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Research Article

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Development and Validation of HPLC Method for the Simultaneous Determination of Dexamethasone sodium phosphate and Prednisolone acetate in Injectable Veterinary Suspension

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Abstract Dexamethasone sodium phosphate and prednisolone acetate are corticosteroids, both the drugs are available in a veterinary injectable suspension dosage form as DMSP 3.291 mg per ml and PA 7.923 mg per ml. The chromatographic separation was carried out on a reversed-phase C18 column (250mm X 4.6 mm, 5µm) in isocratic mode using acetonitrile: phosphate buffer 0.1% (50:50), at pH 3.0 adjusted with diluted orthophosphoric acid as the mobile phase at a flow rate of 1.0 ml/min. Retention times of DMSP and PA were 2.25 min and 4.50 min respectively. Quantification was carried out with UV detector at 254 nm. The linearity of DMSP and PA was in the range of 10.528µg/ml-23.1616µg/ml and 13.392µg/ml-24.106µg/ml respectively. A simple high-performance chromatography method was developed to the simultaneous determination of dexamethasone sodium phosphate and prednisolone acetate. The developed method is economical in terms of the time taken and the amount of solvents consumed for each analysis. The method was validated as per ICH and successfully applied to the simultaneous determination of dexamethasone sodium phosphate and prednisolone acetate in bulk and pharmaceutical dosage forms.

Keywords Dexamethasone sodium phosphate; Prednisolone acetate; RP-HPLC; Validation

Introduction

Corticosteroids are steroid hormones that naturally occurring in the adrenal cortex and take part in various physiological functions such as they decrease bump, inflammation, burning irritation and, allergic reactions in the body. In general, metabolism of carbohydrates, catabolism of protein, inflammatory state regulation, stress, and immune reactions, electrolytes balance in the body. Man-made corticosteroids are similar to the naturally existing steroids and have a stronger effect. Generally, steroids are used in veterinary as well as in human medicines and can also be used as growth promotors of the hormones illicitly. Corticosteroids belonging to group A substances have anabolic effects hence not allowed to using in food-producing animals. However, some synthetic corticosteroids can be used legally in the EU legislation. In Denmark, dexamethasone and prednisolone can be used lawfully for food-producing animals [1].

Dexamethasone is a synthetic glucocorticoid that has an anti-inflammatory effect. It is usually recommended in veterinary clinically as a single active ingredient or in a mixture with other active ingredients for the treatment of breathing, stomach, and intestine diseases. This compound is also recommended and used illegally as a growth

promoter [2]. It is mostly recommended for the treatment of rheumatic, dermal diseases, allergies, chronic lung diseases, croup, and brain pumps with a combination of antibiotics for tuberculosis [3].

Dexamethasone's history was that it is manufactured first in 1957 [4]. It was used for medicinal purposes in 1958 [5]. It is included in the essential medicines list of WHO, hence a very effective and harmless drug required for the human health body system [6].

Dexamethasone a synthetic corticosteroid is usually derived from cortisol (hydrocortisone) and also identified as 1-dehydro-9α-fluoro-16α-methyl hydrocortisone or as 9α-fluoro-11β, 17α, 21-trihydroxy-16α-methylpregna-1, 4-diene-3, 20-Dione. The chemical structures of dexamethasone and dexamethasone sodium phosphate are shown in Figure 1 and Figure 2 [7].

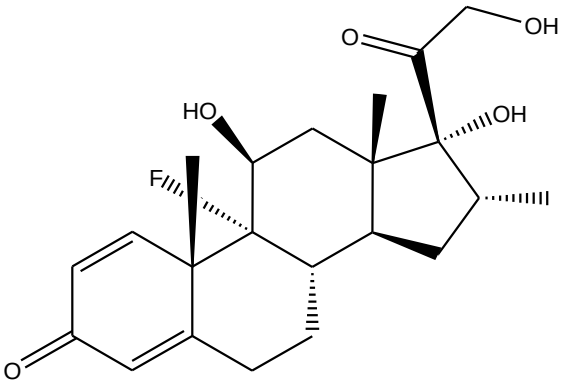

Figure 1: Dexamethasone

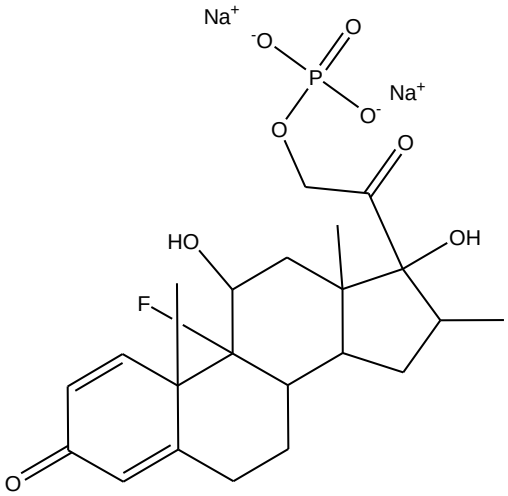

Figure 2: Dexamethasone sodium phosphate

Dexamethasone has an anti-inflammatory action and mostly recommended for the treatment of rheumatoid arthritis and bronchial spasm [8]. Due to immune disorder, there is a decrease in the number of platelets then a condition known as Idiopathic thrombocytopenic purpura occurs. In this condition, daily 40 mg of dexamethasone for 4 days is recommended and can be taken in the fourteen-day cycle. It is doubtful that in this situation whether dexamethasone gives better results than other glucocorticoids [9]. It is also used in small quantities before and after the surgery of teeth. Dexamethasone is also recommended in the treatment of children croup; only one dose is sufficient to moderate the inflammation in the lungs and also recover the breathing system. For treating plantar fasciitis it is injected into heal, but sometimes mixing with triamcinolone acetonide. Its high doses are very effective against allergic anaphylactic shock. Mostly used in certain ophthalmic solution after surgery of the eye, a spray of the nose, and also for ear drops it can be formulated with anti-fungal and antibiotics drugs. FDA approved that dexamethasone can be used as intravitreal steroid implants to cure the ocular diseases like central retinal vein occlusion, uveitis, and occlusion of central retinal vein [10]. In combination with antibiotics dexamethasone is also used for the treatment of severe endophthalmitis (an interior inflammation of the eye) [11]. To decrease the inflammatory reaction of the myocardium, dexamethasone is used to treat transvenous screw-in cardiac pacing. The steroid is released into the myocardium as the screw extended. Normally, 1.0 mg quantity of steroid is present in a lead tip. In the case of bacterial meningitis, dexamethasone may be used before antibiotics. It acts as an anti-inflammatory agent by reducing hearing impairment and neurological destruction [12].

Chemotherapy is a technique used to treat a cancer patient. In this medical technique, dexamethasone is used to minimize the certain side effects in the ant-tumor treatment. It increases the antiemetic effect of setron such as ondansetron [13]. This interaction mechanism is not well clear but it has been considered that this effect may be due to the inhibition of prostaglandin formation, and as the result of anti-inflammatory and immunosuppressive effects [14]. Dexamethasone is used in the brain tumor to reduce the edema growth which could finally affect the structure of the brain. It is administered during spinal cord tumors. Due to its chemotherapeutic action, it can be used in many hematological malignancies, particular for the treatment of multiple myelomas (MM), known as plasma cell

myelomas, in this case, dexamethasone may be used as alone or combined with other therapeutic drug substances such as lenalidomide, thalidomide bortezomib [15].

Dexamethasone is used in rare cases of glucocorticoid resistance disorder [16, 17]. It is usually recommended when adrenal insufficiency and Addison's disease have been observed in the patients. It is used in congenital adrenal hyperplasia in many old adolescents and adults who are unable to produce ACTH. Usually, it is advised to take at night [18]. In order to promote the growth of the fetus's lungs for those women who are at the risk of delivering prematurely. It happens due to low birth weight [19]. It is also used during the pregnancy as off-label in the treatment of congenital adrenal hyperplasia (CAH) in female pregnancy. Some physical abnormalities take place like ambiguous genitalia due to CAH. It was observed that early prenatal CAH treatment reduces certain CAH indications but it prevents the congenital disorder later. It is known that around one in ten of women fetus is at the risk and adverse effects also to be noted from fetal CAH treatment. In Sweden, this practice has been discontinued when one out of five cases are affected by the adverse effects [20]. Dexamethasone is used for brain edema as well as pulmonary edema treatment. High-altitude cerebral edema (HACE), and high-altitude pulmonary edema (HAPE) diseases can be treated by it. It is usually taken up by mountain climbers to help the difficulties of altitude disease [21]. Dexamethasone is also used against nausea and vomiting in the patients whose surgery and post-operative pain of epineural and spinal is prevented with opioids. [22]. It has been found that the combination of dexamethasone with other drugs like setron more effective for the treatment of postoperative vomiting and nausea [23]. Dexamethasone is also used for sore throat treatment its single dose or other steroids more effective for the improvement of sore therapy [24]. The authentic side effects of the dexamethasone are not observed but some adverse effects based on the related corticosteroids adverse effects as skin acne, the vertigo of ear and brain, sleeping insomnia, increase of weight, amnesia, headache, mental euphoria, eye cataract, increased risk of infection, blood pressure, nausea, abnormal skin healing, depression, nausea, heartburn, confusion, and increased intraocular pressure. contraindications of dexamethasone are diabetes mellitus, hyperglycemia, hypothyroidism, and inactive tuberculosis, high cholesterol, cataracts, muscle problems, high blood pressure, ulcer of the stomach, osteoporosis, measles, untreated system fungal infection [25, 26].

Dexamethasone sodium phosphate is a very discriminating glucocorticoid commonly used in ocular inflammation. Its chemical name is 9- fluoro-11b, 17, 21-trihydroxy-16α- methylpregna-1, 4- diene3, 20-dione 21-(dihydrogen phosphate) disodium salt [27]. It is anti-inflammatory and immunosuppressant drug used in the treatment of inflammation, allergy, rheumatic disease, an endocrine disorder, dermal problems, and autoimmune diseases. Dexamethasone sodium phosphate also used to treat oncology patients, including those undergoing chemotherapy. Physically, it is white to creamy white powder, soluble in water, moisture-absorbing character, and its solution has basic properties. Dexamethasone and its derivatives (dexamethasone sodium phosphate, and dexamethasone acetate, etc.) are synthetic glucocorticoids. Dexamethasone and tobramycin combination is mostly used to treat cerebral edema [28].

Prednisolone is a synthetically manufactured drug acts as an immunosuppressant and affects all of the immune systems. Its chemical name is 11, 17- dihydroxy-17-(2-hydroxyacetyl)-10, 13-dimethyl-7, 8, 9, 11, 12, 14, 15,16-octahydro-6H cyclopenta[a]phenanthren-3-one [29]. It is a prominent corticosteroid used to treat many different types of long-lasting and serious disorders, including congenital adrenal hyperplasia, arthritis, breathing diseases, skin allergic diseases, liver diseases, cardiac, infections, some hematological, neurological, metabolic, gastric, and intestinal disease as well as malignant disease and many inflammatory diseases [30-32]. It has a molecular formula $C_{21}H_{28}O_5$. It consists of 21 carbon atoms and 4 rings, three six-carbon rings represented by A, B, C, and D as shown in Figure 4. It exists as anhydrous forms or in hemihydrate. Its molecular weight is 360.45. It is a synthetically produced glucocorticoid and derivative of cortisol. It is an active metabolite used for the treatment of liver failure [33]. In 1955, Prednisolone was introduced and approved for medication [34]. In different disorder conditions such as high level of blood calcium, rheumatoid arthritis, adrenocortical deficiency, inflammation of the skin, bronchitis, multiple sclerosis, prednisolone is usually recommended to treat them. It is administrated through the mouth, intravenous, as a cream for skin, and drops for the eye.

Prednisolone is a prominent glucocorticoid and has low mineralocorticoid activity. The sterile ophthalmic suspension of prednisolone is used in the treatment of an extensive range of inflammatory and autoimmune abnormalities [35] such as uveitis, asthma, [36] urticaria, rheumatoid arthritis, angioedema, ulcerative colitis, pericarditis, multiple-sclerosis [37], and pyoderma gangrenosumis. It is also used to treat eye swelling, eye redness, eye itching, and allergic conditions of the eye [38]. It has been discovered that bacterial keratitis is also treated by prednisolone. It is also used for allergic conditions, as an immunosuppressive for organ transplants [39]. It is taken in a low dose in case of adrenal insufficiency such a disease is known as Addison‘s disease [40, 41]. The chemical structures of prednisolone and prednisolone acetate are shown in Figure 3 and Figure 4 [42].

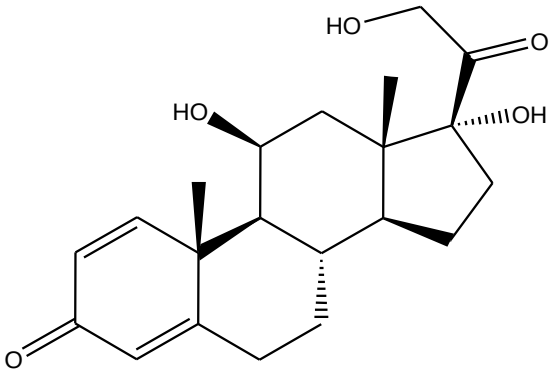

Figure 3: Prednisolone

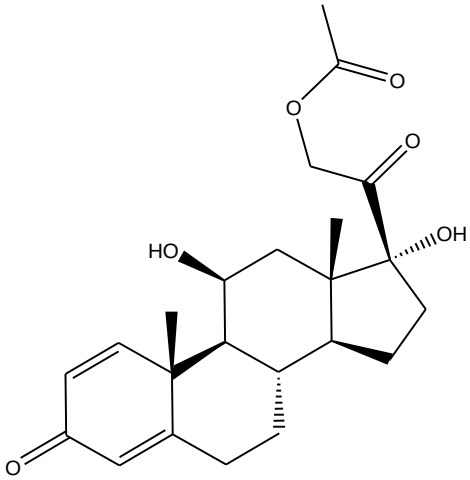

Figure 4: Prednisolone acetate

Experimental

Materials & Methods

Chemicals

Pure Dexamethasone sodium phosphate (origin China) was kindly supplied by Selmore Pharmaceuticals (Pvt.) Ltd, Lahore-Pakistan, its purity was certified to be 100.2% on an anhydrous basis and Prednisolone acetate (origin China) was also kindly supplied by Selmore Pharmaceuticals (Pvt.) Ltd. Lahore-Pakistan, its purity was certified to be 100.8% on an anhydrous basis. The sample of Penacort injectable suspension was purchased from the commercial market. Each ml of the sample contains 3.291mg of Dexamethasone sodium phosphate and 7.923mg Prednisolone acetate.

Methanol (HPLC grade) and Acetonitrile (HPLC grade), Distilled water obtained from distillation water plant (Saturn Pharma, Lahore- Pakistan), Sodium dihydrogen phosphate (AR Grade Merck), Ethanol 96% (Sigma-Aldrich), Methylene chloride (AR Grade) glacial acetic acid (AR Sigma -Aldrich), Sodium hydroxide (AR Grade Sigma-Aldrich), Acetic acid (AR Grade), Hexane (AR Grade), Perchloric acid (AR Grade), Holium oxide (AR Grade), Toluene (AR Grade), Sulphuric acid (AR Grade) and buffers pH-4.0, and pH -7.0 (AppliChem, Germany) were obtained from the Quality Control Laboratory of Saturn Pharmaceuticals (Pvt.) Ltd. Lahore-Pakistan.

Apparatus

Beaker (50 ml), Beaker (100 ml), Beaker (250ml), Beaker (500 ml), Beaker (1000ml), Funnel, Glass rode, Test tubes, Tripod stand, Measuring cylinder (500 ml, 100 ml, 50 ml), Bulb pipettes (1ml, 2 ml, 5 ml 10 ml), Graduated Volumetric flasks (2000ml, 1000ml, 500ml, 250ml, 50 ml, 25 ml), Graduated pipettes (1 ml, 2 ml, 5ml, 10ml), Spatula, Reagent bottles, Whatman filter papers, Polyacrylamide filter 42 mm of 0.45µm (Sartorius Germany), Glass micro syringe 20µl, C18 Column 50mmx4.6µm, 5µm (Suplco, Germany).

Equipment

Incubator (Germany), UV- VIS Spectrophotometer (UV−VIS 1800, Shimadzu, Japan), HPLC (LC −10 AS Shimadzu, Japan & PG Instruments, UK), (FTIR- Carry 600, Agilent), Sartorius Precision Balance (A200S, Germany). Oven Memmert (Germany), Ultrasonic bath (Germany), Magnetic Stirrer (USA), Karl Fischer apparatus, Metrohm (USA), Digital polarimeter (China), pH meter (Henna), and Glass Filtration Assembly (Germany).

Mobile phase preparation

The mobile phase was prepared by mixing, acetonitrile and 0.1% v/v NaH_2PO_4 (50:50) and filtered through a filter of 0.45 µm pore size before use and degassed in Ultrasonic bath in the Quality Control Laboratory of Saturn Pharmaceuticals (Pvt.) Ltd, Lahore, -Pakistan.

Buffer preparation

1.0 g of disodium hydrogen phosphate was dissolved in one-liter distilled water and pH-3.0 adjusted with diluted orthophosphoric acid/ NaOH.

Diluent preparation

The mobile phase was used as diluent.

Filtration & sonication of the mobile phase

The mobile phase was filtered through 0.45µm pore size filter of 42 mm by glass filtration assembly and sonicated the filtered mobile phase in a 250 ml glass bottle for 5-10 minutes and preserved in an airtight flask.

Standard preparation

Accurately weighed 16.54 mg of dexamethasone sodium phosphate working standard and 17.0 mg of Prednisolone acetate working standard was transferred into 50 ml volumetric flask, dissolved well in the diluent and diluted to volume 50 ml with the same solvent. Mixed well and sonicated for 5 minutes. Then 5.0 ml was diluted to 50 ml in a volumetric flask with the mobile phase (diluent).

Test sample preparation

2.0 ml sample was transferred into a 50 ml volumetric flask and cleaned the pipette with the mobile phase (diluent) diluted up to mark with the same solvent. 5.0 ml was diluted to 50ml with the mobile phase and sonicated it for about 5 minutes.

Setting conditions for HPLC system

The following chromatographic conditions were set during this study.

Mobile phase flow rate: 1.0 ml per minute

Column specifications: C18 (250mmx 4.6mm, 5µm)

UV detection: 254 nm

Run time: About 10 minutes

The retention time of dexamethasone sodium phosphate: About 2.5 minutes

The retention time of Prednisolone acetate: About 4.5 minutes

Results and Discussion

Proposed method specificity

By analyzing the standard solution, the specificity of the method was checked. The sharp peaks were observed in the standard solution of dexamethasone sodium phosphate and Prednisolone acetate as shown in Figure 6 that shows the high degree specificity of the method.

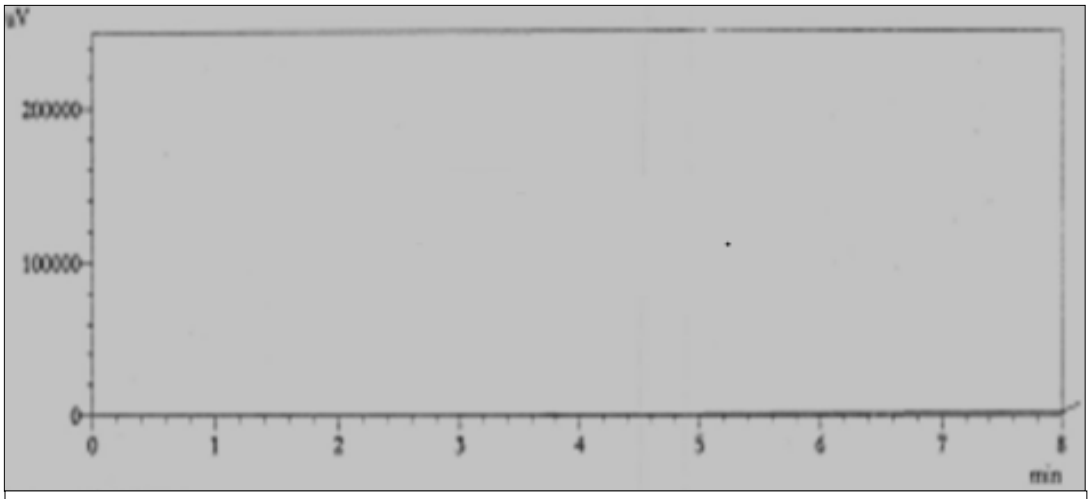

Figure 5: Chromatogram of mobile phase (steady baseline)

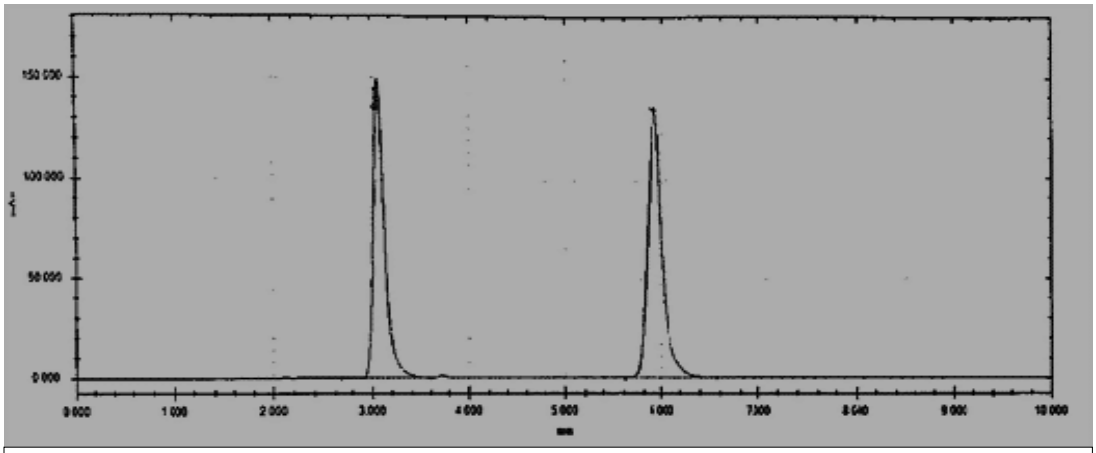

Figure 6: HPLC Chromatogram for standard solution of (1) DMSP (32µg/ml), (2) PA (30µg/ml)

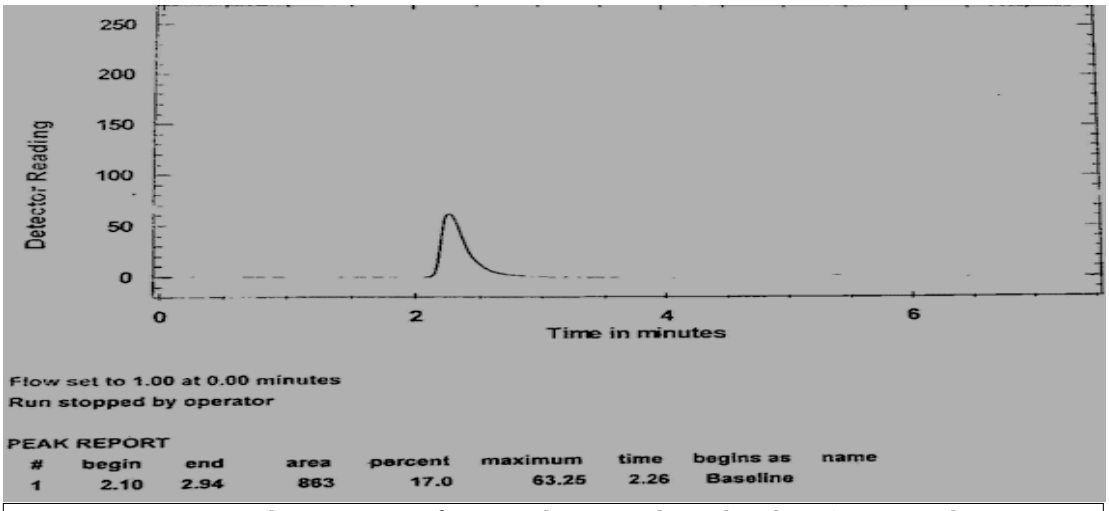

Figure 7: Chromatogram of Dexamethasone sodium phosphate (65.85µg/ml)

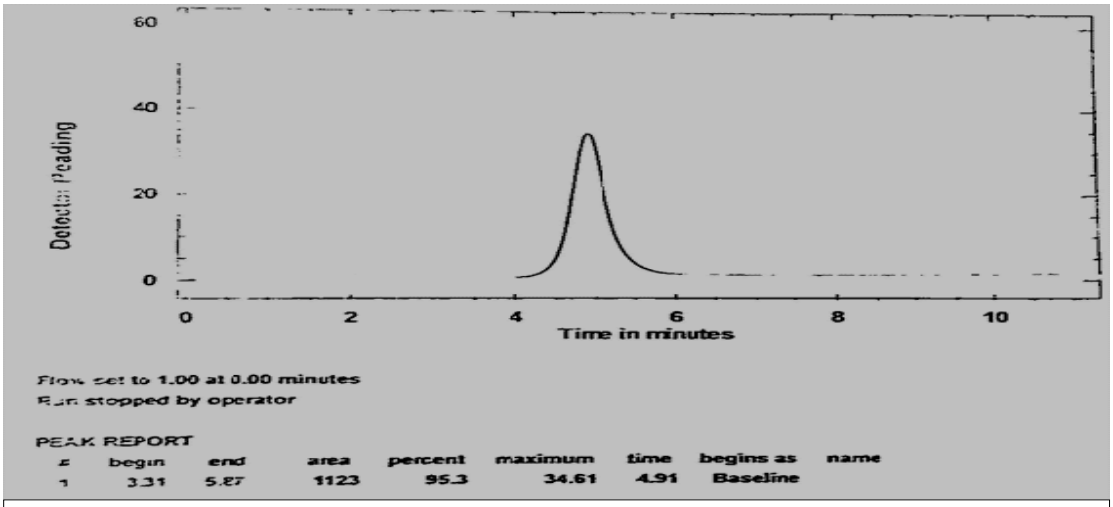

Figure 8: Chromatogram of Prednisolone acetate (20.24µg/ml)

HPLC system specificity

Six replicate injections of dexamethasone sodium phosphate and prednisolone acetate were injected into the HPLC system for system specificity as shown in Table 1(a) & 1(b).

Table 1(a): HPLC system specificity (six replicate injection of DMSP)

No	Conc. (µg/ml)	Injection volume (µl)	Retention time (min.)	Peak area (AU)
1	65.85	20	2.26	863.0
2	65.82	20	2.26	862.0
3	65.82	20	2.26	861.0
4	65.82	20	2.28	863.0
5	65.82	20	2.25	862.0
6	265.82	20	2.28	862.0
Average (X^-)			2.26	863.0
Standard Deviation (SD)			0.012247	0.0816497
(%RSD)			0.542	0.103

Table 1(b): HPLC system specificity (six replicate injection of PA)

No	Conc.(µg/ml)	Injection volume (µl)	Retention time(min.)	Peak area (AU)
1.	40.24	20	4.91	1123.0
2.	40.24	20	4.90	1150.0
3.	40.24	20	4.91	1123.0
4.	40.24	20	4.90	1141.0
5.	40.24	20	4.90	1140.0
6.	40.24	20	4.91	1166.0
Average (X^-)			4.906667	1140.5
Standard Deviation (SD)			0.008165	16.45296
Relative Standard Deviation (RSD)			0.166	1.442

Acceptance criteria: The % RSD of six sample peak areas should not be more than 2.0%.

Linearity

It is the proportionality of measured value to the concentration of a substance." Linearity shows that an increase in the concentration of the sample also increase the measured values as shown in Figure 9. So, the peak area in the chromatogram is proportional to the concentration of the sample. The peak area is the measurement of concentration. Linearity study was performed by using the HPLC system and peaks areas were observed in milli absorbance unit (mAU).

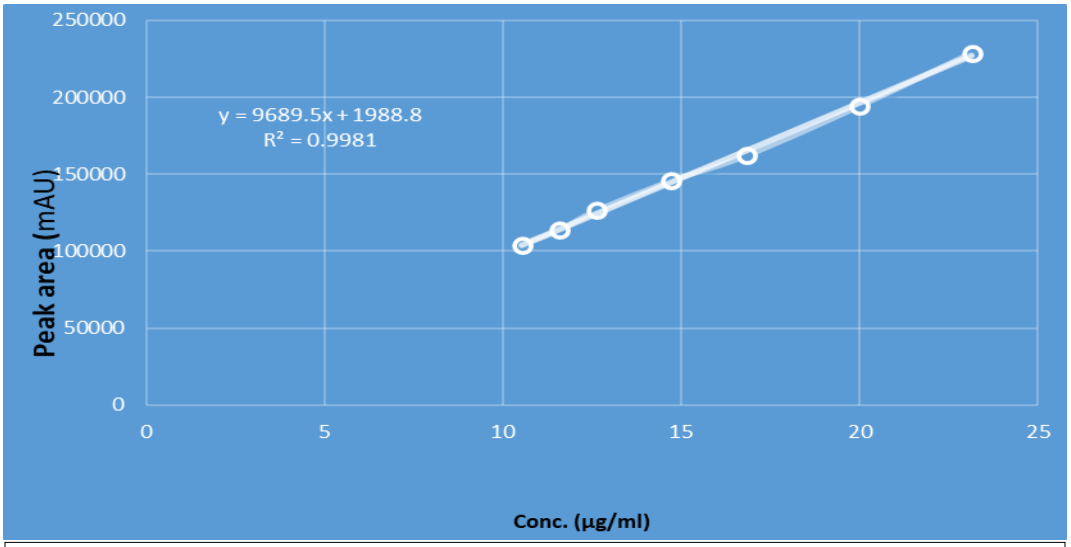

Figure 9: Linearity of Dexamethasone sodium phosphate

Table 2: Calibration data of Dexamethasone sodium phosphate (DMSP)

Sr #	Conc. Dexamethasone sodium phosphate (µg/ml)	Peak area (mAU)	Recovery (%) Found conc. = ---------- X100 Known conc
1.	10.528 µg/ml	104153.5	100.11
2.	11.58 µg/ml	113706.2	99.56
3.	12.644 µg/ml	126442	101.58
4.	14.739 µg/ml	145715.1	100.63
5.	16.844 µg/ml	162048.6	98.07
6.	20.000 µg/ml	194042.9	101.00
7.	23.1616 µg/ml	228699.2	101.02
Regression equation (y*)		9689.5x+1988.8	
Slope (m)		96989.5	
Intercept (y)		1988.8	
Correlation coefficient (R^2)		0.9981	

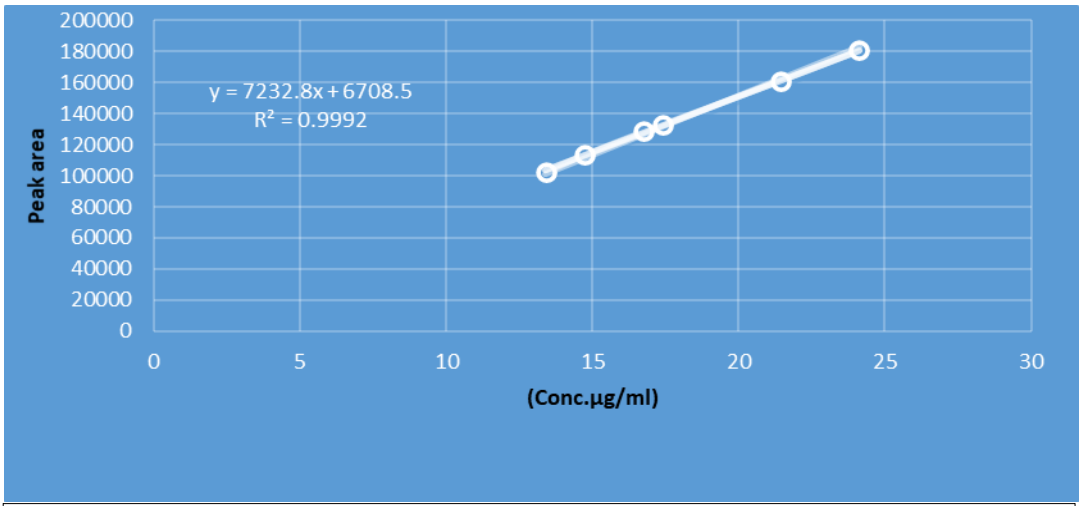

Figure 10: Linearity of Prednisolone acetate

Table 3: Calibration data of Prednisolone acetate (PA)

Sr#	Conc. of Prednisolone acetate (µg/ml)	Peak area (mAU)	Recovery (%) Found conc. = ----------X100 Known conc
1.	13.392 µg/ml	102251.8	98.63
2.	14.7312 µg/ml	113706.2	100.42
3.	16.740µg/ml	128572.2	100.59
4.	17.4096 µg/ml	133357.6	100.57
5.	21.4272 µg/ml	161392.7	99.80
6.	24.106 µg/ml	180705.9	99.79
Regression equation (y*)		7232.8x+6708.5	
Slope (m)		7232.8	
Intercept (y)		6708.5	
Correlation coefficient (R^2)		0.9992	

Precision study

The precision of method was confirmed by repeatability, intermediate, and reproducibility studies. Further, studies were performed by using the HPLC system and peaks areas were observed in absorbance unit (AU).

Repeatability (Intra-day) study

A repeatability study was conducted on the three different sample concentrations in three times on the same day (Intra-day assay precision) as shown in Table 4(a), 4(b), and 4(c).

Acceptance criteria: %RSD of three sample peak areas should not be more than 2.0%.

Table 4(a): Intra-day precision results

Dilution-1: 1.0 ml sample was diluted to 100 ml with mobile phase.

Active ingredient	Analysis Day	Concentration (µg/ml)	Retention time (min.)	Peak area (AU)
Dexamethasone -Na-PO_4 (µg/ml)	Same day	32.91	2.19	428.0
			2.26	428.0
			2.26	434.0
	Mean		2.236667	430.0
	S. D		0.040415	3.464102
	%RSD		1.80	0.805
Prednisolone acetate µg/ml)	Same day	79.23	4.51	2188.0
			4.51	2148.0
			4.50	2229.0
	Mean		4.51	2188.33
	S. D		0.005774	40.50103
	%RSD		0.128	1.85

Table 4(b): Intra-day precision results

Dilution-2: 5.0 ml sample was diluted to 100 ml with mobile phase. 5.0 ml was further diluted to 50ml with the same diluent.

Active ingredient	Analysis day	Concentration (µg/ml)	Retention time (min.)	Peak area (AU)
Dexamethasone -Na-PO_4 (µg/ml)	Same day	16.45	2.30	222.0
			2.30	225.0
			2.28	223.0

	Mean		2.29333	223.0
	S.D		0.011547	1.527525
	%RSD		0.50	0.68
Prednisolone acetate		39.615	4.54	1075.0
(µg/ml)			4.54	1085.0
			4.53	1067.0
	Mean		4.54	1075.6
	S. D		0.017321	9.0185
	%RSD		0.38	0.84

Table 4(c): Intra-day precision results

Dilution-3: 10.0 ml sample was diluted to 50.0 ml with mobile phase. 5.0 ml was further diluted to 50ml with the same diluent.

Active ingredient	**Analysis Day**	**Concentration (µg/ml)**	**Retention time (min.)**	**Peak area (AU)**
Dexamethasone -Na-PO_4	Same day	65.85	2.28	883.0
(µg/ml)			2.25	898.0
			2.26	897.0
	Mean		2.26	892.6
	S. D		0.015275	8.386497
	%RSD		0.676	0.94
Prednisolone acetate	Same day	158.46	4.54	4385.0
(µg/ml)			4.51	4471.0
			4.53	4409.0
	Mean		4.53	4421.667
	S. D		0.015275	44.37717
	%RSD		0.33	1.00

Intermediate precision (Inter-day) study

The intermediate precision of the method was checked by three different analysts on three different days and results were precise as shown in Table 5(a), 5(b), and 5(c).

Table 5 (a): Inter-day precision results

Dilution-1: 1.0 ml sample was diluted to 100 ml with mobile phase.

Active ingredient	**Analysis Day**	**Concentration (µg/ml)**	**Retention time (min.)**	**Peak area (AU)**
Dexamethasone -Na-PO_4	Day-1	32.91	2.26	443.0
(µg/ml)	Analyst-1		2.26	428.0
			2.26	435.0
	Mean		2.26	435.333
	S. D		0	7.505553
	%RSD		0	1.72
Prednisolone acetate		79.23	4.50	2229.0
(µg/ml)			4.50	2180.0
			4.51	2245.0
	Mean		4.50	2218.0
	S. D		0.005774	33.86739
	%RSD		0.13	1.53

Acceptance criteria: % RSD of three sample peak areas should not be more than 2.0%.

Table 5 (b): Inter-day precision results

Dilution-2: 5.0 ml sample was diluted to 100 ml with mobile phase. 5.0 ml is further diluted to 50ml with the same diluent.

Active ingredient	Analysis day	Concentration (µg/ml)	Retention time (min.)	Peak area (AU)
Dexamethasone -Na-PO_4 (µg/ml)	Day-2 Analyst-1	16.45	2.30	197.0
			2.31	202.0
			2.28	201.0
	Mean		2.29	200
	S. D		0.015275	2.645751
	%RSD		0.67	1.32
Prednisolone acetate (µg/ml)		39.615	4.54	1075.0
			4.56	1050.0
			4.53	1067.0
	Mean		4.54	1064.0
	S. D		0.01525	12.76715
	%RSD		0.33	1.20

Acceptance criteria: % RSD of three sample peak areas should not be more than 2.0%.

Table 5 (c): Inter-day precision results

Dilution-3: 10.0 ml sample was diluted to 50.0 ml with mobile phase. 5.0 ml is further diluted to 50ml with the same diluent.

Active ingredient	Analysis Day	Concentration (µg/ml)	Retention time (min.)	Peak area (AU)
Dexamethasone -Na-PO_4 (µg/ml)	Day-3 Analyst-2	65.85	2.26	919.0
			2.26	898.0
			2.25	915.0
	Mean		2.26	910.0
	S. D		0.00774	11.15049
	%RSD		0.26	1.22
Prednisolone acetate (µg/ml)		158.46	4.53	4471.0
			4.51	4409.0
			4.53	4426.0
	Mean		4.52	4435.3330
	S. D		4.52333	32.03644
	%RSD		0.25	0.72

Acceptance criteria: % RSD of three sample peak areas should not be more than 2.0%.

Reproducibility study

Reproducibility of the method was checked by testing the same samples in multiple quality control laboratories (Selmore Pharmaceuticals Multan Road, Lahore-Pakistan, Saturn Pharmaceuticals Raiwind Road, Lahore-Pakistan and Izfaar Pharmaceuticals Sundar Industrial Estate, Lahore-Pakistan. Only 2% result variation was observed**.**

Accuracy (Recovery studies)

Accuracy was studied at three levels at 50%, 100%, and 150% for dexamethasone sodium phosphate and prednisolone acetate. The recovered value of dexamethasone sodium phosphate in the sample was 98.72% to 100.31% while the recovered value of prednisolone acetate was 97.05% to 101.60%.

Table 6: Recovery study of Dexamethasone sodium phosphate

Level	Peak area (AU)	Amount recovered (Y= 13.51x-4.0528)	% Recovery
50% (16.455µg/ml)	1-222.0 2-220.0 3-225.0 Mean:216.0 S.D:2.516611 %RSD: 1.13	16.28µg/ml	99.69%
100% (32.91µg/ml)	1-434.0 2-428.0 3-443.0 Mean: 435.0 S.D:7.549834 %RSD:1.73	32.49µg/ml	98.72%
150% (49.365µg/ml)	1-650.0 2-652.0 3-651.0 Mean: 651.0 S.D:1.000 %RSD: 0.153	49.52g/ml	100.31%

Limit of detection (LOD)

The limit of detection was determined by using the STEYX function to find the standard error of the response and the slope attained from the linearity plot of dexamethasone sodium phosphate and prednisolone acetate well recovered from veterinary injectable suspension. LOD was calculated by applying the formula as 3 x standard error (STEYX)/S, where S is the slope of the regression line. The calculated value of LOD for each analyte was reported in Table 8.

Table 7: Recovery study of Prednisolone acetate

Level	Peak area (AU)	Amount recovered (y=26.659x+48.775)	% Recovery
50% (39.615µg/ml)	1-1075.0 2-1085.0 3-1067.0 Mean:1075.6670 S.D:9.0185 %RSD:0.84	38.45µg/ml	97.05
100% (79.23µg/ml)	1-2188.0 2-2148.0 3-2229.0 Mean: 2188.0 S.D: 40.50103 %RSD:1.85	80.15 µg/ml	101.16
150% (118.84µg/ml)	1-3208.0 2-3209.0 3-3207.0 Mean:3204.667 S.D: 6.658333 %RSD: 0.2077	118.37 µg/ml	99.60
40.24µg/ml	1141.0	40.92µg/ml	101.60

	1142.0			
	1139.0			
	Mean:1141.0			
	S.D:1.527525			
	%RSD: 0.13			

Table 8: Limit of Detection (LOD)

Analytes (API's)	**Concentration (µg/ml)**	**Peak area (response) (mAU)**	**Standard error (STEYX)**	**LOD (µg/ml)**
Dexamethasone	10.528	104153.5	2145.46	
Na-PO_4	11.58	113706.2		= 3x2145.46
	12.644	126442.0		9689.5
	14.739	145715.1		= **0.66µg/ml**
	16.844	162048.6		
	23.1616	228699.2		
Prednisolone acetate	13.392	102251.8	908.8971	
	14.7312	113706.2		= 3x908.89
	16.740	128572.2		7232.8
	17.4096	133357.6		**= 0.37µg/ml**
	21.4272	161392.7		
	24.106	180705.9		

Limit of Quantification (LOQ)

The limit of quantification was determined by using the STEYX function to find the standard error of the response and the slope attained from the linearity plot of dexamethasone sodium phosphate prednisolone acetate well recovered from injectable suspension. LOQ was calculated by applying the formula as 10 x standard error (STEYX)/S, where S is the slope of the regression line. The calculated value of LOQ for each ingredient is reported in Table 9. In this study, LOD for 20µl of dexamethasone sodium phosphate and prednisolone acetate was 0.66µg/ml and 0.37µg/ml respectively. While LOQ was 2.21µg/ml and 1.25µg/ml for Dexamethasone sodium phosphate and Prednisolone acetate respectively. The results show that the proposed method is very sensitive and accurate as shown in Table 9.

Table 9: Limit of Quantification (LOQ)

Analytes (API's)	**Concentration (µg/ml)**	**Peak area (response) (mAU)**	**Standard error (STEYX)**	**LOD (µg/ml)**
Dexamethasone	10.528	104153.5	2145.46	
Na-PO_4	11.58	113706.2		
	12.644	126442.0		= 10x2145.46
	14.739	145715.1		9689.5
	16.844	162048.6		= **2.21µg/ml**
	23.1616	228699.2		
Prednisolone acetate	13.392	102251.8	908.8971	
	14.7312	113706.2		= 10x908.89
	16.740	128572.2		7232.8
	17.4096	133357.6		**= 1.25µg/ml**
	21.4272	161392.7		
	24.106	180705.9		

Ruggedness

The ruggedness of the test method was confirmed by carrying out the precision study in three preparations of the sample on the same batch by different HPLC operators. The results of the intermediate precision study were given in Table 10.

Table 10: Ruggedness Results

Analyte	Conc.(µg/ml)	RT (min.)	Peak area (AU)
Dexamethasone Na-PO_4	16.45µg/ml	2.30	421.0
		2.31	425.0
		2.28	428.0
		Mean: 2.296667	424.6667
		S.D: 0.015275	3.511885
		%RSD:0.66	0.83
Prednisolone Acetate	39.65µg/ml	4.54	1075.0
		4.56	1050.0
		4.53	10.69.0
		Mean: 4.54	1064.0
		S.D:	13.01181
		%RSD	1.23

Robustness

The robustness of the test method was checked by deliberately changing±2% in mobile phase composition, pH-3.0±0.1, flow rate 1.0±0.1 and column temperature variation 30±5^{0}C (25-35^{0}C). The retention time, tailing factor, and %RSD of six replicate injections response were evaluated. In all these experiments results were not affected as given in Table 11.

Table 11: Robustness results

Parameters	RT		Peak area (AU)	
	DMSP (32.8µg/ml)	PA (79.23µg/ml)	DMSP	PA
Mobile phase-1(pH-3.1)	2.25	4.54	443.0	2229.0
Mobile phase-2 (pH-2.90)	2.30	4.56	428.0	2180.0
Flow rate-1 (1.1ml/min.)	2.26	4.51	435.0	2245.0
Flow rate2- (0.9ml)	2.26	4.53	426.0	2190.
Column temperature-1 (26^0)	2.29	4.52	439.0	2218.0
Column temperature (30^{0}C)	2.28	4.50	438.0	2203.0
Mean	2.27333	4.53	434.8333	2210.8333
S.D	0.019664	0.025298	6.615638	24.4738
%RSD	0.86	0.56	1.52	1.12

Assay of a marketed product

The contents of dexamethasone sodium phosphate and prednisolone in the marketed product were found within the stated amount. The method is simple, easy, and accurate in the routine analysis of the pharmaceutical products as shown in Table 12.

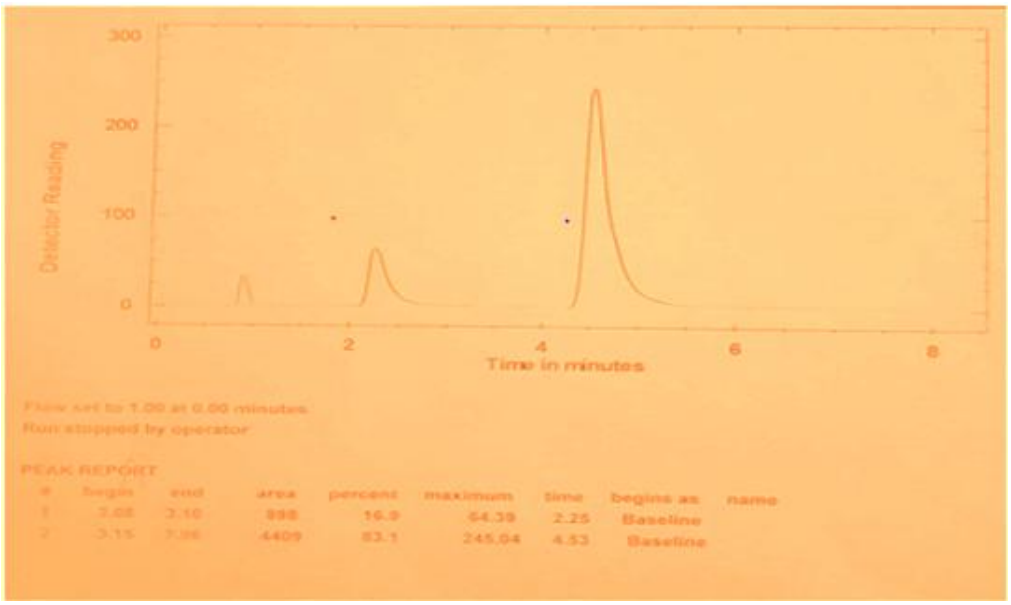

Figure 11: Chromatogram for mixture of Dexamethsone sodium phosphate & Prednisolone acetate in injectable veterinary suspension

Table 12: Assay summary of Product (Penacort Injectable suspension)

Drugs (API's)	**Label claim (mg)**	**Amount found (mg/ml)**	**Assay 90-110% of the labelled amount.**
Dexamethasone Na-PO_4	3.291mg/ml	3.295 mg/ml	100.11%
Prednisolone acetate	7.923 mg/ml	8.04 mg/ml	101.51%

HPLC system suitability

Table 13: Results of HPLC system suitability

Parameters	**Acceptance criteria**	**D-Star HPLC system (USA) Model: DIS-20S Model: DGS-200**
Injection precision for areas (n=6)	RSD≤2%	For DMSP:0.103 For PA: 1.442
Injection precision for retention time (min.)	RSD≤2%	For DMSP: 0.542 For PA: 0.166
Tailing factor for DMSP & PA	T≤2	**1.05**
Theoretical plates (N) for DMSP & PA	NLT:2000	**3595**

A summary of validation parameters results for the proposed HPLC method

Table 14: Summary of the results

Sr #	**Parameter**	**Value to be found**
1.	Linearity range of DMSP	10.528-13.1616µg/ml
	Linearity range of PA	13.392-24.106µg/ml
2.	Correlation coefficient (R^2) of DMS	0.9981
	Correlation coefficient (R^2) of PA	0.9992
3.	% Recovery of DMSP	98.72%-100.31%
	%Recovery of PA	97.05%-101.60%

4.	Intraday precision of DMSP(%RSD)	0.81
	Intraday precision of PA (%RSD)	1.23
5.	Interday precision of DMSP (%RSD)	1.42
	Inter precision of PA (%RSD)	1.15
6.	Ruggedness of DMSP (%RSD)	0.83
	Ruggedness of PA (%RSD)	1.23
7.	Robustness of DMSP (%RSD)	1.52
	Robustness of PA(%RSD)	1.12
8.	LOD of DMSP (µg/ml)	0.66
	LOD of PA (µg/ml)	0.37
9.	LOQ of DMSP (µg/ml)	2.21
	LOQ of PA (µg/ml)	1.25
10.	Assay:	
	The content of DMSP (%age)	103.26%
	The content of PA (%age)	100.77%

Conclusion

The proposed method is simple, economical, reliable and robust with good precision, accuracy, linearity and raggedness. The validated method is specific while estimating the commercial formulations without interference of excipients and other additives. Hence, this method can easily be used in the routine analysis of dexamethasone sodium phosphate and prednisolone acetate from pharmaceutical dosage forms in QC and R&D laboratories.

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