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Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 730–735
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Using of HPLC coupled with coulometric detector for the determination of biotin in pharmaceuticals

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Received 20 April 2007; received in revised form 8 August 2007; accepted 9 August 2007
Available online 15 August 2007

Abstract

The method for the determination of biotin by high performance liquid chromatography (HPLC) coupled with coulometric detector is presented here. Chromatographic and detection conditions were tested. A LiChrospher 60RP-select B column (250 mm × 4 mm; 5 μm) and the mobile phase containing 0.24 mol/L aqueous solution of acetic acid and acetonitrile in the ratio 85:15 (v/v) were found as the most suitable. The flow rate was 1 mL/min and the injected volume of the sample was 20 μL. The hydrodynamic voltammogram of biotin was measured and according to obtained data the detection parameters were set – channel I 600 mV, channel II 900 mV, sensitivity 1 μA. The developed method has been validated. The calibration curve is linear in the range 15–3600 ng/mL, correlation coefficient is 0.9998, limits of detection and quantification are 5 and 15 ng/mL, respectively. Recovery of the spiked samples was 98.67% with R.S.D. 0.255% on average. The developed method has been successfully applied for determination of biotin in pharmaceutical preparations.

Keywords: Biotin; Coulometry; Electrochemical detection; HPLC; Pharmaceutical preparations

1. Introduction

In the last approximately 30 years, the connection of high performance liquid chromatography and electrochemical detection (HPLC–ED) has seen many improvements. Even though questions of HPLC detectors have been widely discussed and developed, electrochemical detection still has some interesting advantages over other detectors used with HPLC. One of the biggest advantages of electrochemical detection is its high selectivity. This is caused by the property of an electrochemical detector to respond only to substances showing electrochemical activity. Seeing that many substances do not readily react electrochemically, the electrochemical detector is employed to advantage when a complex matrix is analyzed. Furthermore, electrochemically active compounds can be detected with much higher sensitivity. Coulometry, a specialized form of amperometry, based on an electrochemical principle, uses measuring of total current as a function of time – that is, the charge needed to the total chemical change of an analyte measured in coulombs [1]. Many applications of the utilization of coulometric detection have been described: application in analytical food chemistry [2,3], determination of antioxidants [4], determination of biogenic amines [5,6]. In addition, owing to the simple instrumentation the determination of biogenic amines was also carried out *in vivo* [7]. A coulometric detector has also been used for determination of illicit drugs [8] and enantioseparations [9]. For its superior selectivity and sensitivity, this technique is usually chosen when compounds of interest are present only at trace concentrations in comparison with other sample components. This is also the case in

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doi:10.1016/j.jpba.2007.08.010

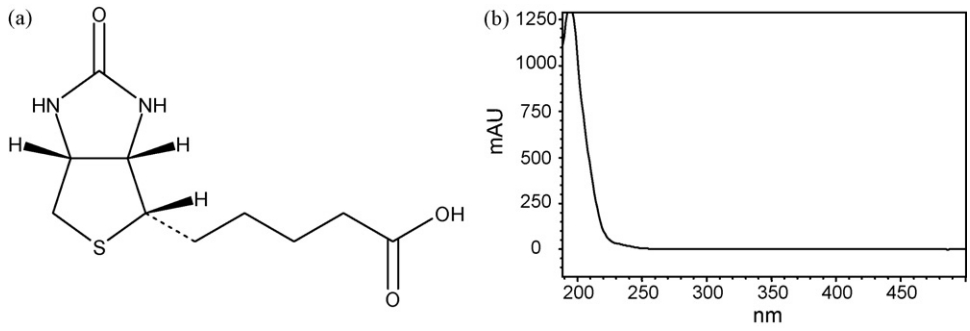

Fig. 1. Structural formula of biotin (a) and its UV-spectrum (b).

the determination of biotin in pharmaceuticals described in this paper.

Biotin (Fig. 1a) 5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydrothieno[3,4-*d*]imidazol-4-yl]pentanoic acid, also known as vitamin H or B_8, is a water-soluble vitamin belonging to the B-complex. It acts as a cofactor responsible for the carbon dioxide transfer in several carboxylase enzymes. It is involved in the biosynthesis of fatty acids, gluconeogenesis, energy production, the metabolism of the branched-chain amino acids and the *de novo* synthesis of purine nucleotides. Recent research indicates that biotin plays a role in gene expression and that it may also act in DNA replication. A sufficient intake is necessary for healthy hair, skin, sweat glands, nerve tissue, bone marrow and it also assists with muscle pain [10,11].

The analytical techniques for biotin determination can be divided into four main categories:

(1) Microbiological methods, based on growth of microorganisms in presence of biotin, are very sensitive but they lack specificity and they are very time-consuming [12].
(2) Biological techniques are based on the animal development curve. These are used mainly for the determination of biotin in food [12].
(3) Binding assays make use of formation of a specific avidin (or streptavidin)–biotin complex [12].
(4) The last group of methods includes all physicochemical methods such as spectrophotometry, polarography, thin layer chromatography, gas chromatography, high performance liquid chromatography and capillary zone electrophoresis [12,13].

HPLC was coupled with UV/VIS [14–17], fluorescence [12] or MS detection [18,19]. There have also been some experiments employing electrochemical detectors. The amperometric detection of biotin was published in 1986 but the detection limit was very similar to limits usually reached with UV detection [20]. A voltammetric detector was also tested for determination of biotin; the experiment was based on avidin–biotin interaction [21]. A coulometric detection was used for determination of two B-group vitamins (pyridoxine and cyanocobalamin) [22].

In our paper, a very sensitive and advantageous analytical method for determination of biotin in pharmaceutical preparations using HPLC coupled with a coulometric detector is described. The defined daily dose of biotin is about 30 μg/day, and thus its content is proportional in pharmaceuticals unlike the amount of other water-soluble vitamins (e.g. vitamin C), in which the content is much higher. This fact together with its non-specific UV-spectrum – maximum at 190 nm (Fig. 1b) causes problems when UV/VIS detection for determination of biotin is used. Therefore, the employment of the coulometric detector is more than suitable. A procedure for extraction of biotin from the pharmaceuticals has been developed, conditions for chromatography with regard to usage of coulometric detection have been optimized and the method has been validated. This developed method was consequently applied to determine the amount of biotin in some pharmaceuticals available on the Czech market.

2. Experimental

2.1. Reagents

All reagents were of analytical or gradient grade. Water was purified in a Milli-Q system (Millipore). Acetonitrile, methanol, propan-2-ol, formic acid and phosphoric acid were supplied by Sigma–Aldrich. Citric acid, acetic acid, potassium phosphate monobasic anhydrous and potassium phosphate dibasic anhydrous were obtained from Fluka. Sodium acetate trihydrate was supplied by Lach-Ner. Biotin and placebo were obtained from Zentiva, a.s. Prague, Czech Republic.

2.2. Materials

Pharmaceuticals or dietary supplements, which were investigated during the experiment, are commercially available in pharmacies in the Czech Republic.

2.3. Instrumentation

Analyses were performed using an Esa Solvent Delivery System (model 582) coupled with Coulochem® III, Esa Inc. (model 5011A High Sensitivity Analytical Cell) working in an oxidation mode. Column temperature was set to 35 °C. Chromatographic software CSW 1.7 was used for data collection and processing. The following chromatographic columns were used – Zorbax SB-Aq 150 mm × 4.6 mm 3.5 μm (Agilent); Symmetry C18 150 mm × 3.9 mm 5 μm (Waters) Pathfinder 150 mm × 4.6 mm 3.5 μm (Shimadzu); BDS HYPERSIL C18

100 mm × 4.6 mm 3 μm (Thermo) and LiChrospher 60RP-select B 250 mm × 4 mm 5 μm (Merck).

2.4. Preparation of mobile phases and extraction buffer

Various mobile phases were tested during searching for the optimal separation and detection conditions. In general, aqueous and organic parts were prepared separately and mixed together in the desired ratio. Acetonitrile and methanol were used as organic solvents. The following aqueous solutions were used (in parentheses there is a way of its preparation): phosphate buffer 0.05 mol/L (3.40 g of potassium phosphate monobasic anhydrous were dissolved in 500 mL of water and then pH was adjusted with phosphoric acid to 3.0; for testing of robustness pH was also adjusted to 2.6 and 3.2), acetate buffer 0.05 mol/L (5.64 g of sodium acetate trihydrate were dissolved in 500 mL of water and then pH was adjusted with formic acid to 3.0), solution of acetic acid 0.24 mol/L pH 3 (7.0 mL of 98% acetic acid were diluted to 500 mL with water), solution of phosphoric acid 0.003 mol/L pH 3 (0.09 mL was diluted to 500 mL with water), solution of citric acid 0.001 mol/L pH 3 (0.096 g was dissolved in 500 mL of water) and solution of formic acid 0.0015 mol/L pH 3 (0.03 mL was diluted to 500 mL with water). All aqueous parts were filtered through a 0.22 μm filter before being mixed with an organic part. The following mobile phase was chosen as the most convenient for a determination of biotin in pharmaceuticals: 0.24 mol/L aqueous solution of acetic acid and acetonitrile in the ratio 85:15 (v/v) (7.0 mL of 98% acetic acid were diluted to 500 mL with water, 75 mL of acetonitrile were put into a 500 mL volumetric flask and it was filled up to the mark with acetic acid solution). The flow rate of the mobile phase 1 mL/min was constant during all analyses. The extraction buffer was prepared by dissolving 1.74 g of potassium phosphate dibasic in 100 mL of water and adjusting pH to 8.0 with phosphoric acid [21]. The use of this buffer was found as suitable because this buffer did not influence coulometric detection.

2.5. Preparation of stock solution, standard solution and solutions for linearity

Stock solution of biotin was prepared in the concentration of 600 μg/mL. The exactly weighed quantity of the compound was dissolved in water. Standard solution was prepared by diluting the stock solution with water to the concentration of 2 μg/mL. Linearity solutions were prepared in the following 12 concentration levels of 15, 30, 50, 100, 200, 400, 600, 1200, 1800, 2400, 3000 and 3600 ng/mL by diluting the stock solution with water.

2.6. Preparation of samples

Fortified samples for accuracy and precision evaluation were prepared as follows. Placebo was spiked with the stock solution to the absolute concentration of 2 μg/mL, this was considered to be 100% of the average content of biotin in tablets. Samples with an 80% and a 120% content of biotin were also prepared: 80% (1.6 μg/mL), 100% (2 μg/mL) and 120% (2.4 μg/mL). These fortified samples and real samples underwent the following procedure. Tablets were powdered and an appropriate amount corresponding to 150 μg of biotin in the sample (approximately 0.4 g) were weighed into a volumetric flask. Then 1 mL of acetonitrile and 10 mL of extraction buffer were added and the sample was placed into an ultrasonic bath for 15 min. After sonication the samples were centrifuged at 3600 rpm for 5 min. Then 1 mL of supernatant was diluted with water to 10 mL. All solutions were filtered through a 0.22 μm filter before the injection to the HPLC. The injected volume of all samples was 20 μL.

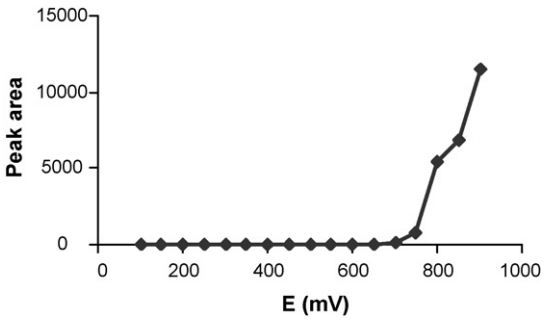

Fig. 2. The hydrodynamic voltammogram of biotin. Mobile phase: acetonitrile–0.24 mol/L acetic acid 15:85 (v/v), flow rate: 1 mL/min, guard cell 1000 mV, channel I 0 mV, channel II from 100 to 900 mV, sensitivity 5 μA.

2.7. Conditions of coulometric detection

The following potentials were set for testing of separation conditions: guard cell 1000 mV, channel I 400 mV, channel II 750 mV, sensitivity 1 μA. The hydrodynamic voltammogram of biotin was measured under the following potentials set to the cells: guard cell 1000 mV, channel I 0 mV, channel II from 100 to 900 mV, sensitivity 5 μA. Validation of the method and determination of biotin in real samples was performed under the following detection conditions: guard cell 1000 mV, channel I 600 mV, channel II 900 mV, sensitivity 1 μA.

3. Results and discussion

3.1. Chromatography and coulometric detection

The optimization procedure was performed with regard to the best possible separation as well as detection of biotin among other water-soluble vitamins commonly present in multivitamin preparations. The experiment started with Waters Symmetry C18 column. The mobile phase contained phosphate buffer and acetonitrile. The percentage of acetonitrile decreased from 25% to 10%, but the separation was either unsatisfactory or the retention times were too long. The next tested column was Agilent Zorbax SB-Aq. Binary and also ternary mobile phases were tested. Binary phases were composed of phosphate buffer and acetonitrile (from 6% to 15%). Ternary mobile phases contained methanol (methanol: acetonitrile:phosphate buffer—26:104:870) or propan-2-ol (propan-2-ol:acetonitrile:phosphate buffer—48:95:857 or 48:124:828). All mentioned ratios were v/v/v. None of the used

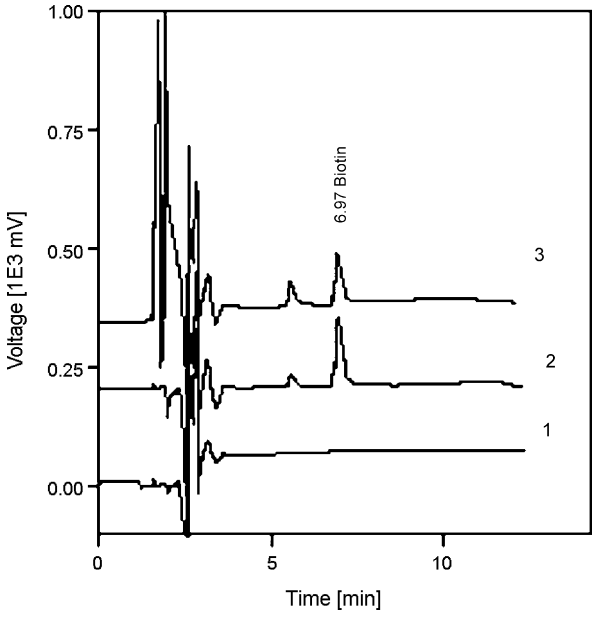

Fig. 3. Chromatograms: placebo (1), standard of biotin (2), sample of Pharmaceutical 3 (3). Column: LiChrospher 60RP-select B 250 mm × 4 mm 5 μm (Merck), mobile phase: acetonitrile–0.24 mol/L acetic acid 15:85 (v/v), flow rate: 1 mL/min, injected volume: 20 μL, guard cell: 1000 mV, channel I: 600 mV, channel II: 900 mV, sensitivity: 1 μA.

combinations provided satisfactory results. Similar results were also obtained on BDS HYPERSIL C18. The fourth employed column was Pathfinder, but the retention was too strong on this sorbent, even with 40% of acetonitrile in the mobile phase. A higher percentage of an organic solvent is not suitable for coulometric detection. The last tested column was LiChrospher 60RP-select B. Its combination with the mobile phase containing phosphate buffer and acetonitrile in the ratio 87:13 (v/v), proved to be the best. When the separation conditions were optimized, robustness was tested. Percentage of acetonitrile (9%, 11% and 15%) and pH of phosphate buffer (2.6 and 3.2) were tested. The results are tabled (Table 1). From these results, it is obvious that the percentage of acetonitrile has a much higher impact on retention time of biotin than pH of the aqueous part.

Table 1
Influence of changes in composition of the mobile phase (the percentage of acetonitrile and pH of phosphate buffer) on retention time of biotin, tailing factor and theoretical plate number (N)

Conditions	RT of biotin (min)	Tailing factor	N
Percentage of acetonitrile			
13% (standard condition)	11.2	1.172	9,954
9%	23.0	1.155	10,652
11%	15.8	1.175	10,204
15%	7.0	1.108	9,411
pH of phosphate buffer			
3.0 (standard condition)	11.22	1.172	9,954
2.6	11.23	1.119	10,135
3.2	11.21	1.141	6,844

Table 2a
Linearity parameters in the whole concentration range

Linearity parameter	Biotin
Concentration range (ng/mL)	15–3600
Regression line	$y = 11.380x + 25.104$
Slope (k)	11.380 ± 0.071
Intercept (q)	25.104 ± 11.711
Residual sum of squares (s)	29.991
Correlation coefficient (r)	0.9998
Limit of detection (ng/mL)	5
Limit of quantification (ng/mL)	15

Table 2b
Linearity parameters of curve of the working range

Linearity parameter	Biotin
Concentration range (ng/mL)	15–400
Regression line	$y = 12.956x + 5.974$
Slope (k)	12.956 ± 0.188
Intercept (q)	15.974 ± 3.549
Residual sum of squares (s)	6.189
Correlation coefficient (r)	0.9996

Table 3a
Accuracy and precision data

Sample	%	Recovery (%)	S.D.	R.S.D. (%)
Spiked placebo	80	97.1	5.436	0.376
	100	98.4	3.535	0.192
	120	100.5	4.431	0.197

As has already been mentioned, the composition of the mobile phase influences not only separation but in case of electrochemical detector also the sensitivity of detection. With regard to this fact, various aqueous solutions were tested but pH (3.0) and the percentage of acetonitrile (13%) were kept constant to preserve separation already developed. Except for the phosphate buffer used in separation experiments, the following aqueous solutions were tested to optimize the sensitivity of the assay: acetate buffer, acetic acid solution, phosphoric acid solution, citric acid solution and formic acid solution. The most sensitive detection was observed using acetic acid solution. The change of the aqueous part shifted the retention time of biotin a little. Therefore, the percentage of acetonitrile was increased to 15%. The separation of biotin from the other vitamins possibly present in multivitamin preparations was not influenced. Under the chosen conditions other B-group vitamins had, due to their positive charge, much shorter retention times than biotin thus the determination of biotin was not interfered with. The only exception was folic acid (possessing negative charge) but

Table 3b
Inter-day precision data

Pharmaceutical 1	Day 1	Day 2
Obtained amount (%)	91.16	92.84
S.D.	1.190	
R.S.D. (%)	1.294	

Table 4
Quantification of biotin in pharmaceuticals

Sample	Declared amount	Determined amount	Obtained amount (%)	S.D.	R.S.D. (%)
Pharmaceutical 1	300 μg/tbl	273.48 μg/tbl	91.16	0.432	0.474
Pharmaceutical 2	75 μg/tbl	73.49 μg/tbl	97.72	0.391	0.400
Pharmaceutical 3	150 μg/tbl	146.66 μg/tbl	98.04	0.373	0.381
Pharmaceutical 4	322 μg/g	335.52 μg/g	103.90	0.426	0.410

its electrochemical properties (high oxidation rate at 600 mV) provided its oxidation at the first channel and thereby its signal was eliminated at the second channel.

When having these conditions developed, the hydrodynamic voltammogram was measured to acquire working potentials of the detection cells for the best possible sensitivity of the method. The hydrodynamic voltammogram was measured from 100 to 900 mV set to the channel II (see Fig. 2). Even though the maximum of the voltammogram was not reached, the experiment was finished, because long duration measuring above 900 mV, which would be necessary for a validation, is not recommended on this instrument. Seeing the behaviour of the hydrodynamic voltammogram the following potentials were set on coulometric cells: guard cell 1000 mV – the highest possible potential to eliminate oxidizable compounds present in the mobile phase, channel I 600 mV – the highest possible potential to eliminate oxidizable impurities in a sample but not so high to oxidize biotin yet, channel II 900 mV – the potential suitable to oxidation of biotin. Fig. 3 shows coulometric chromatograms measured under the optimal conditions.

3.2. Validation

Linearity, accuracy, precision and robustness were tested as validation parameters. The calibration curve was constructed by quadruple injection of 12 concentration levels of linearity solutions. Table 2a summarizes linearity parameters in the whole concentration range such as equation of regression line, slope, intercept, residual sum of squares, correlation coefficient, limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were calculated as three and ten times a signal-to-noise ratio, respectively. Table 2b shows linearity parameters of the curve of the working range from 15 to 400 ng/mL. Sensitivity of 200 nA was adjusted for trying to reach a lower detection limit. Setting this sensitivity LOD of 0.25 ng/mL was reached. Accuracy was measured at three concentration levels (80%, 100% and 120%) of spiked placebo samples. Every concentration level was made in triplicate and every sample was injected three times. Using these samples, precision was tested too. Table 3a presents accuracy and precision results. Precision was also proved on real samples of Pharmaceutical 1. Inter-day precision data was measured also using Pharmaceutical 1 and the obtained data is tabled in Table 3b. Six samples of Pharmaceutical 1 were prepared and each was injected in triplicate (Table 4). Robustness was tested during optimization of separation, see Section 3.1 and Table 1.

3.3. Quantification of biotin in pharmaceutical formulations

The proposed and validated method was applied to determine biotin in pharmaceutical preparations. Two samples of each preparation were prepared and injected in triplicate. The results are summarized in Table 4. As has been mentioned above, Pharmaceutical 1 was also used to prove the precision. The obtained results agree with the labeled amounts of biotin in particular pharmaceutical preparations (Pharmaceutical 1 might be mentioned like the only exception, tablets contained only 91.2% of declared amount). No interferences were found during analyses of the pharmaceutical preparations. The results demonstrate the ability of the proposed method for quantitative determination of biotin in pharmaceuticals and dietary supplements.

4. Conclusion

A novel method for the determination of biotin using HPLC coupled with a coulometric detector has been described in this article. Such a type of detection had not been reported previously for the determination of biotin. The chromatographic and detection conditions have been optimized for this purpose. The developed method has been validated and very good linearity, sensitivity, accuracy, precision and selectivity have been proven. The validated method has been applied for determination of biotin in pharmaceutical preparations.

Using this presented method much lower detection and quantification limits were reached in comparison to previously developed methods using UV and amperometric detection.

Acknowledgement

The financial support of research project MSM No. 002 162 0822 is gratefully acknowledged.

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