Enzymatic Flow Injection Analysis of Salicylate in Blood Serum Using a Permeation Membrane and Colorimetric Detection of Carbon Dioxide

Marta M.D.C. Vila [1], Graciliano de Oliveira Neto [2] and Matthieu Tubino [3]*

[1] Centro de Ciências Biológicas, Exatas e da Saúde, Universidade de Sorocaba, Sorocaba, São Paulo, Brazil
[2] Centro de Ciências Biológicas e da Saúde, Universidade São Francisco, Bragança Paulista, São Paulo, Brazil
[3] Instituto de Química, Universidade Estadual de Campinas, CP 6154, 13084-971, Campinas, São Paulo, Brazil, Fax: +55 19 3788 3133 , e-mail: tubino@iqm.unicamp.br

Abstract

A "Flow Injection Analysis" enzymatic method is proposed for the quantitative analysis of salicylate in blood serum. The method is based on the detection of carbon dioxide formed when salicylate reacts with the enzyme salicylate hydroxylase in the presence of NADH. The solution containing the carbon dioxide formed is merged with a 1.0 mol L^{-1} sulfuric acid solution. This solution passes through a separator containing a PTFE membrane where the CO_2 permeates into a carrier with an acid-base indicator, at pH=7.0, that is conducted to a spectrophotometer. The absorbance is monitored at 580 nm. For recovery studies, concentrations of salicylate, from 1.00×10^{-3} mol L^{-1} to 5.00×10^{-3} mol L^{-1}, were introduced in ten different serum samples. The observed recoveries varied from the minimum of 95.2 % to a maximum of 110.0 %. The mean recovery ± SD is 101.3 ± 4.9 %, considering nine samples analyzed and three determinations for each one. Ten other serum samples containing salicylate were analyzed using the proposed method and the Trinder procedure. The statistical F test and *t*-Student test were applied to compare the results obtained with two independent methods. In all cases complete agreement was observed, in a confidence level of 95% (α=0.05). Considering ten samples analyzed using the proposed method and three determinations for each sample, the observed mean RSD, was 4.3 %. The limit of detection is about 0.12×10^{-3} mol L^{-1} (3×SD of the blank).

Keywords: salicylate, flow injection analysis, salicylate hydroxylase, spectrophotometry

1. Introduction

Salicylates are substances that present anti-inflammatory, analgesic and anti-pyretic properties. Among them, the acetylsalicylic acid (**aas**) is the most employed. Beyond these properties, **aas** has been used to treat and to prevent circulatory and cardiac problems [1]. In Brazil there are 29 medicines containing only **aas** as active substance. It is present yet in other 20 where it is associated with other active substances [2].

Salicylic acid is the most important metabolite of the acetylsalicylic acid and results from its hydrolysis in the body. Its determination in the blood serum is very important as the highest therapeutic level is very close to toxicity. The recommended therapeutic levels in serum are between 1.1×10^{-3} mol L^{-1} and 2.2×10^{-3} mol L^{-1} (150 to 300 mg L^{-1}). Higher values are considered toxic and concentration beyond 4.3×10^{-3} mol L^{-1} (600 mg L^{-1}) can conduct to death [3].

Considering its great availability and low price, the acetylsalicylic acid has a long history of human intoxication caused by accidental or intentional overdose or by prolonged treatments.

From the years 1940 on, it was understood that the control of the salicylate levels in blood is very important. Since then several analytical methods have been proposed for the determination of this chemical species in biological fluids and also in pharmaceutical products. Spectrophotometric, chromatographic, voltammetric, potentiometric and enzymatic analytical procedures have been developed [4]. However, in clinical analysis, the Trinder method [5] continues to be the most used. This method is very simple and has a low cost, however it is subjected to several influences, mainly of aliphatic enolic compounds [6]. The enzymatic methods have appeared as a rapid and selective analytical option for the salicylate determinations in complex matrixes [7-10].

The enzyme salicylate hydroxylase has been used for the determination of salicylate. As it can be seen in the reaction scheme (1), in the presence of molecular oxygen and of the coenzyme NADH this enzyme converts the salicylate in catechol and carbon monoxide.

$$\text{Salicylate} + \text{NADH} + O_2 + 2H^+ \rightarrow \text{catechol} + NAD^+ + CO_2 + H_2O \quad (1)$$

The use of immobilized enzymes increased in analytical chemistry from the end of the years 1980 [11]. This allowed the development of rapid, simple, low cost and specific flow injection analytical methods [12].

Permeation membranes have also been used in flow injection analysis as they allow an increasing in the selectivity of the method by the separation of certain volatile substances [13-16].

In this work it is proposed an enzymatic flow injection analytical method for the quantitative analysis of salicylate in blood serum, based on the determination of CO_2 generated in the reaction of salicylate with the enzyme salicylate hydroxylase.

2. Experimental

2.1. Reagents

Water: was distilled in a glass distiller and then deionized in a Milli Q Plus device. When necessary it was also boiled to eliminate carbon dioxide.

Salicylate solutions: a standard stock solution, 0.0100 mol L^{-1}, was prepared by dissolving 0.1601 g of sodium salicylate in 100.0 mL of phosphate buffer (0.1 mol L^{-1}, pH=7.6). From this solution, adequate dilutions were done, with the same buffer, to

* Corresponding author.

obtain the desired working concentrations.

Iron III solutions: 10 g of $Fe(NO_3)_3.9H_2O$, were dissolved in one liter of 0.10 mol L^{-1} nitric acid.

Bromocresol purple solutions: a stock solution was prepared by dissolving 0.27 g of bromocresol purple (5',5''-dibromo-o-cresol-sulfonephthaleine) in 20 mL of ethanol and diluted to 500 mL with boiled (to remove carbon dioxide) water. From this solution (1.0×10^{-3} mol L^{-1}), by dilution, was prepared the working solution (2.5×10^{-5} mol L^{-1}). The pH of this solution was adjusted to 7.0 by carefully dropping NaOH (0.02 mol L^{-1}) solution [15-16].

Phosphate buffer solutions: to prepare a 0.10 mol L^{-1} solution (pH=7.6), 11.88 g of KH_2PO_4 were dissolved in 1.0 liter of boiled water. 2.27 g of Na_2HPO_4 were dissolved in 250 mL of boiled water. 150 mL of the KH_2PO_4 solution was put in a 1000 mL volumetric flask. The volume was completed to the mark with the Na_2HPO_4 solution. The pH was measured and when necessary it was adjusted to 7.6 by carefully dropping a 0.02 mol L^{-1} NaOH solution.

Coenzyme solution: The solutions of NADH were daily prepared in phosphate buffer (0.10 mol L^{-1}, pH=7.6). 0.532 g where dissolved in 50 mL of the buffer.

Immobilization of the enzyme- 0.2 g of the glass beads were boiled in a 5% v/v HNO_3 solution, for 30 minutes, under constant agitation. Then they were filtered, with the aid of a water aspirator pump, in a Buchner funnel with glass frit, and washed with deionized water and dried in an oven at 95 °C for 2 hours. In sequence, 1.0 mL of 3–aminopropyltriethoxysilane dissolved in 19 mL of xylene were added on the glass beads. The mixture was heated at 100 °C for 3 hours under reflux. The silanized beads were filtered in a Buchner funnel with glass frit, with the aid of a water aspirator pump, and washed with xylene, 97% v/v ethanol and water, in this order. The material was then dried at room temperature for 12 hours. In sequence, it was put in a beaker with 20 mL of a 2.5 % v/v glutaraldehyde aqueous solution, during one hour. Then it was filtered in a Buchner funnel with glass frit and washed with deionized water. Ten unities of the enzyme were dissolved in 3 mL of the phosphate buffer, at 4 °C. The enzyme solution was added on the glass beads, in a beaker, and let to rest for 12 hours at 4 °C. The mixture was filtered in a Buchner funnel with glass frit and washed with cold water (4 °C) to remove the non bounded enzyme. These glass beads with the immobilized enzyme were used to construct the enzymatic reactor.

Serum samples: Blood was collected in a tube without anticoagulant. After natural coagulation the sample was centrifuged during 10 minutes at 3000 rpm. The serum was separated and kept in small tubes closed with rubber caps.

2.2. Apparatus

The FIA system shown in Fig.1, is constituted by the following parts:

Enzymatic reactor (**R**): The enzymatic reactor was constructed by packing the glass beads with the immobilized enzyme in a plastic tube (6 mm outer diameter, 3 mm inner diameter and 3 cm long). In both ends a little of glass wool was used to fix the packed enzymatic material, which corresponded to about 20 mm of the length of the tube. About 5 mm of the tube in each end were used to perform the connection to the FIA system.

Gas diffusion cell (**DC**): Similar to the cell that has been described by van der Linden [17].

Sampling valve (**V**): Has been previously described [18,19].

Sampling inlet (**S**): 20 cm of a polyethylene tube with internal diameter equal to 1 mm.

Reaction coil (**d**): 45 cm of a polyethylene tube with internal diameter equal to 1 mm.

Peristaltic pump (**P**): Ismatec mp13 GJ4.

Spectrofotometer (**SP**): Single-beam Femto model 600.

Chart recorder (**CR**): Barnstead/Thermoline, model LR92425.

Flow cuvette:1.00 cm path length quartz cuvette; volume 60 µL.

Stream of the aqueous 2.5×10^{-5} mol L^{-1} bromocresol purple solution ($\mathbf{A_1}$). Stream of the aqueous 1.0 mol L^{-1} sulfuric acid solution ($\mathbf{A_2}$). Stream of the phosphate buffer solution (0.1 mol L^{-1}; pH=7.6) ($\mathbf{A_3}$).

Pumping tubes: Ismatec two-stop tubes, blue-blue, Tygon®, internal diameter 1.65 mm.

Conducting tubes: Polyethylene tubes, internal diameter 1 mm.

Membrane: commercial polytetrafluoroethylene, PTFE, tape.

Calibration curves: calibration curves were performed using salicylate solutions in phosphate buffer. These solutions, in the range from 5.0×10^{-4} mol L^{-1} to 5.0×10^{-3} mol L^{-1}, were obtained from the above described 0.0100 mol L^{-1} salicylate standard stock solution.

2.3. Preparation of the serum samples

Serum samples were initially treated with a 0.10 mol L^{-1} solution of sulfuric acid, to eliminate carbonates and carbon dioxide, in a proportion of 1.0 mL of serum to 0.5 mL of the acid solution. Then 0.5 mL of the phosphate buffer was added to this solution. 1.0 mL of this solution was mixed with 1.0 mL of a cofactor solution in the buffer. The concentration of the cofactor, NADH, solution in the phosphate buffer, was adequate in order to obtain at least a molar relation 3:1, [NADH] : [salicylate], in the final solution to be introduced in the flow system.

3. Results and discussion

In this method the determination of salicylate in blood serum was based on the detection of the CO_2 generated in the reaction of the enzyme salicylate hydroxylase with the salicylate. The enzyme was immobilized on glass beads of controlled porosity [10] and placed into a plastic tube constituting the reactor that was integrated in the flux system as seen in Fig. 1.

The sample containing the salicylate and the coenzyme is introduced in the analytical system, in the buffer flow (phosphate 0.10 mol L^{-1}; pH=7.6), and transported to the enzymatic reactor. The formed CO_2, remains in the form of HCO_3^- due to the pH. This solution is merged with a sulfuric 1.0 mol L^{-1} solution and passed through a membrane cell where the generated CO_2 permeates to another flow containing the acid-base indicator in pH=7.0. This last solution is then conducted to the spectrophotometer where the absorbance is monitored at 585 nm. The acid-base indicator bromocresol purple changes color from yellow (λ_{max} = 419 nm) to purple (λ_{max} = 585 nm), in the pH range 5.2 to 6.8 (pK = 6.1).

The results obtained with this method were compared with those obtained with the Trinder procedure [5].

In order to optimize the method, were studied the influence of the sampling volume, of the flow rate, of the sulfuric acid concentration and of length of the reaction coil where the sulfuric acid solution is merged with the solution that comes from the reactor. The molar ratio of NADH to salicylate was fixed on 3:1 [10].

The sampling volume was studied from 150µL to 500µL. It was observed that the analytical signal increases accompanying the volume increase, in an almost linear dependence, in the range studied. To avoid long periods of analysis the volume of 250 µL was selected as it is related to an analytical signal of enough intensity.

The intensity of the analytical signal strongly depends on the flow, decreasing with the increase of the flow rate. Considering a compromise between the time of analysis and signal intensity the rate of 0.6 mL min^{-1}, per channel, was selected.

The influence on the analytical signal, of the distance **d** (reaction coil), between the T-form mixer and the diffusion cell, where the solution that comes from the enzymatic reactor merges with the 1.0 mol L^{-1} sulfuric acid solution, was studied. The signal increases almost linearly, with the increase of the length. The results obtained with **d**= 45 cm were considered adequate, considering the signal height and the analytical frequency, and therefore this length was selected to perform the analyses.

The influence of the sulfuric acid concentration was studied from 0.20 mol L^{-1} to 2.0 mol L^{-1}. The signal height increases until the acid concentration *ca.* 1.0 mol L^{-1} remaining almost constant beyond this. Considering that at 1.0 mol L^{-1} the signal presents a good intensity and that above this concentration there is not a great signal increase, this concentration was selected.

In order to test the proposed method it was applied to nine different serum samples where known concentrations of salicylate, from 1.00 × 10^{-3} mol L^{-1} to 5.00 × 10^{-3} mol L^{-1}, were introduced. Table 1 describes the results. The observed recovery varies from the minimum of 95.2 % to a maximum of 110.0 %. These results can be considered satisfactory enough for this kind of analyte and matrix.

Ten other serum samples containing salicylate were analyzed using the proposed method and the Trinder procedure [5]. Fig. 2 shows the analytical and calibration signals. The results are described in Table 2 together with those obtained with the Trinder method. The statistical F test and *t*-Student test [20] were applied to compare the results obtained with two independent methods. As it can be observed there is complete agreement in all cases, in a confidence level of 95% (α=0.05). Considering ten samples analyzed using the proposed method (three determinations for each sample), the observed mean RSD, was 4.3 %. The detection limit is about 0.12×10^{-3} mol L^{-1} (3× SD of the blank).

4. Conclusions

Considering the therapeutic concentration range of the salicylate in blood serum (1.1×10^{-3} mol L^{-1} to 2.2×10^{-3} mol L^{-1}) and the analytical results obtained, it can be concluded that the proposed method can be suggested for the quantitative analysis of salicylate in blood serum.

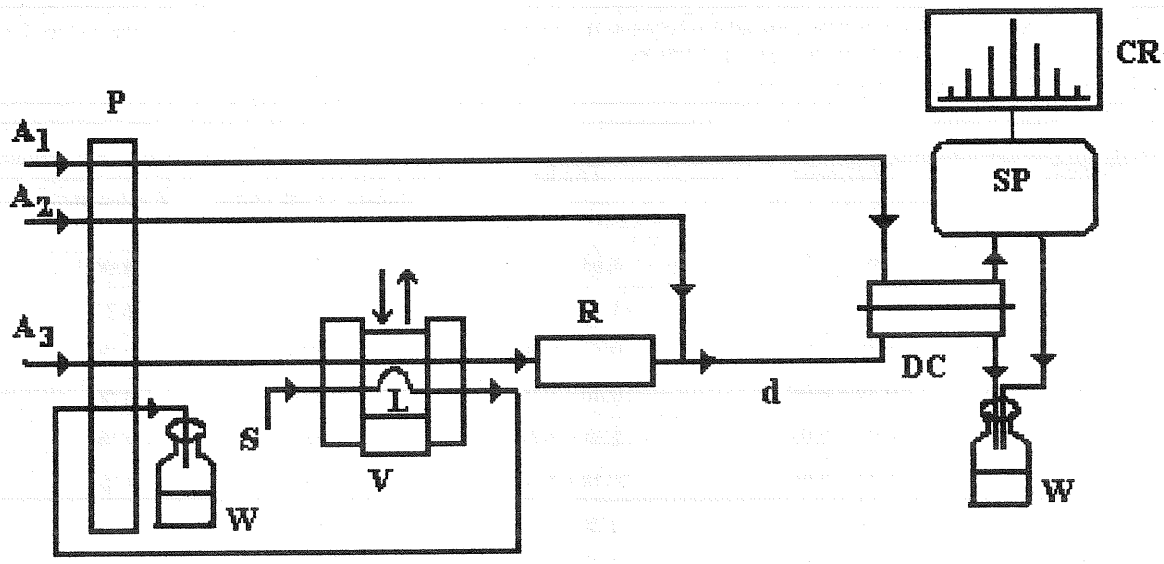

Fig. 1 Flow injection system. A_1 – 2.5×10^{-5} mol L^{-1} BCP solution at pH = 7.0; A_2 – 1.0 mol L^{-1} sulfuric acid solution; A_3 – phosphate buffer solution (0.1mol L^{-1}; pH 7.6); CR- chart recorder; d – (reaction coil) distance from the T–form mixer to the diffusion cell; DC- diffusion cell; L – sampling loop; P– peristaltic pump; R- enzymatic reactor; S- sampling inlet; SP – spectrophotometer ;V – sample valve system; W – waste. Flow rate = 0.6 ml min^{-1} per channel.

Table 1 Recovery of salicylate from blood serum [a].

Samples	Salicylate added / (10^{-3} mol L^{-1})	Salicylate found / (10^{-3} mol L^{-1})	Recovery / (%)
1	5.00	4.91 ± 0.18	98.2
2	4.50	4.49 ± 0.20	99.8
3	4.00	4.02 ± 0.05	100.5
4	3.50	3.48 ± 0.03	99.4
5	3.00	2.95 ± 0.03	98.3
6	2.50	2.38 ± 0.06	95.2
7	2.00	2.03 ± 0.11	101.5
8	1.50	1.63 ± 0.11	108.7
9	1.00	1.10 ± 0.04	110.0
		Mean recovery ± SD of the mean	101.3 ± 4.9

a- Flow rate 0.6 mL min^{-1}; sample loop 250 µL; minimum molar ratio [NADH] : [salicylate] = 3:1; temperature 25°C; buffer pH=7.6; **d**= 45 cm; H_2SO_4 concentration = 1.0 mol L^{-1}.

Table 2 Statistical Student'***t*** -test and ***F*** test comparison between the analytical values, and their respective SD, obtained by Trinder and the enzymatic methods, using. Tabulated ***t*** value is 2.776 for ν= 4* and α= 0.05***.
Tabulated ***F*** value is 19.00 for ν= 2** and α = 0.05***.

Sample	Enzymatic / (1×10^{-3} mol L^{-1})	Trinder / (1×10^{-3} mol L^{-1})	Student's ***t*** calculated	***F*** calculated
1	1.62 ± 0.04	1.63 ± 0.02	0.313	4.00
2	1.15 ± 0.05	1.12 ± 0.02	0.789	6.25
3	0.79 ± 0.05	0.81 ± 0.03	0.500	2.78
4	0.39 ± 0.06	0.40 ± 0.03	0.218	4.00
5	1.49 ± 0.03	1.53 ± 0.04	1.143	1.78
6	1.34 ± 0.06	1.38 ± 0.07	0.514	1.36
7	0.98 ± 0.01	1.00 ±0.04	0.687	16.00
8	1.13 ± 0.03	1.19 ± 0.02	2.400	2.25
9	1.12 ± 0.04	1.15 ± 0.03	0.857	1.78
10	1.86 ± 0.06	1.96 ± 0.03	2.128	4.00
Mean RSD / %	4.3	3.2		

a- Flow rate 0.6 mL min^{-1}; sample loop 250 µL; minimum molar ratio [NADH] : [salicylate] = 3:1; temperature 25°C; buffer pH=7.6; **d**= 45 cm; H_2SO_4 concentration = 1.0 mol L^{-1}. 1 cm in analytical height = 0.1 absorbance.
* ν is the degree of freedom ($n_a + n_b - 2$); n_a=3 is the number of determinations of the enzymatic method; n_b=3, is the number of determinations of the Trinder method.
** ν is the degree of freedom (n-1); n=3 is the number of determinations.
*** Confidence level.

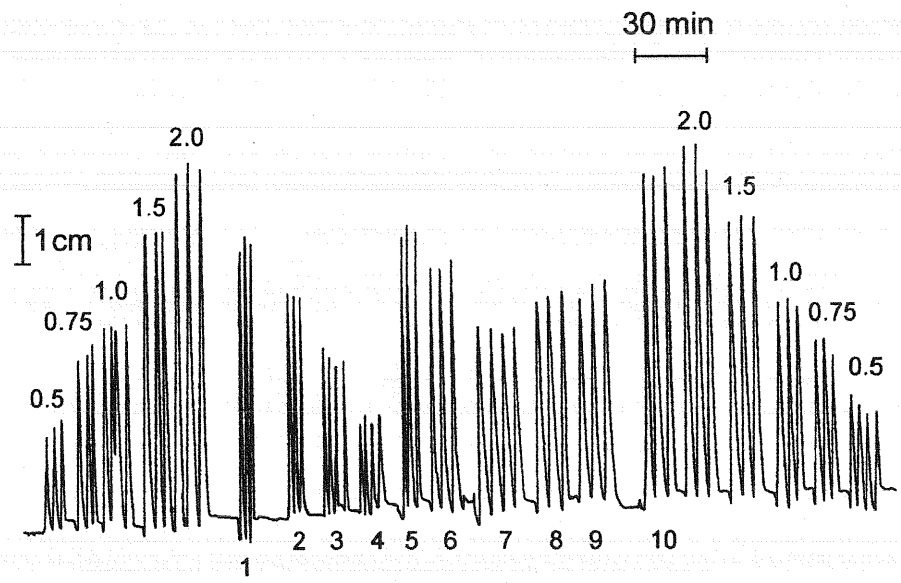

Fig. 2 Calibration and sample runs (1 to 10), for the determination of salicylate concentration in blood serum. The concentrations indicated upon the peaks are in mmol/L. The calibration curve is described by the straight line equation **h** = 0.538 + 3590 **C**, where **h** is the height signal in centimeters and **C** is the salicylate concentration in mol L^{-1}. 1.0 cm in analytical height = 0.1 absorbance. The coefficient of correlation R = 0.995.

5. Aknowlegments

The authors thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, for financial support.

6. References

[1] D .C. Payan, In *Farmacologia Básica e Clínica*, 5th ed., Guanabara Koogan, Rio de Janeiro, 1994, 368-372 p.
[2] J.M.S. Melo, *Dicionário de Especialidades Farmacêuticas*. 30rd ed., Rio de Janeiro, Publicações Científicas Ltda, 2001/2002, 1090 p.
[3] L. Yip, M. Jastremski and R. Dart, *J. Intensive Care Med.*, 1995, **12**, 66.
[4] M. M. D. C. Vila, M. Tubino and G. Oliveira Neto, *Lecta*, 2000, **18**, 9.
[5] P. Trinder, *Biochem. J.*, 1954, **57**, 301.
[6] E. S. Kang, T. A. Todd, M. Capaci, K. Schwenzer and J. T. Jabbour, *Clin. Chem.*, 1983, **29**, 1012.
[7] T.J. Moore, M.J. Joseph, B.W. Allen and L.A. Coury Jr., *Anal. Chem.*, 1995, **67**, 1896.
[8] C. Martín and E. Domínguez, *J. Pharm. Biomed. Anal.*, 1999, **19**, 107.
[9] Oliveira Neto, G., L. Rover Jr. and L. T. Kubota, *Electroanalysis*, 1999, **11**, 527.
[10] M.M.D.C. Vila, M. Tubino and G. Oliveira Neto, *J. AOAC Internat.*, 2001, **84,**1363.
[11] M. De la Guardia, *Mikrochim. Acta*, 1995, **120,** 243.
[12] B. F. Reis, *Quím. Nova*, 1995, **19**,51.
[13] V. Kubán, *Crit. Rev. Anal. Chem.*, 1992, **23**, 323.
[14] P. Pandey and R.S. Chuhan, *Prog. Polym. Sci.*,2001, **26,** 853.
[15] M. Tubino and F.G. Barros, *J. Assoc. Off. Anal. Chem.*, 1991, **74**,176.
[16] M. Tubino and F.G. Barros, *Talanta*, 1992, **117**, 917.
[17] W. E. van der Linden, Anal. Chim. Acta, 1983, **151,** 349.
[18] T. C. Rodrigues, M. Tubino, O. E. S. Godinho and G. Oliveira Neto, *Anal. Sci.*, 2001, **17**,629.
[19] M. Tubino and F. G. Barros, *Quím. Nova*, 1991, **14,** 49.
[20] K. Eckschlager, *Erros, measurement and results in chemical analysis*, Van Nostrand Reinhold, London, 1972, 111-120p.

(Received February 11, 2003)
(Accepted May 2, 2003)