SHORT COMMUNICATION

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Suitability of a malachite green procedure to detect minimal amounts of phosphate dissolved in acidic solutions

Abstract The study describes the suitability of a colorimetric method (malachite green procedure) for detection of minimal amounts of phosphate (7.3–29.1 μmol/L) in different acidic solutions (hydrochloric acid, oxalic acid, maleic acid, perchloric acid, tartaric acid, citric acid, lactic acid and acetic acid) adjusted to pH 2.0. A mixture of the respective phosphate concentrations with distilled water served as control. The experiments were run with ten repeats in series. Assessment of intra- and interassay coefficient of variation and lower limit of quantification revealed that depending on the acid used, the applied method is a reliable and suitable tool to detect and quantify minimal phosphate contents in small samples of acidic solutions that have the potential to cause erosive dental lesions.

Keywords Phosphate · Tooth · Enamel · Erosion · Malachite green

Introduction

A variety of acids, such as hydrochloric acid, phosphate acid, citric acid, oxalic acid, lactic acid, tartaric acid, acetic acid or maleic acid might be involved in the process of dental erosion [16, 27]. The acid attack leads to an irreversible loss of the outermost enamel and dentin layers and demineralisation and softening of the tooth surface. It was shown that patients with erosions had an enamel wear of more than 6 months in a range from 17.6 to 108.2 μm [4].

Erosion-induced surface loss is primarily assessed with physical methods, such as microradiography, surface profilometry, microhardness testing, scanning electron microscopy, atomic force microscopy (AFM) or quantitative light-induced fluorescence determination [2, 4, 6, 9, 11, 15, 20, 22]. Application of these methods requires specific preparation of tooth specimens before and/or after an erosion experiment, so that the specimens could not be used in successive experiments. Moreover, the experiments could often not be performed with native tooth surfaces because the surfaces had to be polished before the experiment. This is especially true when using profilometry for determination of minimal surface loss. Detection of erosive loss in native enamel was successfully described with profilometry only in a range of 50 μm with a high variability [9]. Another disadvantage of this method is that tooth substance loss usually should exceed 1 μm to allow reliable and reproducible quantification with profilometrical analysis, although a single study could determine erosive/abrasive loss in a range of about 0.3 μm with this method [14]. Application of a modified microhardness measurement using the change of the indentation length in enamel due to erosive/abrasive loss allows for detection of surface loss of about 0.24 μm [17, 23]. However, early stages of erosive loss due to dietary acids already occur in a nanometer scale, which is below the sensitivity of most physical methods. Only tapping mode AFM, which is a highly demanding and technique-sensitive procedure, allows for detection of surface loss in these small ranges [18, 19]. We therefore searched for a method that enables quantitative analysis of the acid-induced mineral loss from dental hard tissue below the sensitivity of physical methods.

Dental enamel is a highly mineralized acellular tissue in which microscopic calcium phosphate crystals comprise some 99% of the dry weight. This means, that dental enamel consists of 34–39% m/m (g per 100 g) calcium (dry weight) and 16–18% m/m phosphorus [26]. Therefore, determination of dental enamel dissolution by assessing the amount of calcium or phosphate dissolved from the apatite crystals of dental hard tissue could also be regarded as a possible tool for assessing dental erosions.

In a recent study, we showed that photometrical analysis (Arsenazo III) allows for quantification of small concentrations of calcium in minimal volumes (10 μL) of various acidic solutions, such as hydrochloric acid, maleic acid, phosphoric acid, lactic acid and acetic acid [1]. The method

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was suitable for detection of 12.4–49.4 μmol/L calcium in those acids that were adjusted to pH 2.0, 2.3 and 3.0 and requires only minimal preparation of the solution before measurement. However, oxalic acid, tartaric acid and citric acid did not fulfil the requirements for all applied calcium concentrations or at all pH values. Therefore, for determining small amounts of calcium in chelating acidic agents, other applications, such as using flame photometers, seem to be more suitable. On the other hand, during enamel dissolution induced by acids, phosphate is also released from the enamel and diffuses into the acidic solution.

It was recently shown that the phosphate concentration in acidic beverages could be measured by a colorimetric assessment method [3]. This method, first described by Hohenwallner and Wimmer [13], could be an alternative tool to measure dental hard tissue dissolution in those acids in which calcium determination could not be reliably performed.

As mentioned above, such stages of dental erosion could only be recorded by technique-sensitive and demanding methods, such as AFM. It would be very helpful to have a tool that allows both easy handling and precise measurement of enamel dissolution in tooth samples without requirement of surface preparation. The malachite green procedure has already been applied by Hattab and Linden [12] for determination of phosphate dissolved from enamel after etching with perchloric acid at a range of 0.025–3.0 mM. The authors proved that the method using perchloric acid did not interfere with fluoride concentrations up to 80 μg/mL in the assay. However, it is not known how far the colorimetric malachite green method might be influenced by specific properties of other acids. It is conceivable that acids may form precipitates or complexes, which might interfere with a spectrometical analysis, as previously shown when using a colorimetric method for calcium analysis [1]. Moreover, it is of interest if the method could be applied for detection of minimal amounts (in the micromolar range) of phosphate dissolved from enamel in small volumes of acids during very short contact time (a few seconds) of the solution with the dental substrate. Analysis of phosphate release into small volumes of acidic solutions would allow investigation of surface phenomena during erosion.

Consequently, the aim of the present study was to evaluate the feasibility of the malachite green procedure to determine minimal amounts of phosphate in different acidic solutions with small volume, using the method in microtiter plates according to the latest descriptions made by Fathi et al. [8].

Materials and methods

In the study, phosphate concentrations in various acidic solutions were colorimetrically assessed in a flat microplate reader at 650 nm (Molecular Devices, Ismaning/Munich, Germany). Preparation of the solutions for measuring phosphate concentration was conducted according to the method introduced by Hohenwallner and Wimmer [13] and to further descriptions of the method by Fathi et al. [8]. In this method, malachite green reacts with phosphate in solution to form a green complex. The intensity developed proportional to the phosphate concentration and could be determined photometrically according to Lambert–Beer's law.

Chemicals

Malachite green and ammonium molybdate were from Sigma (Deisenhofen, Germany). All other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

The malachite green reaction solution (R1) was mixed as follows. First, 4.2 g ammonium molybdate was dissolved in 100 ml of 4 M HCl. The malachite green dye solution was prepared by adding one part of this ammonium molybdate solution to three parts 0.045% (w/v) malachite green in aqua bidest. This solution was stirred for 30 min until it became clear. After centrifugation, the supernatant was stable for more than 6 months whilst stored at 4°C. Before its use, the solution was centrifuged again. The standard reagent (R2) was 6 mmol/L phosphate, prepared by mixing 8.52 Na_2HPO_4 with 10 ml aqua bidest.

The following acids were used in the study: hydrochloric acid (HCl), perchloric acid (HClO), oxalic acid (O-A), maleic acid (M-A), tartaric acid (T-A), citric acid (C-A), lactic acid (L-A) and acetic acid (A-A). All acids were adjusted to pH 2.0 by addition of distilled water before further use in the experiment. Concentrations of the resulting acidic solutions were calculated according to the volume of distilled water needed for achieving pH 2.0. The standard reagent R2 was diluted with distilled water, resulting in the following phosphate concentrations: 7.3, 14.5, 21.8 or 29.1 μmol/L.

Analysis

Ten microliters of the diluted solutions of reagent R2 was placed into the wells of the microtiter plate and mixed with 30 μl of the particular acidic solution and 100 μl R1. Reagent R1 was applied rapidly with a multipipette (Eppendorf, Hamburg, Germany). A mixture of the respective phosphate concentrations with distilled water served as control. A mixture of R1 (100 μl), the respective acidic solution (30 μl) and distilled water (10 μl) acted as blank. Absorbance was read at 650 nm in the microplate reader. According to the recommendations given by Fathi et al. [8], absorbance was read exactly after 8 min because the colour stability of the complex is unstable. The measurements were performed at room temperature. The experiments were run with ten repeats in series.

Statistical analysis

The software package Statistica 6.0 (Statsoft, Tulsa, OK, USA) was used for analysis. The phosphate concentrations were calculated by fitting the sigmoid standard curves with polygonal interpolation. Intra- and interassay precision were checked according to the guidance for bioanalytical method validation recently described [24, 25]. Intraassay coefficient of variation of phosphate recovery was calculated for assessing precision of the test since it considers both distribution of the data and slope of the calibration curve. Threshold for acceptable precision was set at a coefficient of variation of ≤10%. For example, an intraassay coefficient of variation of 10% would mean that a phosphate concentration of 10 μmol/L in a respective solution would yield a reading in the range of 9–11 μmol/L.

Precision with time was checked by calculating the interassay coefficient of variation. Therefore, for each solution, ten calibration curves were constructed and recovery of phosphate and interassay coefficient of variation were calculated. Acceptable threshold was set at an interassay coefficient of variation of ≤20%. For example, an interassay coefficient of variation of ≤20% would mean that using different calibration curves, a phosphate concentration of 10 μmol/L in a respective solution would yield a result of 8–12 μmol/L.

Lower limits of quantification (LLOQs, μmol/L) for the respective solutions were calculated as phosphate concentration at the particular point on the calibration curve presenting the following: mean value of blank absorbance+ (5×standard deviation). This procedure is recommended in the literature for assessment of quality in analytical chemistry [7]. For this purpose, absorbances of blank solutions of each acid were determined with ten repeats in series. As a threshold, the lowest standard on the calibration curve (7.3 μmol/L) should be higher than the LLOQ [24, 25].

Results and discussion

The intraassay coefficients of variation (Fig. 1) amounted to ≤10% for all of the acidic solutions at all phosphate concentrations, except for citric acid with phosphate content of 7.3 μmol/L. In most of the cases, the intraassay coefficient of variation distinctly decreased with increasing phosphate concentration of the respective acidic solution. With 29.1 μmol/L, the coefficient of variation was ≤6% for all solutions.

The interassay coefficient of variation for distilled water ranged from 4.1 to 11.2% with a recovery rate of 92.5–98.9%. For all tested acidic solutions, interassay coefficient of variation (Table 1) amounted to ≤20%. The recovery rates were in a range of 90.3 (lactic acid, 7.3 μmol/L) to 118.2% (maleic acid, 7.3 μmol/L). With increasing phosphate concentrations, the recovery rate for most of the solutions came closer to 100% which is the optimal recovery rate.

LLOQ for distilled water amounted to 35.5 μmol/L. For the different acidic solutions, the respective LLOQs are given in Table 1, showing a range from 1.89 (perchloric acid) to 9.03 μmol/L (citric acid). With the exception of citric acid, all LLOQs were distinctly lower than the lowest phosphate concentration (7.3 μmol/L) applied in the experiment.

The aim of this study was to assess the suitability of a colorimetric method as a tool for determining small amounts of phosphate in small volumes of acidic solutions. The method had proven to have an up to fourfold sensitivity in terms of absorbance readings as compared to standard methods when used for detection of alkaline phosphate activity in enzyme immunoassays [5, 10]. In the present study, the pH of the acidic solutions was adjusted to pH 2.0. This pH value is in the lower range of the pH of acidic drinks with different kinds of acids as ingredients, which have the potential to create erosive dental lesions [21]. Different acidic solutions were chosen that might reflect acids in acidic beverages (i.e. citric acid, maleic acid, tartaric acid) or in gastric content (hydrochloric acid). Since the phosphate determinations in the acidic solutions with pH 2.0 already fulfilled the guidance for bioanalytical method validation [24, 25], we did not analyze solutions with higher pH. It might be assumed that when the method works at pH 2.0 it will also work at higher pH values (presumably with an even higher accuracy), as shown in the control group (distilled water). Moreover, pilot experiments showed that the sensitivity of the test improved with increasing pH. The experiments were performed with

Fig. 1 Intraassay coefficient of variation (%) for the different acidic solutions. A logarithmic scale is chosen for the ordinate [hydrochloric acid (*HCl*), perchloric acid (*HClO*), maleic acid (*M-A*), oxalic acid (*O-A*), lactic acid (*L-A*), tartaric acid (*T-A*), acetic acid (*A-A*), citric acid (*C-A*) and distilled water (*dist.*)]. Threshold level is ≤10%
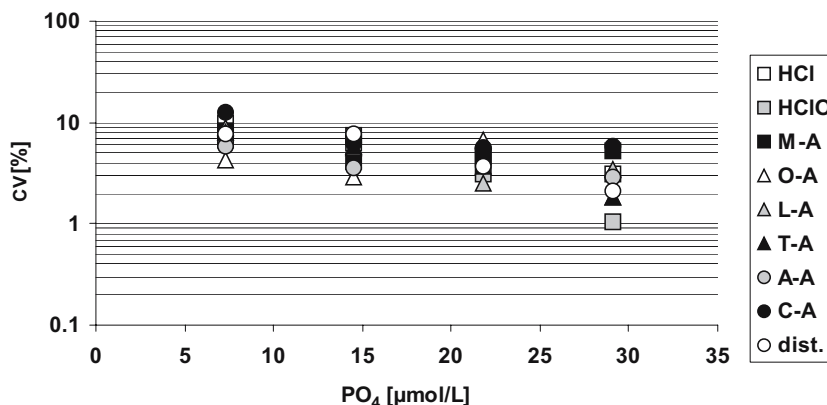
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Table 1 Mean values, interassay coefficients of variation (CV), recovery rates and lower limits of quantification [LLOQs (μmol/L)] for the different solutions with various amounts of applied phosphate

Solution	PO$_4$ (μmol/L)	Mean	CV (%)	Recovery (%)
Maleic acid	7.3	8.6	12.8	118.2
(15.7 mmol/L)	14.5	13.7	7.5	95.1
	21.8	22.5	7.7	103.2
	29.1	31.8	8.3	109.3
	(LLOQ=2.3)			
Hydrochloric acid	7.3	7.6	15.6	104.8
(10.5 mmol/L)	14.5	14.0	7.8	96.9
	21.8	21.3	6.8	98.1
	29.1	28.4	3.7	97.6
	(LLOQ=4.1)			
Perchloric acid	7.3	7.3	15.3	100.7
(10.5 mmol/L)	14.5	14.3	9.1	98.6
	21.8	22.3	6.0	102.3
	29.1	29.9	4.7	102.8
	(LLOQ=1.8)			
Lactic acid	7.3	7.1	10.6	98.1
(292 mmol/L)	14.5	14.1	9.6	97.2
	21.8	21.8	3.5	100.4
	29.1	29.7	5.3	102.4
	(LLOQ=2.7)			
Citric acid	7.3	6.5	15.9	90.3
(95.2 mmol/L)	14.5	13.2	7.6	91.3
	21.8	19.9	7.9	91.4
	29.1	29.0	7.1	99.8
	(LLOQ=9.0)			
Tartaric acid	7.3	6.7	11.0	92.1
(50.0 mmol/L)	14.5	14.7	8.7	101.7
	21.8	21.2	6.4	97.6
	29.1	27.9	2.8	96.2
	(LLOQ=5.7)			
Oxalic acid	7.3	7.4	6.3	102.6
(8.8 mmol/L)	14.5	15.4	7.2	106.5
	21.8	22.8	11.6	104.6
	29.1	29.7	6.5	102.1
	(LLOQ=2.1)			
Acetic acid	7.3	7.6	5.1	105.1
(2214 mmol/L)	14.5	14.6	5.1	100.8
	21.8	21.5	8.0	98.9
	29.1	28.4	4.3	97.8
	(LLOQ=2.5)			
Distilled water	7.3	6.7	9.2	92.5
	14.5	14.1	11.2	97.5
	21.8	21.2	6.2	97.5
	29.1	28.7	4.1	98.9
	(LLOQ=3.5)			

Concentration of respective acid at pH 2 is given in parentheses
Threshold level for CV is ≤20%

acidic solution that were adjusted to the same pH and not in solutions with same acid concentration. Using solutions with same pH allows for better comparisons among the different acids with respect to their specific H$^+$-dependent kinetics. Moreover, solutions with erosive potential, such as beverages, are often characterized by their pH (which could be easily determined) and not by a quantitative analysis of the concentrations of their various acidic components.

The different parameters for checking precision of the tests, such as intra- and interassay coefficient of variation and lower limit of quantification, showed similar results compared to the values determined for distilled water. This underlined the fact that the method could be adopted for determining phosphate in acidic solutions with the same precision as in solutions with neutral pH.

To show the suitability of the method to determine phosphate in small volumes, the applied phosphate concentrations were added to a small sample (10 μl) of the acidic solutions. This small sample size was chosen because it presents an amount suitable to wet a small tooth surface area of about 1 to 2 mm in diameter. This would allow one, in future erosion studies, to measure the phosphate release of small tooth specimens when having short contact with minimal amounts of acidic solutions or to gain insight into erosion processes occurring in the interface of thin films of acidic solution on dental hard tissue. The study showed that the applied method is able to detect and quantify 73 pmol phosphate in a 10-μl (7.3 μmol/L) sample of different acidic solutions. Under the premise that enamel has a density of 2.95 g/cm^2 and a phosphorus concentration of 17% [26], this would hypothetically mean that for a specimen 2 mm in diameter a minimal enamel loss of 1.6 nm could be quantified with this method by determining the phosphate release of this specimen into an acidic solution.

Acceptable values for inter- and intraassay coefficient of variations were set at ≤20 and ≤10%, respectively, to fulfil the guidance for industry bioanalytical method validation [24, 25]. For most of the solutions, inter- and intraassay coefficients of variation were in a range fulfilling this guidance clearly. In addition, most of the solutions showed distinctly lower LLOQs for the different acids than the lowest standard on the respective calibration curves, thereby meeting the suggested criteria for LLOQ. However, citric acid did not fulfil the guidance for all applied phosphate concentrations. Therefore, for determining small amounts of phosphate in citric acid, other applications, such as using flame photometers, seem to be more suitable. However, with increasing phosphate concentrations, citric acid also fulfilled the criteria defined in the guidance for industry bioanalytical method validation.

Hence, it is concluded that depending on the acid used, the malachite green procedure is a reliable and suitable tool to detect and quantify minimal phosphate contents in small samples of acidic solutions that have the potential to cause erosive dental lesions.

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