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Evaluation of a Colorimetric Test for the Determination of α -Amylase with *p*-Nitrophenylheptaoside as Substrate

By A. Scholer

Kantonsspital, Basle, Switzerland and

W. Hohenwallner

Allg. öffentl. Krankenhaus der Barmherzigen Schwestern, Linz, Austria

Participants:

Dr. D. H. den Blaauwen

Dr. J. H. Jansen-Ziekenhuis, Emmeloord, The Netherlands

Prof. Dr. A. Burlina, Dr. P. Rizzotti

Istituti Ospitalieri B. Trento, Verona, Italy

G. Clayes

Kath. Universiteit, Leuven, Belgium

Prof. Dr. J. P. Colombo, Dr. E. Peheim, P. Degiampietro

Inselspital, Berne, Switzerland

Dr. H. J. Gibitz

Landeskrankenanstalten, Salzburg, Austria

Dr. C. van der Heiden

Wilhelmina Kinderziekenhuis, Utrecht, The Netherlands

Priv.-Doz. Dr. E. Henkel

Krankenhaus Oststadt, Hannover, Germany

Dr. W. Hohenwallner

Allg. öffentl. Krankenhaus der Barmherzigen Schwestern, Linz, Austria

Prof. Dr. O. Lalégerie, A. Vassault

Hôpital Necker Enfants Malades, Paris, France

Prof. Dr. K. Lorentz

Institut für Klinische Chemie, Med. Hochschule, Lübeck, Germany

Dr. E. Magid

Bispebjerg Hospital, Copenhagen, Denmark

Dr. K. Mayr

Allg. Krankenhaus, Wels, Austria

Prof. Dr. P. Métais, Prof. G. Ferard

Centre de Traumatologie et d'Orthopédie de Strasbourg, Illkirch-Graffenstaden, France

Dr. A. Noma, Dr. H. Okabe

Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan

Dr. H. Pilgerstorfer, E. Rupprechtsberger

Med. Diagn. Laboratorium, Linz, Austria

Prof. Dr. W. Rick, Dr. E. Schnaith

Zentralinstitut für Klinische Chemie und Laboratoriumsdiagnostik, Düsseldorf, Germany

Dr. S. Rosalki

Royal Free Hospital, London, United Kingdom

A. Scholer

Kantonsspital, Basle, Switzerland

Prof. Dr. N. Tietz, Dr. F. Shuey

University of Kentucky, Lexington, USA

Dr. J. Waldenström

Sahlgrenska Sjukhuset, Göteborg, Sweden

Prof. Dr. L. Weiss, Dr. G. Hoffmann

Krankenhaus München-Harlaching, Munich, Germany

G. Staber, G. Möller

Boehringer Mannheim GmbH, Tutzing, Germany

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Summary: A continuous method for the determination of α -amylase activity with a defined substrate has been published by Rauscher et al. in *Berichte der Österreichischen Gesellschaft für Klinische Chemie* 4, 150 (1981). It offers the following advantages: good practicability, easy adaptation to analysers and short incubation time.

Twenty-one laboratories took part in a multicentric study on this method, working with different instruments and manual procedures at three temperatures. Additionally, all laboratories participated in a laboratory improvement program, in order to discriminate systematic from accidental errors. Data on imprecision, linearity, interferences and comparability of the method have been presented at a workshop.

The resulting coefficients of variation for imprecision in series as well as from day to day were 0.6–6.3% and 1.3–7.7%, respectively. No interferences from bilirubin, glucose, maltose and 37 selected drugs were found. The results from the described method correlate well with those from an UV-test (substrate: maltoheptaose); compared with other methods, however, the expected systematic deviations were observed. Reference values for 25, 30 and 37 °C are indicated in this report.

*Evaluierung eines kontinuierlichen Farbtests zur Bestimmung von α -Amylase mit *p*-Nitrophenyl-maltoheptaosid als Substrat.*

Zusammenfassung: Rauscher et al. beschrieben in Berichte der Österreichischen Gesellschaft für Klinische Chemie 4, 150 (1981) eine kontinuierliche Methode zur Bestimmung der α -Amylase-Aktivität mit einem definierten Substrat. Diese Methode bietet als Vorteile: gute Praktikabilität, Automatisierbarkeit und kurze Inkubationszeit.

Einundzwanzig Laboratorien nahmen an einer multizentrischen Studie über diese Methode teil, wobei verschiedene Automaten und manuelle Arbeitsverfahren bei drei Temperaturen verwendet wurden. Um systematische von zufälligen Fehlern zu diskriminieren, nahmen die Laboratorien zusätzlich an einem Ringversuch teil. Ergebnisse über Ungenauigkeit, Linearität, Störungen und Vergleichbarkeit der Methode wurden auf einem Workshop vorgestellt.

Die erhaltenen Variationskoeffizienten für die Ungenauigkeit in der Serie sowie von Tag zu Tag lagen zwischen 0,6–6,3% bzw. 1,7–7,7%. Störungen durch Bilirubin, Glucose, Maltose und 37 ausgewählte Pharmaka wurden nicht festgestellt. Die Resultate der beschriebenen Methode korrelieren gut mit einem UV-Test (Substrat: Maltoheptaose); im Vergleich mit anderen Methoden traten hingegen die erwarteten Abweichungen auf. Referenzwerte für 25, 30 und 37 °C werden angegeben.

Introduction

According to the German Society for Clinical Chemistry (1), about 200 methods for measuring α -amylase, based on nine different principles, have been developed during the last 100 years. The main requirements, postulated also by other national committees, are the following:

- continuous measurement
- defined substrate of constant quality
- sufficient specificity for endo- α -amylase
- defined reaction mechanism

From practical considerations, the method should satisfy further conditions, such as

- short incubation time
- adaptability to all types of analysers
- no interference by endogenic glucose
- formation of a chromogenic reaction product (2).

For the new method, some of these points have been published separately (3, 4).

The following report presents the results obtained during the evaluation of the continuous colour test, “ α -Amylase PNP”. Twenty-one laboratories in Europe, USA and Japan took part in this study. Thorough examinations of the methodology were made at 25, 30 and 37 °C, according to manual work sheets

as well as on the following analysers: ACP 5040, ABA-100®, Abbott VP, CentrifChem® 400, Cobas® Bio, ENI Gamsac, Hitachi 705, LKB 2086, IL multistat® III. Reference values in serum and urine were established for the three temperatures.

Materials and Methods

Boehringer Mannheim's test kit for automatic analysers, “ α -Amylase¹⁾ PNP”, contains phosphate buffer solution pH 7.1 and lyophilisates of α -D-glucosidase²⁾ (30 U/l) and *p*-nitrophenyl- α -D-maltoheptaoside (5 mmol/l), all in separate bottles.

To improve stability, particularly for samples with extreme pH-values, the buffer concentration was meanwhile increased from 50 mmol/l to 100 mmol/l. Comparison of tests at 25 and 37 °C showed tolerable deviations up to 4%. For the comparison of methods, the following additional reagent kits were used: amyloclastic and chromogenic tests, one reductometric and two UV-methods (substrates: maltoheptaose and maltotetraose) and another kinetic colour test.

The measurements were made on the following analysers according to the instructions of the reagent manufacturers:

- Abbott 100® and Abbott VP (Abbott Scientific Productions Division, S. Pasadena Ca. 91030, USA)
- CentrifChem® System 400 (Union Carbide Inc., Tarrytown, N.Y. 10591, USA)
- I Gamsac Centrifugal Analyzer (Electro Nucleonics, Fairfield, N.J. 07006, USA)

¹⁾ EC 3.2.1.1

²⁾ EC 3.2.1.20

- Cobas® Bio (Hoffmann-La Roche & Cie AG, CH-4002 Basle, Switzerland)
- IL multistat® III (Instrumentation Laboratory Inc., Lexington, Mass. 02173, USA)
- Hitachi 705 (Boehringer Mannheim GmbH, D-6800 Mannheim 31, Germany)
- Clinicon AB 2086 Mark II (Boehringer Mannheim GmbH, D-6800 Mannheim 31, Germany)
- Greiner GSA (Greiner Electronics AG, CH-4900 Langenthal, Switzerland)
- ACP 5040 (Eppendorf Gerätebau, Netheler und Hinz, D-2000 Hamburg, Germany)

- Validate Gödecke AG, D-1000 Berlin, Germany
- Hyland Div. Travenol Laboratories S.A., B-7860 Lessines, Belgium
- Seronorm Nyegaard & Co., AS-Olso, Norway
- Versatol General Diagnostics, Div. of Warner-Lambert Company, Morris Plains, N.J. 07950, USA

Results

Imprecision

The imprecision of the method in series as well as from day to day was measured in human sera of low, medium and high amylase catalytic activity on the different analysers and for three temperatures. The samples were selected by the particular laboratories. Examples are shown in table 1.

The study was realized with fresh and frozen human sera, urine samples, in one case with pancreas fistula fluid, and the following control sera:

Monitrol and Enzatrol Merz & Dade, Dept. American Hospital Supply GmbH Deutschland, D-8000 Munich 50, Germany

Kontrollagen L Behring-Werke AG, D-3550 Marburg, Germany

Tab. 1. Precision of the colorimetric test α -Amylase PNP at 25, 30 and 37 °C.

Instruction sheets for	Precision within run n = 30				Precision between run n = 10 duplicates			
	Catalytic concentration $\bar{x}$ (U/l)	Temperature			Catalytic concentration $\bar{x}$ (U/l)	Temperature		
		25 °C CV (%)	30 °C CV (%)	37 °C CV (%)		25 °C CV (%)	30 °C CV (%)	37 °C CV (%)
ACP 5040 start with substrate	41	2.8	—	—	48	5.4	—	—
	101	4.0	—	—	106	4.5	—	—
	426	0.6	—	—	607	2.3	—	—
ABA® 100	84	—	4.8	—	108	—	7.7	—
	152	—	2.3	—	165	—	6.6	—
	684	—	2.2	—	745	—	4.6	—
Abbott VP	101	—	—	1.7	101	—	—	2.2
	227	—	—	0.6	230	—	—	2.6
	1150	—	—	1.3	1153	—	—	2.0
Centrifichem® 400	52	2.8	—	—	53	2.1	—	—
	126	1.8	—	—	121	1.3	—	—
	450	4.2	—	—	428	1.8	—	—
Cobas® Bio	60	—	1.5	—	65	—	2.5	—
	121	—	1.0	—	121	—	1.8	—
	656	—	0.8	—	655	—	0.9	—
ENI Gemsac®	96	—	—	2.2	108	—	—	5.9
	196	—	—	2.0	192	—	—	3.6
	667	—	—	1.7	651	—	—	2.4
Manual procedure start with sample	65	3.6	—	—	67	3.6	—	—
	139	3.6	—	—	145	3.0	—	—
	491	2.5	—	—	500	2.6	—	—
Hitachi 705	102	—	0.7	—	103	—	3.9	—
	227	—	0.5	—	227	—	1.8	—
	585	—	0.5	—	568	—	2.2	—
LKB 2086	426	—	—	3.5	416	—	—	3.0
	922	—	—	2.1	897	—	—	4.3
Manual procedure start with substrate	147	—	3.9	—	155	—	4.0	—
	651	—	1.3	—	647	—	5.0	—
Multistat® III	52	—	3.5	—	56	—	7.7	—
	127	—	3.9	—	127	—	3.0	—
	485	—	1.4	—	494	—	1.8	—

Behaviour of reaction and linearity

Figure 1 shows the reaction curves of four different sera: one control serum, one icteric, one lipaemic and one serum with increased amylase catalytic activity.

The linearity of the continuous measurement was examined with stepwise dilution of human sera and urine samples with 154 mmol/l (9 g/l) NaCl solution (fig. 2).

For all adaptations on analysers, the linearity up to the indicated dilution limit or higher is given as a function of the sample to reagent ratio (fig. 2).

Fig. 1. Absorbance/time diagram of α -amylase PNP from different samples at 25 °C.

Fig. 2. Dilution series for serum (—) and urine (---) samples (triple determinations) at 25, 30 and 37 °C.

Stability of sample material and reagents

Amylase-containing sera can be stored frozen or at refrigerator temperatures without any problem, whereas the urine samples show a decrease in catalytic activity. The stability of the reagents was tested by storing them for three weeks at +35 °C. Solutions made from these reagents were again stored for five days at +25 °C and for two weeks at +2 to +8 °C. The amylase activities found in two control samples of pathological and normal range were within tolerable limits.

Interference

As one important component of this evaluation, the reliability of the "α-Amylase PNP" test was checked by extensive interference studies.

The following possible interfering substances were examined:

Bilirubin, haemoglobin, glucose, anticoagulants, maltose, 37 drugs, lipaemic sera, turbidity, hydroxyethyl starch. A serum of high bilirubin concentration (225 μ mol/l) and low amylase activity was mixed in several steps with a serum of high amylase activity and low bilirubin concentration. A linear function was found for the amylase activity (fig. 3). The dashed line shows the corresponding bilirubin content.

The influence of *haemoglobin* was tested by diluting a haemolytic with a non-haemolytic serum sample of the same blood donor. Figure 4 shows that samples containing up to 0.15 mmol/l haemoglobin may be used for analysis without problems.

Fig. 3. Relationship between aliquot size (μ l) of a high α -amylase serum added to high bilirubin serum and the resulting α -amylase catalytic activity concentration (closed circles) and bilirubin concentration (closed triangles).

Fig. 4. Influence of haemoglobin (M , 68000) on α -amylase catalytic activity concentration. Results given as fraction $\pm$ 2 SD of the non-haemolytic sample.

A normal serum (approx. 70 U/l) was mixed with increasing quantities of glucose up to 56 mmol/l. No interference was recognized. A maltose concentration of 6.2 mmol/l also had no influence on the test system. On the other hand, decreased values were recorded in citrate, fluoride, EDTA, and oxalate plasmas, since these anticoagulants complex calcium, which is an active component of the enzyme's active centre. For this reason, only serum, urine and heparin plasmas can be used. None of the other drugs examined *in vitro* (5) interfered at therapeutic concentrations. For extremely turbid sera, a predilution is recommended. Interference by hydroxyethyl starch was shown *in vitro* as well as *in vivo*. The *in vitro* influence is most probably based on a removal of the enzyme via absorption by hydroxyethyl starch. *In vivo*, an increase of α -amylase in serum appears after infusions with this plasma substitute, while on the other hand, the excretion of the enzyme in urine is correspondingly reduced.

Accuracy

Laboratory improvement program

The comparability of the results from the particular instruments with those obtained by the original manual working instruction was tested by measurement of two control sera of unknown amylase catalytic activity on five days at 25, 30 and 37 °C.

The results of this comparison program are plotted in histograms (e.g. 30 °C see fig. 5).

Fig. 5. Determination of α -amylase catalytic activity concentration in an unknown control sample by 8 laboratories on 5 days, double determinations, 30 °C.

$N = 80$; $\bar{x} = 584.9$ U/l; median = 585.5 U/l; SD = 55 U/l; CV = 9.5%.

The good results of the study are documented by a fairly symmetric distribution. The deviation of the mean value from the assigned value is $\pm 5\%$. Information about the distribution of the values in the different laboratories is given in the data-structure analysis (6).

This diagram analyses the distribution of the values of the particular laboratories. The position of every laboratory's median is marked by a point. Figure 5 shows e.g. that the classes 654–680 U/l and 680–706 U/l contain only those values from multistat® III. The median is in the class 680–706 U/l.

The systematic differences showed by figure 5 pointed to the need for the correction of the worksheets of multistat® III and Hitachi 705. The Hitachi 705 should be calibrated with a calibration serum, because calibration with *p*-nitrophenol results in values that are too low. After the adaptation of the manual instructions to the multistat® III the recovery in dependence on the sample volume was found to be different. At 25 and 30 °C the most favourable ratio between sample and total volume was 1 + 21 for serum and urine. Smaller sample volumes showed a tendency to higher values.

Quality control

A further item of the laboratory improvement program was the examination of the aptitude of several commercially available control sera for the continuous colour test at different temperatures and according to different instructions. Figure 6 shows an example, using Boehringer Mannheim control sera at 37 °C (Precinorm® U and Precipath® E).

The recovery of the assigned values for Precinorm® U (407 U/l) and Precipath® E (798 U/l) was between 90 and 115%. For both samples the lowest values resulted from the Cobas® Bio and the highest ones from ACP 5040.

Fig. 6. α -Amylase in Precinorm® U (a) and Precipath® E (b) on different instruments at 37 °C: fraction of assigned value. Method: α -Amylase PNP.

Comparison of the methods

The comparability of the results, using the new method on different instruments, can be judged by the values of the multicentric study. The comparability with other methods, however, was investigated by the comparison of the following methods: procedures measuring α -amylase catalytic activity by the decrease of the iodine-starch colour, chromogenic, one reductometric, two UV methods (substrates: maltoheptaose and maltotetraose) and another colorimetric test (substrate: a mixture of maltopentaose and maltoheptaose). Pathological and normal range human sera as well as heparin plasma and urine were used as samples. After the determination on analysers, the results may be evaluated by the means of a computing factor (based on the ϵ -value of *p*-nitrophenol especially determined for this system) or of a

control serum based on human serum. When deviations occur, due to systematic influences, calibration with a control serum is recommended.

The following examples show the correlation of the new " α -Amylase PNP" colour test on different instruments and vs. the compared methods (fig. 7–10).

Fig. 7. Reductometric method, 25 °C (x) vs. α -Amylase PNP on ACP 5040, 25 °C (y). $n = 48$ serum samples. Regression line: $y = -16.3 + 0.53x$. Correlation coefficient: $r = 0.995$.

Fig. 8. Chromogenic colorimetric test (manual), 37 °C (x) vs. α -Amylase PNP on Cobas® Bio, 30 °C (y). $n = 70$ serum samples. Regression line: $y = -8.17 + 0.49x$. Correlation coefficient: $r = 0.994$.

The correlations between the four methods are excellent. According to the respective test conditions (selection of the substrate), all computing factors showed larger or smaller, theoretically expectable systematic differences.

Fig. 9. Continuous chromogenic colorimetric test, 30 °C (x) vs. α -Amylase PNP on Hitachi 705, 30 °C (y). Substrate: a mixture of maltopentaose and maltohexaose. $n = 80$ heparin plasma samples.
Regression line: $y = -5.07 + 2.44x$
Correlation coefficient: $r = 0.989$

Fig. 10. UV-test, substrate: maltoheptaose, 30 °C (x) vs. α -Amylase PNP on Hitachi 705, 30 °C (y). $n = 80$ heparin plasma samples.
Regression line: $y = -3.52 + 0.99x$
Correlation coefficient: $r = 0.997$

For completion, the results at 37 °C are also presented (fig. 11–12). The Abbott VP results were obtained with human sera and urine samples vs. an UV-test (substrate: maltotetraose) and an amyloclastic method on an LKB 2086. The Abbott VP was calibrated with a Precinorm® U control serum. A good correlation was found for the whole measuring range, as well as for the LKB examinations.

Fig. 11. UV-test, substrate: maltotetraose, 37 °C (x) vs. α -Amylase PNP on Abbott VP, 37 °C (y). $n = 65$ urine (●) and serum (○) samples.
Regression line: $y = -1.294 + 1.64x$
Correlation coefficient: $r = 0.983$

Fig. 12. Amyloclastic method on Greiner GSA II, 37 °C (x) vs. α -Amylase PNP on LKB 2086, 37 °C (y). $n = 59$ serum samples.
Regression line: $y = -10.2 + 0.95x$
Correlation coefficient: $r = 0.976$

Tab. 2. Reference intervals of α -amylase in serum and urine with PNP-maltoheptaoside at 25, 30 and 37 °C.

Sample-material	Collective	n	25 °C	n	30 °C	n	37 °C
Serum	♀ + ♂ Selected blood-donors ²⁾	194	34–121 U/l	197	39–158 U/l	83	69–210 U/l
Heparin plasma	♀ + ♂ Selected blood donors ²⁾	—	—	—	—	138	63–228 U/l
Urine portion	♀ + ♂ Selected patients of an ambulatorium and staff of the Basle Kantonsspital ³⁾	91	32–557 U/l	138	57–703 U/l	60	92–1239 U/l
24 h urine ¹⁾	♀ + ♂ Staff of the Basle Kantonsspital	57	34–335 U/24 h	57	57–505 U/24 h	52	68–1021 U/24 h

¹⁾ To be applied only with reservation (see "Stability of sample material and reagents", p. 680)

²⁾ Normal values requested for: aspartate aminotransferase, alanine aminotransferase, total bilirubin, γ -glutamyltransferase, alkaline phosphatase, total protein, albumin, glucose, lipase and α -amylase from a routine method.

³⁾ Normal values requested for all other urine parameters and α -amylase from a routine method

Reference values

In addition to the evaluation of the " α -Amylase PNP" colorimetric test, reference values for serum, heparin plasma and urine had to be determined for different temperatures. Some participants have already presented provisional values since the end of the test period. The final results have been published by W. Hohenwallner & A. Scholer (7) and others (8). The following limits are recommended (tab. 2).

Melzi d'Eril et al. also used the above-mentioned method for the determination of reference values for serum, spontaneous urine and 12 hours-urine at 37 °C (9). For serum (97.5 percentile: 225 U/l) and spontaneous urine (97.5 percentile: 1222 U/l), the results demonstrate an excellent agreement with the defined reference values.

Discussion

Unimportant scattering in series and good reproducibility of the measurements from day to day confirm the practicability of the " α -Amylase PNP" method

with every experienced worksheet. After a lag phase of 3 min at 25 °C and of 2 min at 37 °C, the selected reaction conditions showed zero order reaction kinetics.

As the response for serum is linear up to the tenfold upper reference limit, only few sera have to be diluted.

In view of its excellent comparability with other similarly based methods (e.g. continuous measurements of α -amylase with maltotetraose or a mixture of *p*-nitrophenylpentaoside/*p*-nitrophenylhexaoside as substrates), the new method offers a real alternative. In comparing these methods, particular account should be taken of the lack of interference by secondary reactions, the stability of the reagents and the choice of a defined substrate for the new " α -Amylase PNP" method.

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References

- Lorentz, K. (1979) J. Clin. Chem. Clin. Biochem. 17, 499–504.
- Rauscher, E., von Bülow, S. & Hägele, E. O. (1982) Fresenius Z. Anal. Chem. 311, 454.
- Hägele, E. O., Schaich, E., Rauscher, E., Lehmann, P., Bürk, H. & Wahlefeld, A. W. (1982) Clin. Chem. 28, 2201–2205.
- Rauscher, E., Neumann, U., Schaich, E., von Bülow, S. & Wahlefeld, A. W. (1984) Clin. Chem. in print.
- Staber, G., Busch, E. W. & Koller, P. U. (1982) Med. Lab. 35, 10–13.
- Markowetz, D. (1982) Med. Lab. 35, 53–66.
- Hohenwallner, W., Hägele, E. O., Scholer, A. & Staber, G. (1983) Berichte der Österreichischen Gesellschaft für Klinische Chemie 6, issue 3.
- Hohenwallner, W., Wimmer, E. & Sommer, R. (1983) Berichte der Österreichischen Gesellschaft für Klinische Chemie 6, 31.
- Melzi d'Eril, G. V., Pollini, E. & Mazzicco, M. (1982) Abstract 32, Congresso nazionale AIPAC 03.06.1982.

A. Scholer
Klinisch-chemisches Laboratorium
Kantonsspital
CH-4031 Basel