix Biochemica – Kauniainen, Finland] are used as murine monoclonal antibodies. NS1S011 [kit insert Hybritech – acridinium ester], 84B10 [5, kit insert Roche – murine with a ruthenium complex or horse-radish peroxidase], E17 [14: isotope ^{125}I, kit insert DRG – horse-radish peroxidase], 9602 [kit insert Medix Biochemica] are used as signal monoclonal antibodies.

Four antigenic determinants are known on the γ-polypeptide chain of NSE [kit insert HyTest], of which one epitope is close to the N-terminus [kit insert HyTest]. HyTest offers monoclonal antibodies of the IgG2a sub-classes (5E2, 5A4, 1C1) and of one IgG2b sub-class (5G10).

The α and γ peptides differ in a sequence of amino-acids 271-285, 298-316 and 416-433 [15]. The authors of a published study [8] mentioned the human monoclonal antibody E1-G3 against the γγ-enolase (inhibits γγ enolase by 70 % and αγ by 30 %), and the beef monoclonal antibody B1-D6 against γγ-enolase (inhibits γγ-enolase by 90 % and αγ by 40 %). Both MAbs (monoclonal antibodies) bind to the antigen at a molar ratio of 1:1. The human MAb E1-G3 reacts similarly with γγ- and αγ-enolase, while the response to the beef MAb B1-D6 by γγ-enolase is significantly greater than in αγ-enolase. The authors [8] stress the possibility of great interference when measuring serum samples.

It was found that MAb 22.212 binds to the NSE (MW 47,000) epitope (amino acids 402–423) in patients with Alzheimer disease [10]. The MAb H14 [16] reacts with any γ-subunit although cross-reactivity in the case of αγ is only 41.5% instead of 50% [17]. In some sources MAb specificity against the γγ-homodimer only is stated [18], in others both the γγ-homodimer and the αγ-heterodimer [16, 17, kit insert DiaSorin] or even the αα-homodimer [7] have been described.

Material and Methods

Analytical and Measuring Systems

We used the following analytical systems: Elecsys 2010 (Roche Diagnostics GmbH, Penzberg, Germany), Kryptor (B.R.A.H.M.S. AG, Hennigsdorf/Berlin, Germany), the automated pipetting and measuring system Stratec SR300 (Stratec Electronic GmbH, Birkenfeld-Gräfenhausen, Germany) for two IRMA (immunoradiometric assay) methods (DiaSorin, Stillwater, Minnesota, U.S.A. and Immunotech a Beckman Coulter Company, Prague, Czech Republic), the manual IRMA method (Schering-CIS, Gif/Yvette, France), DELFIA (dissociation enhanced lanthanide fluorescence immunoassay, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) and a manual ELISA (enzyme linked immunosorbent assay) method (DRG Instruments GmbH, Marburg, Germany). These methods are most frequently used in the Czech Republic and Germany.

Characteristics of the methods

All methods are non-competitive, heterogeneous sandwich immunoassays. Kryptor only uses a homogenous assay. A comparison of the basic characteristics of the methods is shown in Table 1. Cut-off values were provided by the manufacturers, and percentages (in parentheses) refer to the group of healthy volunteers.

Table 1. Characteristics of tested analytical systems

Method	MAbs description	Range (μg/l)	Reproducibility (%)		Cut-off (μg/l)
			within-run	between-run	
B.R.A.H.M.S.	not available	0.8–200	1.1–3.7	3.3–5.8	12.5 (93%)
CIS	not available	0.3–100	1.7–2.0	3.1–4.5	12.0 (97.5%)
DiaSorin	not available	0.5–200	7.5–9.8	6.2–11.4	12.5 (95%)
DRG	E21, E17	1–150	1.3–2.8	3.6–4.7	13.0 (95%)
Immunotech	Medix 9601, 9602	0.15–150	2.1–5.3	2.9–5.3	13.4 (97.5%)
PerkinElmer	not available	1–1000	3.0–3.4	2.4–5.6	12.5 (95%)
Roche	18E5, 84B10	0.05–370	0.6–1.6	1.6–3.8	16.3 (95%)

Samples

Native Samples

Sera after routine analysis, obtained from patients with a diagnostic indication for NSE examination were stored at -70 °C prior to the analysis.

The native samples group included 15 specimens with low concentrations of NSE (under 15 μg/l) and 30 specimens with concentrations of NSE between 15 and 200 μg/l. The amount of selected samples was sufficient for duplicate analysis on all evaluated instruments, and the evaluation of the precision of specific methods could be therefore done. All the evaluated methods were run on the same day.

The linear response on dilution was monitored using a specimen with a high concentration of NSE (~ 150 μg/l). It was progressively diluted by two dif ferent diluents. In the first case a native sample with a negligible NSE concentration was used, and in the second one a diluent supplied by the manufacturer with zero NSE concentration was applied. Two series of samples were prepared and analysed.

In order to evaluate the modified recovery, two mixtures were prepared from the analysed specimens. Both components were mixed at a ratio of 1:1. In the first case, another native sample of similar concentration was added to the original specimen. In the second case, control material with the similar NSE concentration as in the specimen was added. The recovery was calculated according to the following formula:

$\{[c(x_1 + x_2) - c(x_1)]/c(x_2)\}$ 100, where c is the concentration, x_1 the specimen and x_2 the material added.

Control Samples

Control materials provided by the manufacturers of the evaluated kits were used, except for DRG and PerkinElmer (which were not available). Four TM EQA materials used in DGKL and SEKK (supplied by DGKL Bonn, Germany); three control materials from BioRef TM (BIOREF, Möbris, Germany) and two Lyphochek Tumor Marker Controls (Bio-Rad Laboratories Diagnostics Group, Hercules, CA, USA) were further analyzed. The results of control sample analyses were compared in order to find the cause of discrepancies of different immunoassays. The long-term experience of hundreds of Czech and German labs show that the results of various immunoassays vary in control samples mostly.

Verification of Calibrations

All calibrations were carried out according to the manufacturers' recommendations immediately prior to the analyses of specimens or, for manual methods, in the same run as specimens. The adequacy of the calibration was verified using control materials supplied by the kit producers or by BioRad controls.

Statistics

Statistical calculations were performed using MedCalc statistical software, Version 4.31.010 by Frank Schoonjans (Mariakerk, Belgium) and Microcal Origin, Version 6.0 (Microcal Software, Northampton, MA, USA) or A Program for Statistical Analysis in Clinical Chemistry, Version 4.2.0 by Kristian Linnet (Risskov, Denmark).

Statistical parameters (mean, median, IQR, 95th percentile values) were used to specify the data acquired for the evaluated immunoassays. Correlation analysis, Passing-Bablok linear regression and box plots were used for mutual comparison of individual pairs of methods.

Results

Measurement Precision

For each pair of analytical results of 84 samples performed in duplicate (66 native samples and 18 control materials), the mean and the percent differences between duplicates were determined. Precision was assessed using precision profiles based on the percent differences plotted against the corresponding average concentrations of NSE. The differences of the measured values should not be influenced by the matrix of the analysed material, and therefore the analytical results of the patient sera and control samples were put into a single group. The Elecsys 2010 analyzer gave very good results with a variability of 3% even in the range of the lowest concentrations. These outcomes correspond to data reported in literature [6]. Similar precision was observed when Kryptor analyzer was used, except for the interval of normal values, where the variability was higher (but lower than 6%). The precision of other methods was at a level of 7 – 8%, with no differences observed between the

manual and automated IRMA methods. These procedures can be arranged according to decreasing precision as follows: DRG, Immunotech, CIS, DiaSorin and finally PerkinElmer.

Linearity on Dilution

The linear response on dilution was assessed, where the specimen with a high NSE concentration was diluted using either a serum with zero NSE concentration, or a diluent supplied by the manufacturer (Table 2). Some negligible effects of the choice of diluent were observed using the CIS and DiaSorin kits. DELFIA technique gave a less accurate linear response on dilution in our experiment, which does not matter in practice. The wide range of this method (up to 1000µg/l) allows routine measurements without any dilution.

Table 2. Response on dilution

Method	Diluent	
	Native sample with low NSE	NSE zero standard
B.R.A.H.M.S.	y = 7.6 + 0.79x	y = 9.7 + 0.89x
CIS	y = 10.2 + 0.81x	y = 20.0 + 0.67x
DiaSorin	y = 7.1 + 0.87x	y = 13.4 + 0.69x
DRG	y = 4.2 + 0.79x	y = 6.5 + 0.80x
Immunotech	y = 8.9 + 0.81x	y = 11.6 + 0.76x
PerkinElmer	y = 5.7 + 0.82x	y = 7.6 + 0.83x
Roche	y = 6.7 + 0.82x	y = 9.5 + 0.80x

Modified Recovery

As no standard with a defined amount of NSE is available, the evaluation of accuracy was carried out using modified recovery (Fig. 1). It was mainly used to study the role of different matrices. Modified recovery levels of 100% for both types of matrices were found in the Roche (Elecsys 2010), B.R.A.H.M.S. and Immunotech methods, while in the other procedures the difference from 100% for these values (due to the matrices) was 18–35%.

Comparability of the Immunoassays

Assessment of the comparability of the methods evaluated can be carried out in several ways.

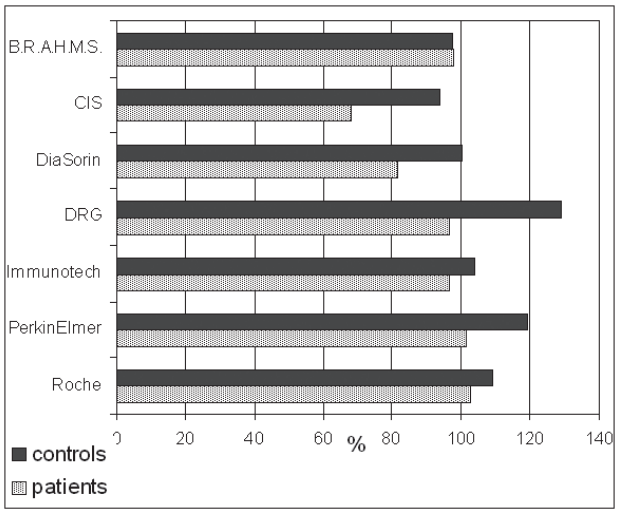

Fig. 1. Modified recovery (%) of native and control samples

Comparison of the results of independent controls (four EQA SEKK, three BioRef, and two BioRad) was performed. The ELISA method (DRG) and the electrochemiluminiscence system (Roche) gave very low results for NSE in the case of TM EQA samples by DGKL and SEKK, compared to the IRMA method or homogenous fluorescence procedure (B.R.A.H.M.S.). The data received from the DELFIA method (PerkinElmer) lie in between. The differences in the values obtained using individual systems were even more significant when BioRef control samples were used. The differences depend on the amount of NSE in the given sample. The third type of control materlals came from BioRad. At concentration level 2, results from Roche or the DRG method were again low, but the lowest results came from the DiaSorin procedure.

Table 3 shows the close correlation between the Roche and the other systems. The former was chosen as the reference system because of its best precision. Moreover, this system is the most widely used in the EQA DGKL and SEKK. The system of homogenous fluorescence B.R.A.H.M.S seems to compare best with the Roche electrochemiluminiscence technique.

All seven evaluated immunoassays were also compared mutually (Table 4). The closest correlation was found between the IRMA methods (CIS, Immunotech, DiaSorin) and also B.R.A.H.M.S. Table 4 shows the systems with the relatively closest correlations ($r > 0.9$).

Table 3. Parameters of Passing-Bablok regression compared to Roche

Method	Intercept		Slope		Cut-off (µg/l)	
	a	95 % CI	b	95 % CI	r	95 % CI
B.R.A.H.M.S.	3.56	2.98–4.07	0.71	0.67–0.75	0.975	0.966–0.981
CIS	4.64	4.04–5.27	0.61	0.57–0.66	0.957	0.943–0.968
DiaSorin	5.40	4.78–6.23	0.62	0.56–0.67	0.960	0.946–0.970
DRG	0.90	0.22–1.67	0.54	0.49–0.60	0.926	0.902–0.945
Immunotech	2.14	1.33–2.84	0.77	0.70–0.82	0.962	0.949–0.972
PerkinElmer	1.87	1.35–3.37	0.76	0.70–0.83	0.945	0.927–0.959

CI - confidence interval

Table 4. Selected correlations in immunoassays

Comparison (Y vs X)	Slope	Intercept	r
CIS vs. Immunotech	0.810	3.257	0.994
Immunotech vs. DiaSorin	1.214	-4.464	0.993
CIS vs. DiaSorin	0.979	-0.571	0.988
B.R.A.H.M.S. vs. DiaSorin	1.145	-2.812	0.985
B.R.A.H.M.S. vs. Immunotech	0.927	1.919	0.982
PerkinElmer vs. Immunotech	0.951	0.689	0.980
PerkinElmer vs. CIS	1.214	-3.181	0.979
B.R.A.H.M.S. vs. CIS	1.124	-1.409	0.978
B.R.A.H.M.S. vs. Roche	0.707	3.564	0.975
PerkinElmer vs. DiaSorin	1.186	-3.774	0.973
DRG vs. Immunotech	0.705	-0.36	0.972
DRG vs. CIS	0.863	-3.198	0.971
PerkinElmer vs. DRG	1.389	0.974	0.964
PerkinElmer vs. B.R.A.H.M.S.	1.053	-1.564	0.962
Immunotech vs. Roche	0.766	2.139	0.962
DiaSorin vs. Roche	0.624	5.403	0.960
DRG vs. DiaSorin	0.853	-3.773	0.957
CIS vs. Roche	0.610	4.641	0.957
B.R.A.H.M.S. vs. DRG	1.301	2.279	0.947
PerkinElmer vs. Roche	0.758	1.381	0.945
DRG vs. Roche	0.539	0.902	0.926

Discussion

The precision allows differentiating between automated and manual analysis. The reproducibility stated by manufacturers (see Table 1) corresponds to the results achieved in this study.

The accuracy from the point of linear response on dilution could be assessed as very good in most cases (see Table 2), regardless of the diluent used. The monitoring of method accuracy using modified recovery shows that some of the studied methods can be more sensitive to the influence of the material's matrix composition (see Fig. 1). Permanent result discrepancies, confirmed by the analysis of EQA TM DGKL and SEKK samples, were one of the reasons that led to the initiation of this study. A similar trend may be observed in the results of the BioRef controls, prepared in a different way. The NSE concentrations of BioRef 1 and BioRad 1 samples were inadequately low, and the interpretation of these results is therefore questionable. In the immunoassays with target NSE concentrations given by a manufacturer of controls, the results corresponded well with the declared values. Manufacturers admit that the results of assays using their controls vary widely depending on the methods used. For example BioRef 3 for Roche system Elecsys 2010 declares an NSE concentration of 8.2 µg/l, while for IRMA CIS the stated NSE concentration is 40 µg/l (i. e. differences of 388% for the same control). Compared to the cut-off value of the Roche system, this concentration corresponds to the normal value, while in CIS this result is significantly higher than the cut-off value. Even more bizarre is the fact that the cut-off values stated by CIS are actually lower than those stated for the Roche method (see Table 1, Fig. 1). In the BioRad 2 sample, the differences between the measured values and the stated NSE concentrations were smaller compared to BioRef 3. For example, the manufacturer of BioRad control states the NSE concentration of 18.9 µg/l for the Roche kit and the concentration of 27.7 µg/l (+47%) for CIS (proved by our experiment).

It is evident from these examples that the results from the individual immunochemical kits are considerably dependent on the matrix of the samples analyzed. This is without any doubts due to the fact that the degree of affinity of antibodies to the αγ- and γγ isoenzymes of enolase used in the specific methods varies [8]. According to the data provided by the manufacturers, this effect cannot be quantified. However, it is highly unlikely that the occurrence of these isoenzymes in the analyzed controls is constant.

As the information provided by the manufacturers does not include the antibody specificity (i. e. no description of the epitopes recognized), we can assume that the differences are caused by the fact the some antibodies bind to the γγ-homodimer only, while others also bind to the αγ-heterodimer. The differences of MAbs at low and high NSE concentrations may be due to the changes in the ratio of αγ-and γγ-dimers when the concentration alters [13], as well as to a different affinity of the individual antibody paratopes to the αγ- and γγ-dimers.

Conclusion

In conclusion, all the evaluated immunoassays are efficient and the diagnostic plausibility of the individual analytical kits is comparable. The analytical results for certain low-concentration specimens agree well while others do not (up to fivefold difference), especially in the case of control samples prepared synthetically. The results of DRG are almost always the lowest of all. This raises the question about the appropriateness of their use. In the cut-off range, the difference in immunoassays does not exceed its double values for native samples. At values over 100 µg/l, the difference between the values of specific methods is lover than 40%. The DRG kits do not always provide the lowest results at high concentrations. These data confirm the expected interference of the matrix that is especially apparent for NSE concentrations within the normal range and cut-off levels. A different affinity of kits to the αγ- and γγ-enolase isoenzymes might be another source of discrepancy. In general, there are no doubts about these discrepancies [8], but since most manufacturers did not provide more detailed information about their MAbs, they cannot be quantified. Both of these interferences

have a significant effect on the analysis of TM controls in EQA DGKL and SEKK and they might also cause the observed differences in the results of various immunoassays. The analytical quality of these assays could be improved if control materials of comparable interference with various immunochemical kits were produced, and if the manufacturers of immunochemical kits were more open to the unification of procedures. The kit dependent outcomes will be evident in the EQA systems until the preparation of control materials is standardized.

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Adresa pro korespondenci:
Doc. RNDr. Petr Štern, CSc.
ÚKBLD VFN a 1. LF UK
Karlovo nám. 32
121 11 Praha 2
e-mail: petr.stern@vfn.cz