Department of Drug Form Technology[1], Karol Marcinkowski Medical Academy in Poznań, and Department of Applied Pharmacy[2], Institute of Technology and Chemistry of Drugs, Medical Academy in Łódź, Poland

Physicochemical and microbiological properties as well as stability of ointments containing aloe extract (*Aloe arborescens* Mill.) or aloe extract associated to neomycin sulphate

A. Kodym[1] and T. Bujak[2]

The aim of the study was to work out methods of quality assessment of ointments containing dry extract from fresh leaves of *Aloe arborescens* Mill. (Lilliaceae) and also of ointments containing both of dry extract and neomycin sulphate. The stability of the ointments, stored at 20 °C, was studied and the following criteria were considered: chromatographic analysis (TLC), pH of the ointments, the content of the substances in the dry extract converted to aloenin, the content of aloenin and aloin, anti-microbial activity of neomycin in the ointments, the size of the particles of the dry extract and of neomycin sulphate in the ointment suspension and the sterility of the ointments. After two years of storage at 20 °C, the ointments prepared with the anhydrous lipophilic base, did not change their physicochemical characteristics and neomycin in those ointments retained almost 100% of starting anti-microbial activity. Water or propylene glycol significantly decreased the stability of the biologically active substances of the dry extract in the ointments. Besides, in the ointments containing the dry extract and neomycin sulphate, the presence of water or propylene glycol induced degradation of the biologically active substances of the dry extract and a decrease in the anti-microbial activity of neomycin in the ointments. Considering the physicochemical and microbiological stability, the most advisable base for the ointments with aloe and neomycin sulphate was composed of white vaseline, liquid paraffin, solid paraffin, cholesterol.

1. Introduction

Despite the relatively well-known chemical composition and the therapeutic properties of several species of *Aloe*, there are only few aloe dosage forms that are scientifically described and registered on the world's pharmaceutical market. *Aloe* drugs are most often oral forms of drugs (tablets, solutions) of purgative activity and regulating the intestinal transit, in which the active substances are anthranoids, mostly aloine. The formulation of aloe dosage forms, e.g. ointments, is not an easy process. Problems result from chemical diversity of numerous biologically active substances contained in aloe, their chemical instability and pharmaceutical interactions with both inactive and therapeutic substances.

The therapeutic properties of fresh leaves of *Aloe arborescens* Mill. justify its use in the production of dermatological ointments of anti-inflammatory and regenerative activity that enhance the processes of healing skin lesions.

The scientific literature lacks data concerning the quality assessment of the ointments containing *Aloe* and also information about the influence of the components of the ointment base on the stability of biologically active *Aloe* substances in the ointments.

Subject of our research were ointments from the dry extract of fresh leaves and neomycin sulphate.

The dry extract obtained by spray drying [1] contains biologically active substances typical for fresh leaves [2]. As opposed to fresh juice, it is characterised by significant chemical stability because of low water content: after three years of storage at 8−10 °C the dry extract maintained the zero content of protein substances, aloenin, aloin and the majority of amino acids i.e. Lis, Arg, Glu, Ala, Ileu + Leu, Tyr and Phe [2]. According to the literature biologically active substances include lectins [3, 4], carboxypeptidase (C Pase) of bradykininase activity [5, 6], mucous substances, aloin, aloenin [7–9], aloesin and its esters: 2″-*O*-*p*-coumaroylaloesin and 2″-*O*-feruloylaloesin, aloe-emodine [2, 7], lactates of magnesium and calcium [7, 10]. Neomycin, an aminoglycoside antibiotic, which is especially effective in the infections caused by *Staphylococcus* and *Enterobacter*, was added to the ointments containing *Aloe*. It is a polar compound of a cationic character and it is only slowly adsorbed, which enables its use in the topical therapy of e.g. skin and eyes. The procedure of *Aloe* ointment production was registered in 1999 in the Patent Office of the Republic of Poland. The clinical studies of the ointments containing *Aloe* and neomycin proved their efficiency among others in therapy of various ulcerations, also those with secondary bacterial infections [11]. The ointments with the dry extract alone proved to be efficient emollients in the therapy of dry skin and its symptoms in different forms of eczema and dermatoses cured with the use of UV irradiation. Besides, they were efficient in the treatment of psoriasis and other diseases with keratogenesis disorders as a preliminary therapy or the addition to basic therapy [12]. *Aloe* substances in the ointments have anti-inflammatory properties, diminish itching, show cosmetic qualities [12] and protect against UV light [13]. The combining of the anti-inflammatory and regenerative biologically active substances of *Aloe* dry extract with antibacterial substance (e.g. neomycin) can be justified in case of bacteria induced dermatitis or inflammations accompanying mycotic and viral infections i.e. in pathological states, when applying corticosteroids is contradicted. *Aloe arborescens* applied topically can show some side effects in patients suffering from allergy, stimulating contact dermatitis. Contribution factors are phenolic compounds such as aloin, which in normal conditions is characterised by strong anti-inflammatory properties [14]. The activity mechanism is based on slowing down the degranulation of mast cells and inhibiting the release of histamine from cells [9].

The aim of the study was to work out the methods of quality assessment of the ointments containing biologi-

cally active constituents of the dry extract from the leaves of *Aloe arborescens* Mill. and also of the ointments containing both the dry extract and neomycin sulphate.

2. Investigations and results

The physicochemical features of the dry extract used in the ointments were determined according to the procedures described earlier [1]. The analytical data of the dry extract were as follows: loss after drying: $5.013 \pm 0.002\%$, pH: 5.13 ± 0.01, content (converted to aloenin): $18.331 \pm 0.002\%$, content of aloenin: $5.013 \pm 0.014\%$, content of aloin: $4.507 \pm 0.012\%$, content of nitrogen (N_2): $0.3983 \pm 0.0002\%$.

Standard substances: aloenins, aloins, aloesins, coumaroylaloesins, feruloylaloesins and aloe-emodins were extracted from the dry extract from fresh leaves of *Aloe arborescens* Mill. as described [2].

The ointment was extracted with distilled water, the eluate was concentrated to dryness, the residue was dissolved in methanol. The separation of the chemical substances contained in the methanol solution was carried out on chromatographic plates covered with silica gel. The results are presented in Table 1.

5.0 g of the ointment were extracted with 80 ml of double distilled water at 90 °C. After separation of the solidified ointment base on a Filtrak filter, the pH was measured to be 5.00–5.24. The results of determining the content of biologically active substances converted to aloenin are presented in Table 2. The precision of the method is characterised by the parameters of statistical evaluation (Table 3).

The concentration of aloin and aloenin was determined by the chromatographic-spectrophotometric method. The results of the studies are presented in Table 2.

To validate the extraction method of both chemical compounds from ointments, 6 reference ointments were prepared using the base of the composition similar to the one of the studied ointments (Table 4). Strictly defined content of the dry extract (about 3%) was inserted into the ointments. The studies show that the extraction indicator (% efficiency) of both compounds from reference ointments was very similar in spite of the differences in their base composition.

The results of the precision evaluation of chromatographic – spectrophotometric method of determining aloenin and aloin in the ointments were between $CV = 0.7\%$ and 3.2%. On the basis of the obtained data it can be concluded that the suggested method is accurate and precise.

The anti-microbial activity of neomycin in the ointments was determined by means of the cylinder-plate procedure according to P. Ph. V. The results of the studies are presented in Table 5.

The sterility of the ointment was confirmed according to P. Ph. V using thioglikolane medium (PB1) and also the medium with casein hydrolysate and soya (PB2). In the studies of the ointments containing substances that inhibit microbial growth, i.e. neomycin sulphate, bronopol and polyethylene glycol (1_A, 1_{AN}, 2_A, 2_{AN}), the membrane filtration was applied, while in the case of the ointment 3_A which does not contain those substances the direct inoculation method was used.

The size of particles of the dry extract and neomycin sulphate suspended in the ointments 3_A, 3_{AN} was determined using the projection microscope (see Table 6).

Table 1: Biologically active substances of aloe adsorbed on chromatograms of freshly prepared ointments (TLC)

R_f	Name of a biologically active substance on a chromatogram	The colour of spots in UV_{356}	
		1	2
0.35	aloesin	blue	bright blue
0.46	aloenin	blue	willow green
0.51	aloin	orange	bright yellow
0.58	coumaroyloaloesin feruloyloaloesin	violet	weakening violet
0.87	aloe-emodine	orange	pink-red

1 – an undeveloped chromatogram
2 – a chromatogram developed with the fumes of concentrated ammonia

Table 3: Statistical evaluation of the determination results of active substances in ointments (converted to aloenin) using the direct spectrophotometric method

Ointment code	Parameters of statistical evaluation	Ointment code	Parameters of statistical evaluation
1_{AN}	$\bar{x} = 0.560$ $s = 0.00476$ $S_{\bar{x}} = 0.00194$ $\mu = 0.560 \pm 0.005$ $CV = 0.85\%$	2_{AN}	$\bar{x} = 0.556$ $s = 0.00190$ $S_{\bar{x}} = 0.00077$ $\mu = 0.556 \pm 0.002$ $CV = 0.34\%$
1_{AN}	$\bar{x} = 0.515$ $s = 0.00381$ $S_{\bar{x}} = 0.00155$ $\mu = 0.515 \pm 0.004$ $CV = 0.74\%$	3_A	$\bar{x} = 0.550$ $s = 0.00285$ $S_{\bar{x}} = 0.00116$ $\mu = 0.550 \pm 0.003$ $CV = 0.52\%$
2_A	$\bar{x} = 0.528$ $s = 0.00095$ $S_{\bar{x}} = 0.00038$ $\mu = 0.528 \pm 0.001$ $CV = 0.18\%$	3_{AN}	$\bar{x} = 0.560$ $s = 0.00190$ $S_{\bar{x}} = 0.00077$ $\mu = 0.560 \pm 0.002$ $CV = 0.34\%$

$n = 6$, $f = 5$, $\alpha = 0.05$, $t\alpha_f = 2.571$

Table 2: Content of the complex of active substances converted to aloenin, aloenin and aloin in the ointments

Ointment code	Complex of substances (converted to aloenin)		Aloenin (%)		Aloin (%)		Loss (%) after two-year storage of ointments		
	1	2	1	2	1	2	Complex	Aloenin	Aloin
1_A	0.560 ± 0.005	0.356 ± 0.002	0.165 ± 0.003	0.164 ± 0.003	0.149 ± 0.004	0.025 ± 0.001	36.61	0.61	83.22
1_{AN}	0.515 ± 0.004	0.313 ± 0.002	0.158 ± 0.004	0.157 ± 0.006	0.143 ± 0.003	0.018 ± 0.001	39.23	0.64	87.42
2_A	0.528 ± 0.001	0.309 ± 0.004	0.150 ± 0.005	0.123 ± 0.003	0.139 ± 0.004	0.041 ± 0.002	41.48	18.00	70.51
2_{AN}	0.556 ± 0.002	0.302 ± 0.003	0.151 ± 0.004	0.120 ± 0.004	0.139 ± 0.004	0.024 ± 0.002	45.69	20.53	82.61
3_A	0.550 ± 0.003	0.549 ± 0.004	0.147 ± 0.002	0.146 ± 0.001	0.134 ± 0.002	0.132 ± 0.001	0.19	0.68	1.49
3_{AN}	0.560 ± 0.002	0.559 ± 0.002	0.151 ± 0.002	0.151 ± 0.001	0.140 ± 0.001	0.139 ± 0.001	0.18	0.00	0.72

1 – freshly prepared ointments
2 – ointments after two-year storage

Table 4: Composition of the ointments containing aloe and the ointments containing aloe and neomycin

Ointment bases		Aloe ointments			
No.	Ingredients	Ointment code	Content (%)		Physico-chemical type of the ointment
			Dry extract	neomycin	
1	Anhydrous eucerine, white vaseline, liquid paraffin, distilled water, bronopol	1_A	3.00	–	Emulsion w/o
		1_{AN}	3.00	0.5	
2	White vaseline, liquid paraffin, solid paraffin, propylene glycol, cholesterol	2_A	3.00	–	Emulsion w/o
		2_{AN}	3.00	0.5	
3	White vaseline, liquid paraffin, solid paraffin, cholesterol	3_A	3.00	–	Suspension
		3_{AN}	3.00	0.5	

Table 5: Anti-microbial activity of neomycin in the ointments containing *Aloe*

Ointment code	Activity in comparison to standard (%)	
	1	2
1_{AN}	99.37	60.49
2_{AN}	99.38	81.36
3_{AN}	99.37	99.38

1 – freshly prepared ointments
2 – ointments after two-year storage

Table 6: Size of the particles of the dry extract and neomycin sulphate in the suspension of the ointments: 3_A and 3_{AN}

Categories of particle size (μm)	Content of particles in the ointment (%)			
	Dry extract		Neomycin sulphate	
	1	2	1	2
1–5	69.2	51.6	92.0	86.5
5–10	22.0	31.6	5.2	3.8
10–20	4.8	11.0	2.8	9.7
20–30	4.0	5.8	–	–

1 – freshly prepared ointments
2 – ointments after two-year storage

3. Discussion

Both the freshly prepared ointments and those after a two-year storage were sterile. On the chromatograms of the freshly prepared ointments visualised by UV, there were present the spots of the main chemical constituents characteristic for the fresh leaves and the dry extract from the fresh leaves i.e. aloenin, aloin, aloesin, coumaroylaloesin and feruloylaloesin and also aloe-emodine (see Table 1). After two years of storage, the UV chromatograms of the ointments prepared in the anhydrous, lipophilic base (series 3_A and 3_{AN}) did not change. On the contrary, the chromatograms of the ointments containing water or propylene glycol (series 1_A, 1_{AN} and 2_A, 2_{AN}) were characterised by a significant decrease in fluorescence of the aloin spots and the disappearance of spots of coumaroylaloesin and feruloylaloesin.

In the ointments of the series 3_A and 3_{AN}, the dry extract and neomycin were suspended in the ointment base in the form of micronised particles and after 2 years of storage there appeared to be little agglomeration (see Table 6).

The pH of the freshly prepared ointments was similar to the natural pH of the skin and was in the range of 5.00−5.13. After a two-year storage the pH did not change significantly. In the complex of *Aloe* biological substances there are chemical compounds that are characterised by the absorbance at the wavelength $\lambda = 310$ nm i.e. aloenin, aloin, aloesin, coumaroylaloesyn, feruloylaloesin and aloe-emodine. Our studies show that in the complex of aloe active substances the main component is aloenin, which makes approx. 28.9% of the complex and aloin 26.1%. In the freshly prepared ointments the content of this complex (converted to aloenin) was 0.6%. After a two-year storage there was a significant decrease in the content of the complex of the active substances and aloin in the ointments containing water (series 1_A, 1_{AN}) or propylene glycol (series 2_A, 2_{AN}). Aloenin in the same ointments was much more stable than aloin. The presence of water in the ointment base did not cause the degradation of aloenin [16] while the presence of glycol decreased its stability to a lesser degree than in the case of aloin. After two years of storage, in the ointments that did not contain water or propylene glycol (series 3_A, 3_{AN}) the loss of the complex of the active substances (converted to aloenin) and the decrease of the content of aloenin and aloin in the ointments were not detected.

The studies of the anti-microbial activity of neomycin showed that it was considerably stable in the ointment prepared with the lipophilic and anhydrous base and not containing propylene glycol (series 3_{AN}). After two-years of storage, neomycin retained almost 100% of its original anti-microbial activity (see Table 5). At the same time, in the ointments of the series 1_{AN} and 2_{AN} in which chemical compounds of *Aloe* and neomycin sulphate were dissolved in the hydrophilic phase of the emulsion of the water-in-oil type, a significant decrease of the anti-microbial activity of neomycin was detected. In the ointment of the series 1_{AN}, the activity in the relation to the standard sample decreased from 99.4% to 60.5%. The loss in the activity of neomycin was also detected in the ointment with glycol (series 2_{AN}) i.e. from 99.38% to 81.36% (see Table 5).

The loss of the anti-microbial activity of neomycin in the ointments with *Aloe* of the series 1_{AN} and 2_{AN}, which appeared in parallel with the change of the colour from light green-yellow to dark brown could be explained by pharmaceutical interaction. It could proceed similarly as the Maillard reaction i.e. between a sugar and an amino acid. Neomycin as an aminoglycoside would perform the role of the sugar and react with amino acids and proteins (lectins) of the dry extract [2]. The conditions in which this interaction can proceed in the ointments with *Aloe* and neomycin demand the presence of water and propylene glycol while in the ointment that did not contain those substances the interaction did not happen. It is confirmed by the unchanged colour of the ointment of the series 3_{AN} after two years of storage.

On the basis of the studies it can be concluded that the presence of a pharmaceutical reaction between chemical

substances of *Aloe* and neomycin sulphate depends not only on the presence of water or propylene glycol but also on the concentration of interacting components. In the eye drops containing the liquid extract of fresh leaves and neomycin sulphate the pharmaceutical interaction did not occur [17], because the concentration of biologically active substances converted to aloenin and aloin was ten times lower in the eye drops than in the ointments.

Considering the physicochemical and microbiological stability, the most advisable medium for ointments containing *Aloe* and neomycin sulphate turned out to be the anhydrous, lipophilic one composed of white vaseline, liquid paraffin, solid paraffin, cholesterol (Table 4).

The proposed analytical methods can be used in the quality assessment of the ointments containing either aloe or aloe and neomycin, both freshly prepared and during their storage, and show the required accuracy and precision.

4. Experimental

4.1. Material for investigation

Ointments containing the dry extract of fresh leaves and ointments containing both the dry extract and neomycin sulphate, prepared in aseptic conditions on the bases presented in Table 4. The dry extract from fresh leaves was prepared in the following way [1]: extraction of leaves' pulp with the distilled water of the temperature 20 °C (dynamic maceration), filtration of the extract, condensation in a vacuum evaporator at the temperature 50–60 °C and pressure 0.8–0.9 kg/cm^2 to the content of dry residue about 5%, spray drying of the condensed extract (the temperature of air inlet 160 °C, of the outlet 90 °C).

4.2. Reagents

Methanol pure p.a. (Przedsiębiorstwo Chemiczne "Odczynniki", Lublin, Poland), rectified 95% spirit (Polmos, Poznań, Poland), ethyl acetate pure p.a. (P.P.H. "Polskie Odczynniki" Gliwice, Poland); DC – Fertigplatten Kieselgel 60 ohne Fluoreszenzindikator (20 × 5 cm) (Merck).

4.3. Apparatus

Spectrometer Specord UV-Vis M40 Carl Zeiss Jena, pH-meter N-512 Mera Elmat, UV lamp Emita VP−60, projection microscope MP3 520 Warszawa, apparatus for membrane filtration (Sartorius).

4.4. Methods

4.4.1. TLC analysis

2.0 g of each ointment were extracted with 80 ml of three-times distilled water at 90 °C. After cooling, the solidified ointment base was separated on a Filtrak 389 filter and the eluate was concentrated under reduced pressure at 75 °C to dryness. The residue was dissolved in methanol. The methanol solution was transferred to a 10 ml volumetric flask and filtrated through Filtrak 389 filter. On the start line of chromatographic plates, ointment solution (0.4–0.5 ml) was applied linearly with a microsyringe. The chromatograms were developed in the system: ethyl acetate−methanol−water (100 : 16,5 : 13,5) [15]. After drying at room temperature, the plates, both undeveloped and developed with the fumes of concentrated ammonia, were visualised at UV_{356}. Simultaneously, the chromatograms of reference substances were prepared by applying linearly 0.08 ml of a reference solutions (0.1%).

4.4.2. Spectrophotometric content determination

Ointment (0.5 g) was extracted with 100 ml of distilled water at 90 °C. After cooling and solidifying of the ointment base, the eluate from the ointment was filtered to a 100 ml volumetric flask. The absorbance of the solution was measured at $\lambda = 310$ nm against distilled water as a blank sample. The percentage content of the complex of substances (converted to aloenin) was calculated from the eq. (1).

$$x = \frac{E \cdot 100}{A^{1\%}_{1cm} \cdot a} \quad (1)$$

x − content of the complex of substances (converted to aloenin) (%), E − absorbance, $A^{1\%}_{1cm} = 221.535 \pm 2.234$ (100 cm$^2 \cdot$ g^{-1}) − the aloenin extinction coefficient determined by the direct spectrophotometric method [16], a − the weighted amount of the ointment (g).

4.4.3. Chromatographic-spectrophotometric content determination

The preparation of the sample solution of the ointment, the application at the TLC plates, chromatographic separation and visualisation of studied substances at the chromatograms in UV_{356} was performed as described above for the TLC analysis. The layers of gel with adsorbed compounds were transferred to separate test tubes and eluted with 760 g/l concentration of ethanol. Then, the gel was separated by centrifugation (95 °C). The absorbance of the solutions was measured at the spectrophotometer (1 cm, $\lambda = 310$ nm (aloenin) or $\lambda = 300$ nm (aloin)) against water as a blank sample. The concentration of aloin and aloenin was calculated from eq. (2).

$$x = \frac{6 \cdot E \cdot 10}{a \cdot b \cdot A^{1\%}_{1cm}} \quad (2)$$

x − content of aloenin or aloin in the ointment (%), E − absorbance, 6 − volume of ethanol used for the extraction of the gel layers (ml), 10 − volume of the flask with the sample solution (ml), a − amount of the ointment (g), b − volume of the sample solution applied at the TLC plate (ml), extinction coefficients determined by chromatographic-spectrophotometric method (1): $A^{1\%}_{1cm}$ (aloenin) = 166.626 ± 12.115 (100 cm$^2 \cdot$ g^{-1}), $A^{1\%}_{1cm}$ (aloin) = 155.187 ± 0.246 (100 cm$^2 \cdot$ g^{-1}).

4.4.4. Anti-microbial activity of neomycin sulphate

For the assay, 1.0 g of the ointment was taken, filled up with emulsion base PJ4 and heated on a water bath at 45 °C for 15 min. The standardised cylinders were installed on Petri dishes with PA4 medium inoculated with a test microorganism (*Bacillus pumilus* NCTC 8241). Then, 1.0 g of the ointment and the standard solution were introduced into those cylinders. The dishes were left for 2 h for the diffusion of antibiotic solution into the medium and after that, for 18 h in the incubator at the temperature 37 °C. Afterwards, the diameters of the areas of inhibited growth of the test bacteria were measured and they were compared with the standard.

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Dr. hab. farm. Anna Kodym
ul. Grunwaldzka 6
60–780 Poznań

Dr. farm. Teresa Bujak
ul. Muszyńskiego 1
90–151 Łódź
Poland