

UV - SPECTROMETRIC METHOD FOR DETERMINATION OF BENZYL PENICILLIN SODIUM (PENICILLIN G) IN MODEL MIXTURES WITH DIFFERENT MILKS

Dobrina D. Tzvetkova*, Danka P. Obreshkova, Ivanka P. Pencheva

Addresses of authors: Medical University - Sofia, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Dunav str. N : 2, 1000, Sofia, Bulgaria

* Corresponding author. E-mail: dobrinkadoncheva30@yahoo.com

Summary: An investigation of 11 different milks from Bulgaria, Greece and France is carried out and parameters of milks are compared. The quantity of Benzylpenicillin Sodium (Penicillin G) in model mixtures with the mentioned milks is obtained. For the investigation of milks are used the followed methods: alkalimetric titration for determination of acidity and content of casein; lactodensitometry for measuring of density; bromthymol method for study of alkalinescence; boiling tests for examination of freshness and stability. For determination of content of Penicillin G in model mixtures with milks is applied an existing UV - spectrometric method, after hydrolysis of antibiotic with solution of hydrochloric acid (pH = 4). The experimental results for the investigated parameters acidity, content of casein, density, alkalinescence, freshness and stability correspond to the regulated standards for milk. Analytical parameters repeatability (precision) and accuracy are determined by the uncertainty of the result, in which are included: standard deviation (SD), relative standard deviation (RSD) and confidential interval ($\bar{X} \pm t.S\bar{X}$). The accuracy is determined by the degree of recovery (R).

Key words: milk-analysis; drug residues; penicillium G; spectrometry, ultraviolet

Introduction

The residues of β - lactam antibiotics in milk, obtained from animals with mastitis, may produce an allergic responses (1). The investigation of residues is conditioned by the requirements to certify the quality of milk. This problem is being realized by means of confirmation of correspondence of the eventual antibiotic residues in the milk with regulated from the European Community a highest degree of admissible antibiotic residues (2).

During the past few years Benzylpenicillin Sodium is the favorite agent for intramammary treatment of the mastitis in the veterinary practice (3). The most widespread method for analysis of Penicillin G in milk is HPLC (2, 4, 5).

Materials and methods

- I. Fresh cow's milk (not pasteurized):
Danone (Sofia - 17.05.2001; Batch. N:1222200);
Elby (Sofia - 28.05.2001; Batch. N:0512025);
Filipopolis (Sofia - 05.06.2001; Batch. N:4737261);
Formalat (Dolni Lozen - 10.06.2001; Batch. N:002532);
Home milk (Kasichene - 17.06.2001; --);
Markelli (Kasanlak - 19.06.2001; Batch. N:924200);
Zdravetz (Svishtov - 21.06.2001; Batch. N:0412002);
Triki (Greece - 13.08.2001; Batch. N:5989);
Family (Greece - 13.08.2001; Batch. N:979123);
Family light (Greece - 14.08.2001; Batch. N:854769);
Candia (France - 28.05.2001; Batch. N:002526).
- II. Drug product: Benzylpenicillin Sodium.
- III. Reagents:
 1. Distilled water, 1 % solution of phenolphthalein in 95 % ethanol, 0.1 M solution of sodium hydroxide (used in methods IV. 1. and IV. 2.).
 2. 2.5 % solution of cobalt sulphate in water, 35 % solution of formaldehyde (used in method

Table 1: Results for absorbance of hydrolyzed Benzylpenicillin Sodium and for Shovene ' s criterion for model mixtures (I - IV) with milk Danone

N	Absorbance			
	A 150	A 200	A 250	A 300
1.	0.10647	0.13911	0.18057	0.21040
2.	0.10432	0.14171	0.17651	0.21436
3.	0.10266	0.14060	0.17568	0.21946
4.	0.10683	0.13735	0.17602	0.21189
5.	0.10387	0.14464	0.17957	0.22024
6.	0.10581	0.14354	0.17387	0.21262
	Shovene ' s criterion			
	Sh C 150	Sh C 200	Sh C 250	Sh C 300
1.	0.9056	0.7519	1.3939	1.0806
2.	0.4120	0.2016	0.2067	0.1138
3.	1.4249	0.2067	0.5335	1.1296
4.	1.1245	1.4005	0.3994	0.7163
5.	0.6867	1.2765	1.0000	1.3205
6.	0.5021	0.8734	1.2486	0.5394

A 150; A 200; A 250; A 300 - absorbance of hydrolyzed Benzylpenicillin Sodium (benzylpenicillenic acid) - respectively in model mixtures I (150 µg/ml); II (200 µg/ml), III (250 µg/ml); IV (300 µg/ml) with milk Danone.

Sh C 150; Sh C 200; Sh C 250; Sh C 300 - Shovene ' s criterion for the obtained quantity of hydrolyzed Penicillin G - respectively in model mixtures I (150 µg/ml); II (200 µg/ml), III (250 µg/ml); IV (300 µg/ml) with milk Danone.

N - number of the individual measurements

IV. 2.).

3. 0.2 % solution of bromthymol blue in 95 % ethanol (used in method IV. 4.).

4. 6.81 % solution of potassium dihydrogen phosphate in water, 1 % solution of calcium chloride in water (used in method IV. 5.).

5. 95 % ethanol, solution of hydrochloric acid (pH = 4) (used in method V.).

All reagents are chemical pure and with analytical great quality.

IV. Methods for control of parameters of milk [6].

1. Alkalimetric titration for determination of acidity.

2. Method of Karunina - Stilovich - alkali-metric titration for determination of content of casein.

3. Lactodensitometry for measuring of density.

4. Bromthymol method for study of alkalence.

5. Boiling tests for examination of stability.

6. Boiling test for examination of freshness.

V. Method for determination of Penicillin G in mixtures with milks by spectrometry.

Standard preparation: Accurately weighed quantity of Benzylpenicillin Sodium (0.015g,

0.020g, 0.025g, 0.030g) is dissolved in solution of hydrochloric acid (pH = 4) and is diluted to 100.0 ml with the same solvent, to obtained respectively the standard solutions (1 - 4) with the followed concentrations of antibiotic: 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml.

Test preparation: The model mixtures (I - IV) are prepared by mixing of 1.0 ml milk with 0.015g, 0.020g, 0.025g and 0.030g Penicillin G respectively. The mixtures are treated with 95 % ethanol and are filtered. The filtrates are diluted to 100.0 ml with solution of hydrochloric acid (pH = 4), to obtained correspondingly model mixtures (I - IV) with the followed concentrations of antibiotic: 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml. After 60 min. of hydrolysis, for standards and for samples are measured at 322 nm the absorbances of hydrolyzed antibiotic (benzylpenicillenic acid) (7).

Results

I. Experimental data for parameters acidity, content of casein and density.

The obtained results for acidity of milks are: Danone (16.8°T), Elby (20.4°T), Filipopolis (18.5°T), Formalat (19.0°T), Home milk (16.2°T),

Table 2: Content of hydrolyzed Benzylpenicillin Sodium in model mixtures (I - II) with milk Danone

N	Model mixture I		Model mixture II	
	C 150	R 150	C 200	R 200
1.	151.67	101.11	197.66	98.83
2.	148.60	99.07	201.35	100.68
3.	146.24	97.49	199.77	99.89
4.	152.18	101.45	195.15	97.58
5.	147.96	98.64	205.51	102.76
6.	150.73	100.49	203.95	101.98
$\bar{X} \pm \text{RSD}$	149.56 \pm 1.56	99.71 \pm 1.55	200.57 \pm 1.93	100.29 \pm 1.93
SD	2.33	1.55	3.87	1.94
RSD	1.56	1.55	1.93	1.93
$S \bar{X}$	0.95	0.63	1.58	0.79
P [%]	99.00	99.00	99.00	99.00
t	4.03	4.03	4.03	4.03
$t \cdot S \bar{X}$	3.83	2.54	6.37	3.18
$\bar{X} \pm t \cdot S \bar{X}$	145.73 \div 153.39	97.17 \div 102.25	194.20 \div 206.94	97.11 \div 103.47
E [%]	0.64	0.63	0.79	0.79

C 150; C 200 - concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [$\mu\text{g/ml}$] - respectively for model mixture I (150 $\mu\text{g/ml}$) and for model mixture II (200 $\mu\text{g/ml}$).

R 150; R 200 - degree of recovery (%) - respectively for model mixture I (150 $\mu\text{g/ml}$) and for model mixture II (200 $\mu\text{g/ml}$).

N - number of the individual measurements

SD - standard deviation

RSD - relative standard deviation

$S \bar{X}$ - mean quadratic error

P - confidential possibility (%)

t - coefficient of Student

$\bar{X} \pm t \cdot S \bar{X}$ - confidential interval

E - relative error (%).

Markelli (18.0 $^{\circ}$ T), Zdravetz (17.6 $^{\circ}$ T), Triki (16.5 $^{\circ}$ T), Family (17.0 $^{\circ}$ T), Family light (18.0 $^{\circ}$ T), Candia (16.0 $^{\circ}$ T). The content of casein in milks is: Danone (3.20 %), Elby (4.00 %), Filipopolis (2.20 %), Formalat (2.23 %), Home milk (3.40 %), Markelli (2.75 %), Zdravetz (3.96 %), Triki (2.27 %), Family (2.64 %), Family light (2.60 %), Candia (3.02 %). The results for parameter density are: Danone (1.0302), Elby (1.0283), Filipopolis (1.0309), Formalat (1.0267), Home milk (1.0255), Markelli (1.0318), Zdravetz (1.0272), Triki (1.0274), Family (1.0296), Family light (1.0308), Candia (1.0296).

II. Quantity analysis of Penicillin G in model mixtures (I - IV) with milks.

In spectra of the placebo solution isn't exist peak at 322 nm and by this fact is confirmed the specificity of method. The linearity is studied and the concentration interval, where Beer's law is valid, is determined - 150 $\mu\text{g/ml}$ \div 300 $\mu\text{g/ml}$. For standard solutions (1 - 4) of Penicillin G the measured absorbances of benzylpenicillenic acid at 60 min. after the beginning of hydrolysis are respectively : 0.10530, 0.14076, 0.17715, 0.21539.

The experimental results for the absorbances of benzylpenicillenic acid in model mixtures (I - IV) with milk Danone (A 150, A 200, A 250, A 300) and the data for the Shovene's criterion for the obtained quantity of hydrolyzed Penicillin G in every sample are presented in table 1. The results

Table 3: Content of hydrolyzed Benzylpenicillin Sodium in model mixtures (III - IV) with milk Danone

N	Model mixture III		Model mixture IV	
	C 250	R 250	C 300	R 300
1.	254.83	101.93	293.05	97.68
2.	249.10	99.64	298.57	99.52
3.	247.93	99.17	305.67	101.89
4.	248.41	99.36	295.13	98.38
5.	253.42	101.37	306.76	102.25
6.	245.37	98.15	296.14	98.71
$\bar{X} \pm \text{RSD}$	249.84 \pm 1.43	99.94 \pm 1.43	299.22 \pm 1.91	99.74 \pm 1.90
SD	3.58	1.43	5.71	1.90
RSD	1.43	1.43	1.91	1.90
$S \bar{X}$	1.46	0.58	2.33	0.78
P [%]	99.00	99.00	98.00	98.00
t	4.03	4.03	3.37	3.37
$t \cdot S \bar{X}$	5.88	2.34	7.85	2.63
$\bar{X} \pm t \cdot S \bar{X}$	243.96 \div 255.72	97.60 \div 102.28	291.37 \div 307.07	97.11 \div 102.37
E [%]	0.58	0.58	0.78	0.78

C 250; C 300 - concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [g/ml] - respectively for model mixture III (250 $\mu\text{g/ml}$) and for model mixture IV (300 $\mu\text{g/ml}$).

R 250; R 300 - degree of recovery (%) - respectively for model mixture III (250 $\mu\text{g/ml}$) and for model mixture IV (300 $\mu\text{g/ml}$).

N - number of the individual measurements

SD - standard deviation

RSD - relative standard deviation

$S \bar{X}$ - mean quadratic error

P - confidential possibility (%)

t - coefficient of Student

$\bar{X} \pm t \cdot S \bar{X}$ - confidential interval

E - relative error (%).

for the obtained quantity of hydrolyzed Benzylpenicillin Sodium in model mixtures (I - II) and (III - IV) with milk Danone, are pointed respectively in table 2 and table 3. The content of hydrolyzed antibiotic is calculated by using the data for the absorbances from table 1. For every kind of the examined mixtures are prepared 6 samples. In table 2 and table 3 are indicated for all mixtures: N - number of the individual measurements (1 \div 6); concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [$\mu\text{g/ml}$] - respectively: C 150, C 200 - for model mixtures (I - II) (table 2); C 250, C 300 - for model mixtures (III - IV) (table 3); degree of recovery (%) - respectively: R 150, R 200 - for model mixtures (I - II) (table 2); R 250, R 300 - for model mixtures (III - IV) (table 3); P - confidential possibility (%), t - coefficient; mean

quadratic error ($S \bar{X}$); relative error (E). The results for the quantity of Penicillin G in the investigated model mixtures (I - IV) with other milks and the data about the degree of recovery are summerized in table 4 and table 5. The content of antibiotic is calculated by using the data for the absorbances.

Discussion

The obtained results show that the acidity for milks Elby (20.4° T), Filipopolis (18.5° T) and Formalat (19.0° T) is higher than 18° T and for other milks corresponds to the regulated standards (16 \div 18° T). The data for the content of casein in milks suit standard requirements (2.2 % \div 4.0 %). Parameter density for milks Home milk (1.0255)

Table 4: Degree of recovery and content of hydrolyzed Benzylpenicillin Sodium in model mixtures (I - IV) with milks

N:	$\bar{X} \pm \text{RSD}$	$\bar{X} \pm t \cdot S \bar{X}$	$R \bar{X}$ [%]	SD
Milk	E L B Y			
I	145.74 ± 1.85	142.91 ÷ 148.57	97.16	2.70
II	218.36 ± 0.44	217.36 ÷ 219.36	109.18	0.96
III	257.27 ± 0.38	256.24 ÷ 258.30	102.91	0.97
IV	307.22 ± 0.25	306.42 ÷ 308.02	102.41	0.76
Milk	FILIPOPOLIS			
I	151.81 ± 0.92	150.35 ÷ 153.27	101.21	1.39
II	195.60 ± 0.35	194.88 ÷ 196.32	97.80	0.68
III	271.03 ± 0.26	270.28 ÷ 271.78	108.41	0.71
IV	294.96 ± 0.31	293.98 ÷ 295.94	98.32	0.92
Milk	FORMALAT			
I	142.02 ± 0.17	141.76 ÷ 142.28	94.68	0.24
II	197.50 ± 0.23	197.01 ÷ 197.99	98.75	0.46
III	245.14 ± 0.54	243.75 ÷ 246.53	98.06	1.33
IV	292.89 ± 0.80	290.42 ÷ 295.36	97.63	2.35
Milk	HOME MILK			
I	147.45 ± 0.33	146.94 ÷ 147.96	98.30	0.48
II	190.40 ± 0.38	189.63 ÷ 191.17	95.20	0.73
III	251.37 ± 0.47	250.11 ÷ 252.63	100.55	1.19
IV	286.38 ± 1.25	282.60 ÷ 290.16	95.46	3.59
Milk	MARKELLI			
I	143.75 ± 0.42	143.13 ÷ 144.37	95.83	0.60
II	187.33 ± 0.35	186.64 ÷ 188.02	93.67	0.65
III	260.23 ± 0.57	258.69 ÷ 261.77	104.09	1.48
IV	301.89 ± 0.30	300.91 ÷ 302.87	100.63	0.92

\bar{X} - concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [$\mu\text{g/ml}$] - respectively for model mixtures: I (150 $\mu\text{g/ml}$); II (200 $\mu\text{g/ml}$), III (250 $\mu\text{g/ml}$); IV (300 $\mu\text{g/ml}$).

$R \bar{X}$ - degree of recovery (%) - correspondingly for model mixtures I (150 $\mu\text{g/ml}$); II (200 $\mu\text{g/ml}$), III (250 $\mu\text{g/ml}$); IV (300 $\mu\text{g/ml}$).

N - number of model mixture

SD - standard deviation

RSD - relative standard deviation

$S \bar{X}$ - mean quadratic error

t - coefficient of Student

$\bar{X} \pm t \cdot S \bar{X}$ - confidential interval

and Formalat (1.0267) is lower than 1.0270 and for other milks corresponds to the regulated standards (1.0270 ÷ 1.0320). The origin of all examined milks from healthy animals is confirmed by the obtained yellow colour by bromthymol sample. The freshness and the stability of milks are proved by the lack of coagulation, obtained from boiling tests. The Shovene's criterion: $U_{\text{Sh}} \text{ C } 150$, $U_{\text{Sh}} \text{ C } 200$, $U_{\text{Sh}} \text{ C } 250$, $U_{\text{Sh}} \text{ C } 300$ for samples is lower than standard Shovene's criterion: $U_{\text{Sh}} \text{ St } = 1.73$ (N

= 6). Analytical parameters repeatability (precision) and accuracy are determined by the uncertainty of the result, in which are included: standard deviation (SD), relative standard deviation (RSD) and confidential interval ($\bar{X} \pm t \cdot S \bar{X}$). The accuracy is determined by the degree of recovery (R) [8].

From the experimental results is confirmed, that for milks Candia, Family light, Family, Triki and Formalat with 150 $\mu\text{g/ml}$ antibiotic, the

Table 4: Degree of recovery and content of hydrolyzed Benzylpenicillin Sodium in model mixtures (I - IV) with milks

N:	$\bar{X} \pm \text{RSD}$	$\bar{X} \pm t \cdot S \bar{X}$	$R \bar{X} [\%]$	SD
Milk	Z D R A V E T Z			
I	153.97 ± 1.12	152.17 ÷ 155.77	102.65	1.72
II	191.98 ± 0.32	191.34 ÷ 192.62	95.99	0.62
III	248.24 ± 0.10	247.96 ÷ 248.52	99.30	0.26
IV	294.78 ± 0.36	293.67 ÷ 295.89	98.26	1.06
Milk	T R I K I			
I	141.74 ± 1.25	139.88 ÷ 143.60	94.49	1.78
II	199.38 ± 0.47	198.40 ÷ 200.35	99.69	0.93
III	245.89 ± 0.94	243.45 ÷ 248.32	98.35	2.32
IV	281.77 ± 0.80	279.41 ÷ 284.14	93.92	2.25
Milk	F A M I L Y			
I	141.08 ± 0.39	140.50 ÷ 141.66	94.06	0.55
II	185.45 ± 0.74	184.01 ÷ 186.88	92.72	1.37
III	232.13 ± 0.20	231.65 ÷ 232.61	92.85	0.46
IV	286.28 ± 0.45	284.93 ÷ 287.62	95.43	1.28
Milk	F A M I L Y L I G H T			
I	140.92 ± 1.33	138.97 ÷ 142.87	93.95	1.87
II	191.28 ± 0.31	190.66 ÷ 191.90	95.64	0.59
III	246.79 ± 0.38	245.81 ÷ 247.77	98.72	0.94
IV	280.12 ± 1.22	276.55 ÷ 283.69	93.37	3.41
Milk	C A N D I A			
I	137.12 ± 0.82	135.94 ÷ 138.30	91.41	1.12
II	194.41 ± 1.36	191.63 ÷ 197.19	97.21	2.65
III	243.27 ± 1.37	239.77 ÷ 246.77	97.31	3.34
IV	298.23 ± 0.47	296.77 ÷ 299.69	99.41	1.39

\bar{X} - concentration of hydrolyzed Benzylpenicillin Sodium at 60 min. after the beginning of hydrolysis [$\mu\text{g/ml}$] - respectively for model mixtures: I (150 $\mu\text{g/ml}$); II (200 $\mu\text{g/ml}$), III (250 $\mu\text{g/ml}$); IV (300 $\mu\text{g/ml}$).

$R \bar{X}$ - degree of recovery (%) - correspondingly for model mixtures I (150 $\mu\text{g/ml}$); II (200 $\mu\text{g/ml}$), III (250 $\mu\text{g/ml}$); IV (300 $\mu\text{g/ml}$).

N - number of model mixture

SD - standard deviation

RSD - relative standard deviation

$S \bar{X}$ - mean quadratic error

t - coefficient of Student

$\bar{X} \pm t \cdot S \bar{X}$ - confidential interval

recovery is lower than the recovery for standard (150 $\mu\text{g/ml}$) respectively with 8.59 %, 6.05 %, 5.94 %, 5.51 %, 5.32 %. For mixtures of 200 $\mu\text{g/ml}$ Penicillin G with milk Elby, the enhancement in relation to standard (200 $\mu\text{g/ml}$) is 9.179 %. For milks Family and Markelli with 200 $\mu\text{g/ml}$ Benzylpenicillin Sodium, the recovery is lower than the recovery for standard (200 $\mu\text{g/ml}$) with 7.28 % and 6.33 %. For mixtures of 250 $\mu\text{g/ml}$ Penicillin G with milks Markelli and Filipopolis the

enhancement in relation to standard (250 $\mu\text{g/ml}$) is 4.093 % and 8.414 %. For milk Family with 250 $\mu\text{g/ml}$ Benzylpenicillin Sodium the recovery is lower with 7.15 % than the recovery for standard (250 $\mu\text{g/ml}$).

Conclusion

All parameters of the examined milks correspond to the regulated standards. The obtained results confirm the possibilities for using the

applied UV - spectrometric method for the determination of a high level of residue of Penicillin G in milk, namely, levels above 100 mg / ml. Method is not enough sensitive to detect Benzylpenicillin Sodium at the MRL level (4 µg /kg).

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UV-SPEKTROMETRIČNA METODA ZA DOLOČANJE BENZILPENICILIN NATRIJA (PENICILINA G) V VZORČNIH MEŠANICAH RAZLIČNIH VRST MLEKA

D. D. Tzvetkova, D. P. Obreshkova, I. P. Pencheva

Povzetek: V raziskavi smo analizirali 11 različnih vrst mleka in Bolgarije, Grčije in Francije. Uporabili smo alkalimetrično titracijo za določanje kislinske stopnje in vsebnosti kazeina, laktodensitometrijo za merjenje gostote, bromtimolno metodo za proučevanje alkalinescence in vrelne teste za preverjanje svežine in stabilnosti. V proučevanih vrstah mleka smo tudi določili količino benzilpenicilin natrija (penicilina G). Pripravili smo vzorčne mešanice, antibiotik hidrolizirali s solno kislino (HCl, pH = 4) in uporabili obstoječo UV-spektrometrično metodo. Eksperimentalni rezultati kislinske stopnje, vsebnosti kazeina, gostote, alkalinescence, svežine in stabilnosti so odgovarjali predpisanim standardom za mleko. Analitska parametra ponovljivosti (natančnosti) in točnosti smo določili z merilno negotovostjo rezultatov, v kar smo vključili standardni odklon (SD), relativni standardni odklon (RSD) in interval zaupanja ($\bar{X} \pm t.S\bar{X}$). Točnost rezultatov smo določili s stopnjo izplena (R).

Ključne besede: mleko-analize; zdravila, ostanki; penicilin G; spektrometrija, ultravioletna