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Adsorptive stripping voltammetry of vitamin D₃ at glassy carbon electrode

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Adsorptive stripping voltammetry (AdSV) of cholecalciferol at the glassy carbon electrode (GCE) was developed and based on existing conventional approaches employing anodic oxidation in pure organic electrolytes or aqueous-organic mixtures. Despite a rather poor reproducibility of the adsorption-driven process (with the RSD of ca. 10 %), significantly higher sensitivity was achieved. As optimum conditions, the respective procedure comprised an accumulation of cholecalciferol from 40% ethanol at 300 min⁻¹ for 8 min followed by electrochemical detection in the Britton-Robinson buffer (pH 4.0) using square-wave voltammetry at a potential step of 5 mV, amplitude of 35 mV, and frequency of 40 Hz

Introduction

Vitamin D (VD) as one of four fat-soluble vitamins has two forms; namely, ergocalciferol (VD₂) and cholecalciferol (VD₃), which are obtained from the endogenous environment (exposure to sunlight) and exogenous environment (foodstuffs), respectively [1,2]. Avitaminosis of VD plays an important role in the

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development of osteoporosis because it is liable for the increase of intestinal absorption of calcium and other minerals [3]. Nowadays, a few scientific journals report on the deficiency of VD may also cause cardiovascular disease, immunological abnormalities, and cancer [4,5]. For the proper adsorption of calcium, adequate amount of VD (10–25 µg) is highly recommended as a daily intake. In this case, it is necessary to mention that the increased VD intake has no therapeutic effect in the development of osteoporosis and thus it serves only to prevent.

Ordinarily, VD_3 is synthesized endogenously from 7-dehydrocholesterol after ultraviolet irradiation or it is exogenously absorbed from the diet, such as fresh salmon, cod liver oil, egg yolk, and some food supplements. For determination of ergocalciferol and cholecalciferol in milk and other foodstuffs, standard analytical method, ČSN EN 12821 (560047) is used. When being based on high-performance liquid chromatography (HPLC) with normal phase and UV detection, it can be characterized by some disadvantages, such as time consuming sample preparation (including usually saponification and extraction into an organic solvent) and high acquisition costs.

Since 1990, four different electroanalytical methods have been developed [6–9]. They are based on anodic oxidation of VD_3 at the glassy carbon electrode (GCE) – either as bare or modified with a suitable reagent and realized in pure organic electrolytes or aqueous-organic mixtures containing lithium perchlorate. From literature [6,10], it is supposed that VD_3 is irreversibly hydroxylated on the C8 and C7 atoms that are part of a conjugated system with three double bonds (see Fig. 1). Only one of them can be succesfully applied for VD_3 determination in the human blood and serum [6]. The main problem is the required sensitivity which could be solved using adsorptive stripping voltammetry (AdSV [11,12]). VD_3 is a fat-soluble steroid that is readily adsorbed from aqueous-organic mixtures onto a nonpolar carrier represented by the freshly polished GCE.
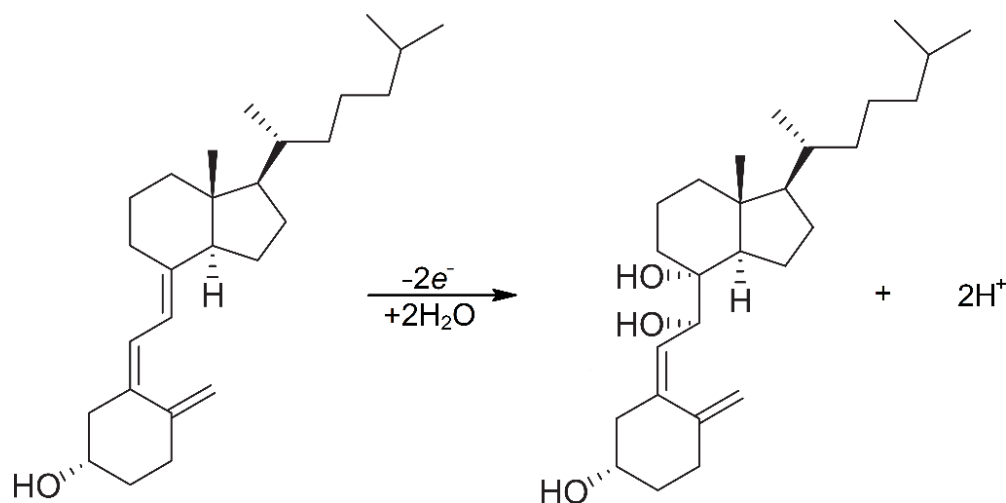
Fig. 1 *Reaction pathway proposed for anodic oxidation of VD₃*

This process ensures a sufficient accumulation of VD_3 on the electrode surface which can then be placed into an aqueous supporting electrolyte and oxidized during the sensitive pulse voltammetric scan. The aim of this study was to clarify whether this analytical approach can be used to analyze samples with the trace content of VD_3.

Materials and methods

Chemicals and reagents

Analytical standard of crystalline cholecalciferol was purchased from Merck. Water-soluble organic solvents were used, namely: 99.9% acetonitrile (AN, Sigma-Aldrich), 99.9% acetone, 99.7% isopropanol, 99.5% methanol, and 96% ethanol from Lach-Ner (Neratovice, Czech Republic). Furthermore, 0.01 mol L^{-1} cholecalciferol stock solutions in the corresponding organic solvents were used and stored in a refrigerator at 5 °C. Universal 0.1 mol L^{-1} Britton-Robinson buffer (BRB) prepared by mixing equivalent amounts of boric acid, 98% acetic acid, 85% phosphoric acid, and sodium hydroxide from the Lach-Ner was used in the study of pH effect. All the mentioned solutions were prepared from demineralized water (with electric resistivity >18.3 MΩ cm) when using Milli-Q purification system (Millipore, Burlington, MA, USA).

Preatment of the working electrode

Surface of the GCE (type 6.1204.600) with a diameter of 2 ± 0.1 mm (Metrohm Prague, Czech Republic) was renewed on polishing pad in the presence of water suspension of Al_2O_3 powder (particle size: 1.0 μm) for 30 s. After the subsequent rinsing of the surface with demineralized water, the GCE was ready for next electrochemical experiment. This procedure was in accordance with the manual from distributor. To ensure the proper pretreatment of the electrode, blank measurement (only with the supporting electrolyte) was performed before each experiment.

Instrumentation

All electrochemical measurements were carried out in a 50 mL glass cell containing usually 10 mL of detection aqueous medium at 25 °C. Conventional three-electrode system consisting the GCE (working), a Ag/AgCl (reference), and platinum wire (auxiliary) electrode was used connected to a potentiostat (Autolab PGSTAT101; Metrohm, Prague, Czech Republic), and compatible with software Nova version 1.11 (Metrohm, Prague, Czech Republic).

Method

Adsorptive square-wave voltammetry (AdSWV) of cholecalciferol at GCE was realized in two separate steps. At first, the adsorption onto the GCE surface was done by immersing the working electrode in aqueous-organic solution chosen containing typically 100 µmol L^{-1} cholecalciferol at 300 min^{-1} for 8 min. Secondly, adsorbed vitamin D_3 was anodically oxidized by SWV at the following conditions: potential window from 0 to +1.4 V, $E_{step} = 5$ mV, potential of amplitude $E_{ampl} = 35$ mV, and frequency $f = 40$ Hz. All voltammetric measurements were repeated at least 5×. If not stated otherwise, all changes in experimental conditions are shown in the legends of the corresponding figures.

Results and discussion

Adsorptive accumulation of the target analyte or products of electrochemical reaction onto the surface of the electrode material is a very frequent process in electroanalysis. In many cases, a deterioration of repeatability of the results within the same experiments occurs. However, in stripping voltammetry, the adsorption can be used as an accumulation step of non-electrolytic nature. Electroanalytical methods based on this kind of accumulation represent usually very sensitive tools suitable for the determination of lipophilic compounds; especially, fat-soluble vitamins [12]. Generally, efficiency of adsorption depends on numerous factors and the respective conditions require optimization.

Selection of suitable aqueous-organic mixture

Lipophilic VD_3 is easily soluble in several polar organic solvents, as well as in their aqueous mixtures. It is not surprising that finding of optimum type and content of the solvent in the aqueous mixture is a crucial task. The content of each organic solvent in the form of aqueous mixture was expressed as the volume fraction ($\varphi = V_{org} / (V_{org} + V_{aq})$. In principle, the adsorption of lipophilic analyte onto nonpolar electrode surface (solid phase) is an equilibrium process which can be characterized by Henry adsorption constant (K_H). In a narrower sense, this constant describes a distribution of target analyte between the solid phase and mobile phase (aqueous-organic mixture).

In this study, the adsorption was performed from different aqueous-organic mixtures containing always 100 mol L^{-1} VD_3 at stirring rate of 400 min^{-1} for 10 min and subsequently, during the stripping step, measured in 0.1 mol L^{-1} BRB (pH 4.0) at $E_{step} = 5$ mV, $E_{ampl} = 25$ mV, and $f = 20$ Hz. It has been found that all the tested organic solvents can be theoretically used for adsorptive accumulation of VD_3 (see Fig. 2).

In terms of the organic solvent content and its effect, it was observed that VD_3 was not absorbed from aqueous mixtures with values of the volume fraction higher than 0.7. A 40% ethanolic solution was chosen as optimum due to the required volatility, guaranteeing a constant composition of the water-organic mixture during the adsorption process.
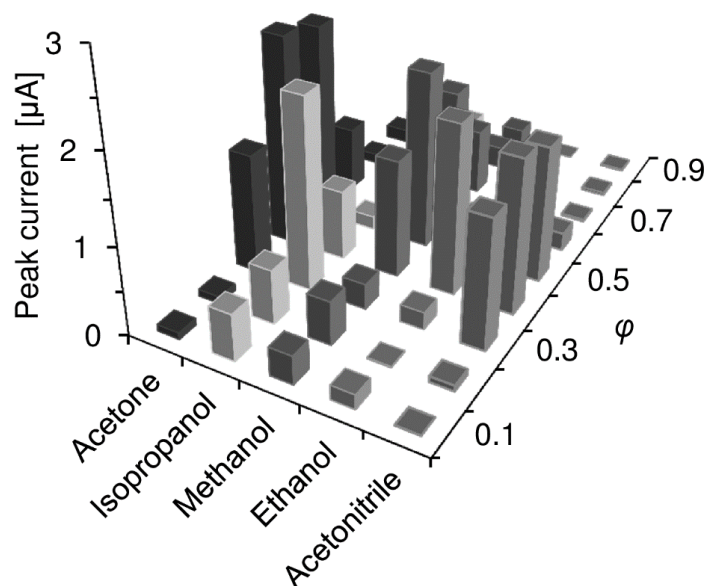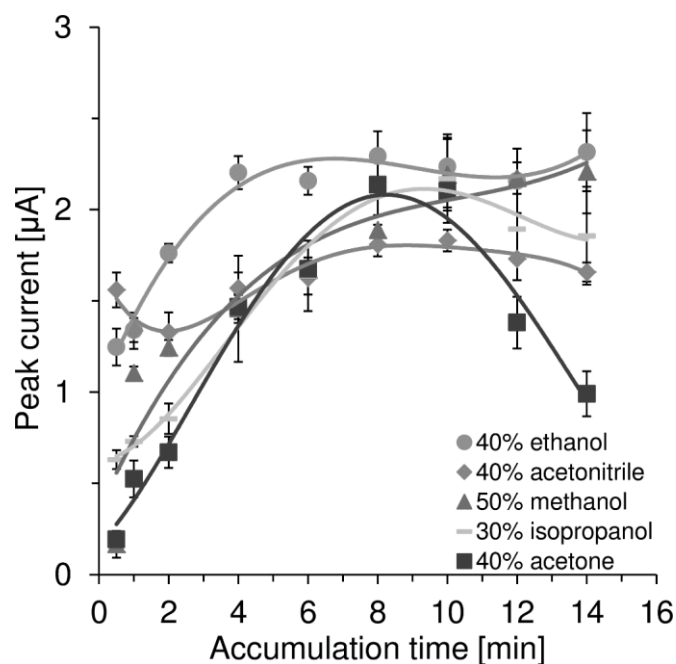

Fig. 2 *Dependence of anodic peak current for VD₃ on the different content of selected organic solvents in the corresponding aqueous mixtures*

Effect of stirring rate

The intensity of stirring significantly affects the rate of VD_3 transport to surface of GCE, where the adsorption takes place. Within this study, it was observed that the values of stirring rate of magnetic stir bar (1.2×0.3 cm) being higher than 300 min^{-1} did not have any evident effect on the increase of final current response. Therefore, this value 300 min^{-1} was chosen as optimum.

Effect of adsorption time

The adsorption can be defined as the adhesion of molecules from gas or liquid to a surface creating a film. This process is usually described by Langmuir isotherm to be the amount of accumulated substance as a function of its concentration at a constant temperature. From electrochemical point of view, the oxidation peak current is linearly proportional to the accumulated amount of VD_3. For that reason, it should be evident that a curve describing dependency of the current intensity on accumulation time forms an isotherm (see Fig. 3), when 8 min. has represented a sufficient time duration to achieve the equilibrium.

Fig. 3 *Dependence of VD₃ anodic peak current on different accumulation time*

Adsorption of 100 μmol L^{-1} VD$_3$ onto surface of GCE from different aqueous-organic mixtures. Detection in 0.1 μmol L^{-1} BRB (pH 4.0) using SWAdSV at E_{step} = 5 mV, E_{ampl} = 25 mV, and f = 20 Hz.

Electrochemical detection of cholecalciferol using square-wave voltammetry

A set of BRBs (pH 2 to 14) made 0.1 mol L^{-1} in concentration was used as detection medium to choose the optimum supporting electrolyte. VD$_3$ provided satisfactory current response at a potential of +1.15 V in weakly acidic buffers. For more basic or alkaline buffers (pH 9–14), a significant decrease of the current was observed. Moreover, the corresponding peak potentials were shifted to more positive values. According to obtained results shown in Fig. 4, BRB with pH 4 was selected as optimum detection medium.

Square-wave voltammetry (SWV) together with differential pulse voltammetry (DPV) belong to pulse voltammetric techniques eliminating the charging current and they are commonly used in electroanalysis. The peak height of VD$_3$ oxidation response was affected by three main parameters, such as potential step (E_{step}), potential amplitude (E_{ampl}), and frequency (f). Sensitivity (slope of calibration curve; k) usually increased with higher values of E_{ampl} and scan rate (v) which can be calculated as E_{step} multiplied by f. The parameter "v" is affected only by f at constant E_{step}. For that reason, optimization of SWV was focused on finding proper potential amplitude and frequency. By setting higher values than 35 mV for amplitude and 40 Hz for frequency, one did not achieve any evident increase of the current signal and the above-stated values were therefore chosen as optimum.
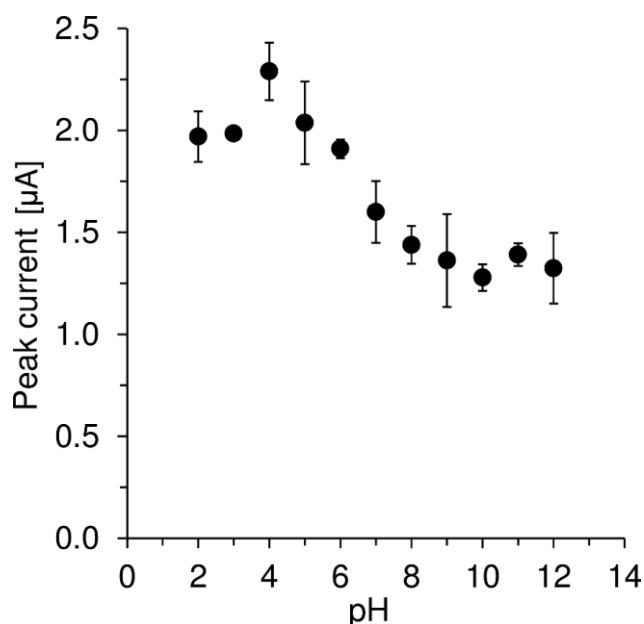
Fig. 4 *Effect of supporting electrolyte pH on peak current*

Results obtained for 100 μmol L^{-1} VD$_3$ accumulated from 40% ethanol onto GCE surface at 400 min^{-1} for 8 min and then detected in different 0.1 mol L^{-1} BRBs by SWV at $E_{step} = 5$ mV, $E_{ampl} = 25$ mV, and $f = 20$ Hz.

Usually, electroanalytical methods based on adsorptive accumulation of target analyte onto the surface of solid GCE with the subsequent electrochemical detection using pulse voltammetric technique represents very sensitive analytical tools. However, it is necessary to consider that the detection and quantification limits (LOD and LOQ, respectively) are completely dependent on the magnitude of standard deviation (s) calculated for at least five repetitions ($n = 5$). Values of LOD and LOQ are being estimated according to the following equations: $LOD = 3\,s/k$ and $LOQ = 10\,s/k$, respectively. Obtained results show that values of relative standard deviation (RSD) have dramatically increased with the decreasing VD$_3$ concentration. An explanation on why the repeatability of adsorption (for 1.0 μmol L^{-1} VD$_3$) expressed by the RSD was nearly 14% and not 6% like for other lipophilic vitamins, can be due to the molecular structure which misses long nonpolar alkyl chain (only six carbons). In addition, a value of 0.4316 for intercept (q) was calculated. These two factors caused that the desired LOD for determination of VD$_3$ in the cow milk (~0.7 μmol L^{-1}) and human blood (0.05–0.1 μmol L^{-1}) could not be achieved. This is also the reason why conventional voltammetric methods (see Table 1) are comparable with the adsorptive approach presented herein.

**Table 1** *Comparison of SWAdSV with conventional voltammetric methods developed for determination of VD₃ since 2000*

Sensor	Technique	k [µA L mol^{-1}]	q [µA]	R^2	Reference
GCE/SiO$_2$/GO/Ni(OH)$_2$	DPV	1.090	9.200	0.9970	[6]
GCE	DPV	0.851	0.025	0.9980	[7]
GCE/poly(ARS)MWCNTs	SWV	0.614	3.197	0.9988	[8]
GCE	DPV	0.119	0.034	0.9974	[9]
GCE	SWAdSV	0.131	0.4316	0.9942	This work

k, q, and R^2 represent slope, intercept, and coefficient of determination, respectively.
GCE/poly(ARS)MWCNTs – poly (Alizarin red S)/multi-walled carbon nanotubes modified glassy carbon electrode,
GCE/SiO$_2$/GO/Ni(OH)$_2$ – amorphous nickel (II) hydroxide particles onto a hybrid material composed of silica and graphene oxide modified glassy carbon electrode.

Typical anodic voltammograms together with the corresponding calibration curve are shown in Fig. 5. A linear range from 1.0 to 50 µmol L^{-1} VD$_3$ was achieved at optimum conditions.
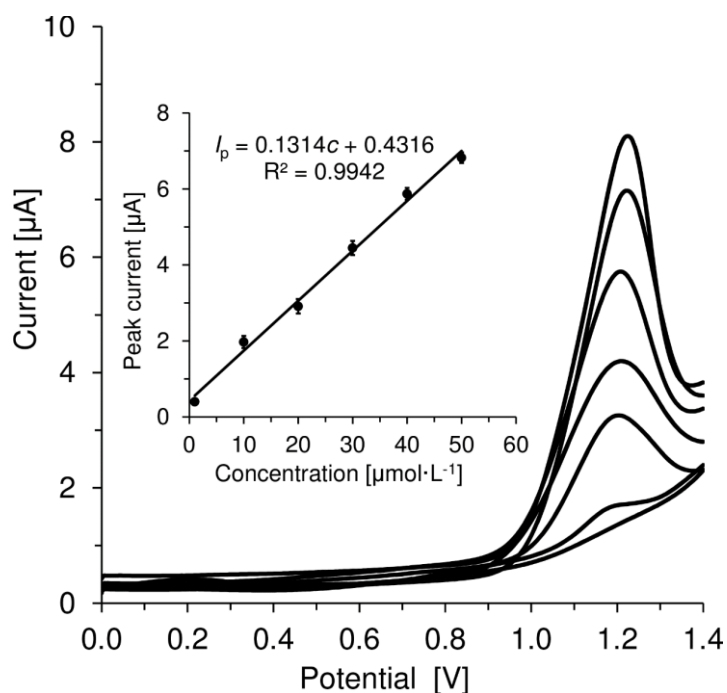
Fig. 5 *SWAdSV of 0, 1, 10, 20, 30, 40, and 50 µmol L^{-1} VD$_3$ together with corresponding calibrations (insets)*

Adsorption from its 40% water-ethanol mixture onto GCE surface at stirring rate 300 min^{-1} for 8 min, electrochemical detection in 0.1 mol L^{-1} BRB (pH 4.0) at $E_{step} = 5$ mV, $E_{ampl} = 35$ mV, and $f = 40$ Hz.

If needed, the linear range can be extended by selecting appropriate working conditions; however, a certain loss of the overall sensitivity should be expected. Such an additional linear range can be assumed for concentrations lower than 1 μmol L^{-1} due to a relatively high value of intercept.

Generally, it can be concluded that a shorter linear range for AdSV than for direct voltammetric approaches were found. For example, similar linear range (from 5 to 50 μmol L^{-1}) was obtained for adsorption approach employing DPV with a sophisticated configuration GCE/SiO$_2$/GO/Ni(OH)$_2$ sensor and stripping in 0.1 mol L^{-1} NaOH [6]. In the contrary, three to seven times longer linear ranges were achieved by using SWV at the GCE modified with poly(ARS)MWCNTs in a surfactant solution [8] and using DPV at the bare GCE performed in water-ethanol mixtures containing lithium perchlorate [9].

Conclusions

This paper represents an initial electroanalytical approach proposed for the determination of biologically active VD$_3$. Adsorption of this analyte onto freshly polished surface of the GCE from an aqueous-organic mixture and the subsequent electrochemical detection by SWV in the anodic range and in a solution of acidic Britton-Robinson buffer represented two main steps which had to be optimized.

Unfortunately, at present, one has to state that the non-conventional way developed in the framework of this study does not offer a satisfactory sensitivity for clinical analysis. As found out, the major problem was a low repeatability of the adsorption process, indicated via 14% RSD for 1 μmol L^{-1} VD$_3$. Due to this, lower concentrations of VD$_3$ have not been yet measured and such a detection will be of interest in the near future.

Acknowledgement

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