CHAPTER-III

NEW ANALYTICAL METHODS FOR THE DETERMINATION OF DISODIUM PAMIDRONATE IN BULK AND PHARMACEUTICAL FORMULATIONS
DISODIUM PAMIDRONATE

3.01 Introduction

In the present investigation DSP, which is an anti osteoporotic agent has been selected. It's status has been presented in table 1.01, p.3.

The characteristics, therapeutic importance, chemical names, structure, analytically useful functional groups and commercially available formulations of DSP are presented in tables 3.01 - 3.03, p.170-172.

A very few physico-chemical methods appeared in the literature for the determination of DSP in pharmaceutical formulations. The methods so far reported include HPLC \textsuperscript{260,261}, IEC \textsuperscript{262,263}. The analytically important functional groups (Table 3.01, p.170) of DSP were not properly exploited for designing suitable spectrophotometric methods for the determination of DSP.

Hence there is a need to develop sensitive and flexible visible spectrophotometric methods, which prompted the author to choose DSP for the investigation. Based on the different chemical reactions nine methods have been developed. These methods are based on the reactivity of DSP with reagents such as MB, SFN-O, Brucine-IO\textsubscript{4}, DCQC, VN, PDAB, NH/AA, NQS and SNP/Acetone (Table 1.03, p.15-17) and were used to produce coloured species of reasonable stability, paving possibility for visible spectrophotometric determination of DSP and it's pharmaceutical formulations. The author has developed a simple and sensitive UV method (CH\textsubscript{3}OH as Solvent) and adopted it as a reference method for comparing accuracy of the results obtained by the proposed methods.
<table>
<thead>
<tr>
<th>SL No.</th>
<th>Generic name</th>
<th>Chemical name</th>
<th>Analytically important moieties/functional groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DISODIUM PAMIDRONATE (DSP)</td>
<td>Disodium 3-amino-1-hydroxypropyldenediphosphonate</td>
<td>Bis phosphonate, Aliphatic primary amine and hydroxyl.</td>
</tr>
</tbody>
</table>
MODE OF ACTION AND THERAPEUTIC USE

It is used to prevent bone loss, and treat osteoporosis. It is also used to strengthen bone in Paget's disease, to prevent bone loss due to steroid use, and in certain cancers with high propensity to bone, such as multiple myeloma. In multiple myeloma, it is usually administered as an intravenous infusion, lasting about 3 hours. The therapy is repeated monthly, and lasted for the life of the patient. Due to its ability to sequester calcium in bone, it is also used to treat high calcium levels. It is also used as an experimental treatment of the bone disorder known as osteogenesis imperfecta, or brittle bones.

CHARACTERISTICS

**Molecular formula:** C₃H₉NNa₂O₇P₂
**Molecular weight:** 279.03
**Colour:** white powder
**Soluble in water**

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>CHARACTERISTICS</th>
<th>MODE OF ACTION AND THERAPEUTIC USE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti osteoporotic agent</td>
<td>Molecular formula: C₃H₉NNa₂O₇P₂</td>
<td>It is used to prevent bone loss, and treat osteoporosis. It is also used to strengthen bone in Paget’s disease, to prevent bone loss due to steroid use, and in certain cancers with high propensity to bone, such as multiple myeloma. In multiple myeloma, it is usually administered as an intravenous infusion, lasting about 3 hours. The therapy is repeated monthly, and lasted for the life of the patient. Due to its ability to sequester calcium in bone, it is also used to treat high calcium levels. It is also used as an experimental treatment of the bone disorder known as osteogenesis imperfecta, or brittle bones.</td>
</tr>
<tr>
<td></td>
<td>Molecular weight: 279.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colour: white powder</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soluble in water</td>
<td></td>
</tr>
<tr>
<td>S.NO</td>
<td>Proprietary name</td>
<td>Pharmaceutical Concerned</td>
</tr>
<tr>
<td>------</td>
<td>-----------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>AREDIA</td>
<td>Novartis (Biochemie)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AREDRONET</td>
<td>Sun (Inca)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BONAPAM</td>
<td>Alkem(cytomed)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PAMIDRIA</td>
<td>Cipla</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 3.04
PROCEDURE FOR THE ASSAY OF DSP IN FORMULATIONS

<table>
<thead>
<tr>
<th>Pharmaceutical formulations</th>
<th>Reference method</th>
</tr>
</thead>
<tbody>
<tr>
<td>INJECTIONS</td>
<td>A volume of injection equivalent to 25 mg of the drug was dissolved in 5 ml of MeOH, shaken well and filtered. The filtrate was diluted to 25 ml with MeOH to get 1 mg/ml solution of the drug in formulations. Five ml of this solution was further diluted to 50 ml to get 100 µg/ml solution. The absorbance of the solution was determined at $\lambda_{\text{max}}$ 242 nm (Fig.3.01, p.173). The quantity of the drug was computed from the Beer’s law plot (Fig. 3.02, p.173) of the standard drug in MeOH.</td>
</tr>
</tbody>
</table>

![Absorption spectrum of DSP in CH₃OH](image1)

![Beer's Law plot of DSP in CH₃OH](image2)

Fig 3.01: Absorption spectrum of DSP in CH₃OH (UV reference method)

Fig 3.02: Beer's Law plot of DSP in CH₃OH (UV reference method)
3.02 Experimental

i. Instruments used

SHIMADZU UV-1601 UV-visible digital spectrophotometer with 1 cm matched quartz cells were used for the spectral and absorbance measurements. Digital pH meter systronic 361 was used for pH measurements.

ii Preparation of standard solution.

For methods $M_3$, $M_4$, $M_{13}$, $M_{15}$, $M_{27}$, $M_{28}$ and $M_{32}$

The stock solution (mg/ml) of Disodium pamidronate (DSP) was prepared by dissolving 100 mg of it in 100 ml of distilled water. A portion of this stock solution was diluted stepwise with the same solvent to obtain the working standard DSP solution of concentrations of 40 $\mu$g/ml ($M_3$, $M_4$), 80 $\mu$g/ml ($M_{28}$, $M_{32}$), 100 $\mu$g/ml ($M_{15}$, $M_{27}$) and 250 $\mu$g/ml ($M_{13}$).

For methods $M_{24}$ and $M_{25}$

The stock solution (1 mg/ml) of DSP was prepared by dissolving 100 mg of it in 100 ml of methanol. This solution was further diluted step wise with the same solvent to obtain working standard solution of concentrations of 40 $\mu$g/ml ($M_{24}$) and 80 $\mu$g/ml ($M_{25}$).

(iii) Preparation of reagents

All the chemicals and reagents were of analytical grade and the solutions were prepared in triply distilled water.
For Method M₃

MB Solution (Fluka, 0.01%, 3.12 x 10⁻⁴M)

: Prepared by dissolving 10 mg of methylene blue in 100 ml of distilled water and washed with CHCl₃ to remove chloroformic soluble impurities.

Buffer solution (pH 9.8)

: Prepared by mixing 7gms of ammonium chloride with 56.8ml of liquor ammonia solution and diluted to 100ml with distilled water and pH was adjusted to 9.8.

Chloroform (Qualigens)

: AR grade chloroform was used

For Method M₄

SFN-O Solution (Fluka, 0.01%, 2.857 x 10⁻⁴M)

: Prepared by dissolving 10 mg of safranin-O in 100 ml of distilled water and washed with CHCl₃ to remove chloroformic soluble impurities.

Buffer Solution (pH 9.8)

: Prepared by mixing 7gms of ammonium chloride with 56.8ml of liquor ammonia solution and diluted to 100ml with distilled water and pH was adjusted to 9.8.

For Method M₁₃

Brucine solution (0.2%, 5.0x10⁻³M)

: 200 mg of brucine was first dissolved in few drops of conc. sulphuric acid then diluted to 100 ml with distilled water (the overall acidity with respect to sulphuric acid was 0.16M).

NaIO₄ solution (BDH, 0.2%, 9.35X10⁻³M)

: Prepared by dissolving 200mg of NaIO₄ in 100ml of distilled water and standardized iodometrically.

For Method M₁₅

DCQC solution (Loba, 0.04%, 1.90 x10⁻³M)

: Prepared by dissolving 40 mg of DCQC in 100 ml of isopropanol.

Buffer solution (pH 9.4)

: Prepared by mixing 250 ml of 0.2 M boric acid with 160 ml of 0.2 M NaOH solution and diluted to 1000 ml with distilled water and pH was adjusted to 9.4.

For Method M₂₄

Vanillin solution (BDH, 0.4%, W/v, 2.63x10⁻²M)

: Prepared by dissolving 400 mg of vanillin in 100 ml of methanol.

H₂SO₄ (concentrated) (Qualigens)

: Used as it is.
For Method M25

PDAB solution (BDH, 0.4%, 2.68x10^{-2}M)  : Prepared by dissolving 400 mg of PDAB in 100 ml of CH₃OH.

H₂SO₄ (Merck, conc.)  : Used as it is.

For Method M27

Ninhydrin solution (BDH, 1%, 5.605x10^{-2}M)  : Prepared by dissolving 1 gm of ninhydrin in 100 ml of acetone.

Ascorbic acid solution (BDH, 0.1%, 5.678x10^{-3}M)  : Prepared by dissolving 100 mg of ascorbic acid in 100 ml of distilled water.

Buffer solution (pH -5.0)  : Prepared by dissolving mixture of 200 ml of 0.5M citric acid and 200 ml of 1.0M NaOH solution to 500 ml with distilled water and pH was adjusted to 5.0.

For Method M28

NQS solution (Loba, 0.5%, 1.92x10^{-2}M)  : Prepared by dissolving 500mg of NQS in 100 ml of distilled water.

NaOH solution (E-Merck, 20%, 5.0M)  : Prepared by dissolving 20 gms of NaOH in 100 ml of distilled water.

For Method M32

SNP solution (E-Merck, 10%, 3.38x10^{-1}M)  : Prepared by dissolving 10gms of the sodium nitroprusside in 100 ml of distilled water.

Saturated solution of sodium tetra hydroborate decahydrate  : Saturated solution of sodium tetra hydroborate decahydrate solution was prepared.

Acetone solution  : Used as it is.

(iv) Recommended Procedures:

After systematic and detailed study of the various parameters involved, as described under results and discussion in this chapter, the following procedures [Methods: (M3) MB, (M4) SFN-O, (M13) Brucine-IO₄, (M15) DCQC, (M24) VN, (M25) PDAB, (M27) NH/AA, (M28) NQS and (M32) SNP/Acetone] were
recommended for the determination of DSP in bulk samples and pharmaceutical formulations.

(a). For Bulk samples

**Method M₃ & M₄**

Into a series of 125 ml separating funnels containing aliquots of standard DSP solution [0.5-2.5 ml, 40 µg/ml (M₃) and 0.5-2.5 ml, 40 µg/ml (M₄)], 1 ml of pH 9.8 buffer and 5.0 ml of dye solution MB (M₃) or SFN-O (M₄) were added. The total volume of aqueous phase in each separating funnel was adjusted to 10 ml with distilled water and 10 ml of CHCl₃ was added. The contents were shaken for 5 min. The two phases were allowed to separate and the absorbances of the separated organic layer were measured at 650 nm (M₃) or 520 nm (M₄) against a similar reagent blank. The amount of DSP was deduced from the calibration curve (Fig. 3.12-3.13, p.200).

**Method M₁₃**

Into a series of 25 ml calibrated tubes, aliquots of standard DSP solution (0.5-2.5ml, 250 µg/ml) were taken. Then 3.0 ml of brucine solution and 1.5 ml of IO₄⁻ solution were added successively. The flasks were kept aside for 2 min. and then 2.0 ml of sulphuric acid was added and then heated the contents on boiling water-bath for 15 min. The flasks were cooled to the room temperature and made up to the mark with distilled water. The absorbance of the coloured species were measured after 5 min. at 520 nm against the reagent blank. The amount of DSP was computed from its calibration graph (Fig. 3.14, p.200).

**Method M₁₅**

Into a series of calibrated tubes, aliquots of standard drug, DSP solutions (0.5-2.5 ml, 100µg/ml), 5 ml of buffer pH 9.4, 2 ml of DCQC solution was added. The volume in each tube was made upto 25 ml with distilled water. The
absorbances were measured at 620 nm against a similar reagent blank. The amount of DSP was estimated from its calibrations curve (Fig. 3.15, p.200).

**Method M\(_{24}\)**

To each one of 10 ml calibrated tubes, aliquots (0.5-2.5 ml, 40\(\mu\)g/ml) of methanolic standard DSP solution, 2.0 ml of Vanillin and 3.0 ml of conc. sulphuric acid were added successively and the total volume in each tube was brought to 9 ml by the addition of methanol and placed in a hot water bath for 15 min. Then the flasks were cooled and made up to the mark with methanol and the absorbances were measured after 5 min. at 560 nm against a reagent blank prepared in a similar way. The amount of DSP present in sample solution was computed from its calibration curve (Fig. 3.16, p.201).

**Method M\(_{25}\)**

To each one of 10 ml calibrated tubes, aliquots (0.5-2.5 ml, 80\(\mu\)g/ml) of methanolic standard DSP solution, 2.0 ml of PDAB and 3.0 ml of conc. sulphuric acid were added successively and the total volume in each tube was brought to 9 ml by the addition of methanol and placed in a hot water bath for 15 min. Then the flasks were cooled and made up to the mark with methanol and the absorbances were measured after 5 min at 480 nm against a reagent blank prepared in a similar way. The amount of DSP present in sample solution was computed from its calibration curve (Fig. 3.16, p.201).

**Method M\(_{27}\)**

Aliquots (0.5-2.5ml, 100\(\mu\)g/ml) of the standard DSP solution were transferred in a series of calibrated tubes containing 4 ml of buffer (pH -5.0), 1 ml of ninhydrin solution and 0.5 ml of ascorbic acid solution. The volume in each tube was adjusted to 8 ml with distilled water and were kept in a boiling water bath. After 15 min. tubes were removed and chilled in ice water. The solution in
each tube was made upto 10 ml with distilled water. The absorbances were measured at 560 nm after 10 min. against a reagent blank prepared similarly. The amount of DSP was calculated from Beer’s law plot (Fig. 3.18, p.201).

Method M\textsubscript{28}

Aliquots of DSP solution (0.5-2.5 ml, 80 μg/ml), were placed in series of 10 ml of calibrated tubes. Then 0.5 ml of NQS and 2.0 ml of NaOH solutions were added to each tube and kept a side for 2 min. at lab temperature. The solutions were made upto the mark with distilled water. The absorbances were measured at 480 nm against a reagent blank prepared simultaneously. The amount of the drug in sample was calculated from the calibration graph (Fig. 3.19, p.201).

Method M\textsubscript{32}

To each one of 10 ml calibrated tubes, neutral aqueous solution of the DSP (0.5- 2.5 ml, 80μg/ml), 0.5 ml of acetone, 0.3 ml of 10% aqueous solution of sodium nitroprusside and 2 ml of saturated aqueous solution of sodium tetra hydro borate decahydrate were added. The tubes were kept a side for one hour in the dark. The solutions were made upto the mark with distilled water. The absorbances were measured at 550 nm. against a reagent blank prepared similarly. The amount of DSP was calculated from the appropriate calibration graph (Fig. 3.20, p.202).

(b) For pharmaceutical formulations:

The tablet powder equivalent to 100 mg of DSP was taken and triturated with (3x25 ml) portions of chloroform. The combined chloroform extract was made upto 100 ml with the same solvent to get mg/ml stock solution.
For methods M₃, M₄, M₁₃, M₁₅, M₂₇, M₂₈ and M₃₂.

From one portion of chloroform extract (25 ml), CHCl₃ was gently evaporated. The residue was dissolved in distilled water and subsequently brought the volume to 50 ml with the same solvent to get 500 μg/ml. It was further diluted stepwise with the same solvent as described under standard solution preparation to obtain 40 μg/ml (M₃, M₄), 80 μg/ml (M₂₈, M₃₂), 100 μg/ml (M₁₅, M₂₇) and 250 μg/ml (M₁₃).

For methods M₂₄ and M₂₅

In second portion of chloroform extract (25 ml), CHCl₃ was gently evaporated. The residue was dissolved in it initially in 5 ml of methanol, followed by dilution to 50 ml of methanol to get 500 μg/ml. This solution was further diluted stepwise with methanol to obtain 40 μg/ml (M₂₄) and 80 μg/ml (M₂₅).

Then the procedures given under bulk samples were followed for the assay of DSP in formulations.

3.03 Results and Discussion

(i) Spectral Characteristics

In order to ascertain the optimum wavelength of absorption (λₓₐₓ) of the coloured species formed in each one of nine spectrophotometric methods, specified amounts of DSP in final solution (10 μg/ml, 10 μg/ml, 25 μg/ml, 10 μg/ml, 10 μg/ml, 20 μg/ml, 25 μg/ml, 20 μg/ml and 20 μg/ml) for methods M₃, M₄, M₁₃, M₁₅, M₂₄, M₂₅, M₂₇, M₂₈ and M₃₂ respectively) were taken and the colours were developed (or diminished) separately following the above mentioned procedures individually. The absorption spectra were scanned on a spectrophotometer in the wavelength region of 360-900 nm against corresponding reagent blanks. The reagent blank absorption spectrum of each method was also recorded against distilled water. The results are graphically presented in Figs. 3.03.
to 3.11, p.183-187. The absorption curves of coloured species formed in each method showed characteristic absorption maximum (Table 3.13, p.206-207) where as the blank in each method has low or no absorption in this region.

(ii) Parameters fixation

In developing these methods, a systematic study of the effects of various relevant parameters in the methods concerned were undertaken by varying one parameter at a time and controlling all other parameters to get maximum colour development, minimum blank colour, reproducibility and reasonable period of stability of final coloured species formed. The following studies were conducted.

**Method M₃, M₄**

The optimum conditions in these methods were fixed, basing on the study of the effects of various parameters such as type of buffer, concentration of dye (MB for method M₃, and SFN-O for method M₄), choice of organic solvent, ratio of organic phase to aqueous phase, shaking time and temperature, intensity and stability of the coloured species in organic phase by measuring the absorbances at appropriate λ<sub>max</sub> (Method M₃, 650 nm and Method M₄, 520 nm). The optimum conditions developed and the actual conditions chosen for the procedure are recorded in table 3.05, p.191-192.

**Method M₁₃**

The coloured species formation with maximum absorbance in this method depends on various factors such as volume of brucine, volume of NaIO₄, volume of H₂SO₄, time and temp, solvent for final dilution and stability period after dilution. The author performed control experiments varying one and fixing the other parameters and the resulting optimum conditions are presented in table 3.06, p.193.
Method $M_{15}$

This method involves the reaction of DSP with DCQC. The optimum conditions were fixed basing on the study of the effects of various parameters such as volume of DCQC solution and temperature for maximum colour development, solvent for final dilution and the stability of the coloured species after the final dilution. The author made a detailed study of above effects by measuring the absorbance at 620 nm and results were incorporated in table 3.07, p.194.

Method $M_{24}$

This method involves the condensation of DSP with vanillin in acid medium. The effect of various parameters, such as concentration and volume of vanillin, nature and strength of acid, order of addition of reagents, solvent for final dilution were studied. The optimum conditions developed and actual conditions chosen for the procedure are recorded in table 3.08, p.195.
Fig 3.03: Absorption spectrum of DSP with MB system ($M_3$)

$[\text{DSP}] = 4.25 \times 10^{-5} \text{M}$

$[\text{MB}] = 1.56 \times 10^{-5} \text{M}$

$pH = 9.8$

Fig 3.04: Absorption spectrum of DSP with SFN-O system ($M_4$)

$[\text{DSP}] = 4.25 \times 10^{-5} \text{M}$

$[\text{SFN-O}] = 1.43 \times 10^{-5} \text{M}$

$pH = 9.8$
Fig 3.05: Absorption spectrum of DSP with Brucine-IO$_4^-$ system ($M_{13}$)

- Test
- Blank

$[DSP] = 1.06 \times 10^{-4} M$

$[Brucine] = 6.00 \times 10^{-4} M$

$[H_2SO_4] = 1.84 \times 10^{-1} M$

Fig 3.06: Absorption spectrum of DSP with DCQC system ($M_{13}$)

- Test
- Blank

$[DSP] = 4.25 \times 10^{-3} M$

$[DCQC] = 1.52 \times 10^{-4} M$

$pH = 9.4$
Fig 3.07: Absorption spectrum of DSP with VN system (M24).

\[
\begin{align*}
[DSP] & = 8.50 \times 10^{-4} \text{M} \\
[PDAB] & = 5.36 \times 10^{-2} \text{M}
\end{align*}
\]

Fig 3.08: Absorption spectrum of DSP with PDAB system (M).
**Fig 3.09:** Absorption spectrum of DSP with NH/AA system ($M_{z1}$).

- Test: $[\text{DSP}] = 4.25 \times 10^{-5} \text{M}$
- Blank: $[\text{NH}] = 5.605 \times 10^{-3} \text{M}$
- $[\text{AA}] = 2.84 \times 10^{-4} \text{M}$

**Fig 3.10:** Absorption spectrum of DSP with NQS system ($M_{28}$).

- Test: $[\text{DSP}] = 8.50 \times 10^{-5} \text{M}$
- Blank: $[\text{NQS}] = 9.6 \times 10^{-7} \text{M}$
Fig 3.11: Absorption spectrum of DSP with SNP/Acetone system (M₃₂)

[DSP] = 8.50 \times 10^{-5} M

[SNP] = 1.01 \times 10^{-2} M
Method M25

This method involves the condensation of the DSP with PDAB in acid medium. The effect of various parameters, such as concentration and volume of PDAB, nature and strength of acid, order of addition of reagents, solvent for final dilution were studied by means of control experiments by varying one parameter at a time. The optimum conditions developed and actual conditions chosen for the procedure are recorded in table 3.09, p.196.

Method M27

This method involves the reaction between DSP and ninhydrin reagent to produce blue colour development. The conditions were fixed basing on the study of effects of various parameters such as volume of ninhydrin, nature and concentration of reducing agent, pH and volume of the buffer, heating time and temperature, order of addition of the reagents, solvent for final dilution and stability of the coloured products after final dilution. The optimum conditions were established by measuring the absorbances at 560 nm and the results are presented in table 3.10, p.197.

Method M28

In developing this method, a systematic study of the effects of various parameters were undertaken by varying one parameter at a time and controlling all others fixed. The effect of various parameters such as time, volume and strength of NQS and NaOH, solvent for final dilution on the stability and intensity of coloured species were studied by measure absorbance at 480 nm and the optimum conditions are incorporated in Table 3.11, p.198.

Method M32

This method involves the reaction of DSP with SNP in the presence of acetone. The optimum conditions in this method were fixed based on the study of
the effects of the various parameters such as volume of SNP solution, volume of acetone, nature and volume of base, order of addition of reagents, time and temperature of the reaction, solvent for final dilution, the intensity and stability of coloured species formed. The optimum conditions developed and the actual conditions chosen for the procedure are incorporated in table 3.12, p.199.

(iii) Optical Characteristics

In order to test whether the coloured species formed (or diminished) in the methods \(M_3, M_4, M_{13}, M_{15}, M_{24}, M_{25}, M_{27}, M_{28}\) and \(M_{32}\) adhere to Beer's law, the absorbance at appropriate wavelength of a set of solutions containing different amounts of DSP and specified amount of reagents (as described in the recommended procedures for each method) were noted against appropriate reagent blanks or distilled water. The Beer's law plots of the system were illustrated graphically in Fig. 3.12-3.20, p.200-202. Least square regression analysis was carried out for the slope, intercept and correlation coefficient. Beer's law limits, molar absorptivity, Sandell's sensitivity and optimum photometric range (Fig.3.21-3.29, p.203-205) for DSP with each of mentioned reagents were calculated. The optical characteristics are presented in table 3.13, p.206-207.

(iv) Precision

The precision of each one among nine proposed spectrophotometric methods were ascertained separately from the absorbance values obtained by actual determination of six replicates of a fixed amount of DSP \([M_3, M_4, M_{13}, M_{15}, M_{24}, M_{25}, M_{27}, M_{28}\) and \(M_{32}\) \([10 \mu g/ml, 10 \mu g/ml, 25 \mu g/ml, 10 \mu g/ml, 10 \mu g/ml, 20 \mu g/ml, 25 \mu g/ml, 20 \mu g/ml and 20 \mu g/ml]\) in final solution. The percent relative standard deviation and percent range of error (at 0.05 and 0.01 confidence limits) were calculated for the proposed methods and are presented in table 3.13, p.206-207.
(v) Accuracy

To determine the accuracy of the proposed methods, different amounts of bulk samples of DSP within the Beer’s law limits were taken and analyzed by the proposed methods. The results (percent error) are recorded in table 3.13, p.206-207.

(vi) Analysis of formulations

Commercial formulations (Table 3.03, p.172) containing DSP were successfully analyzed by each proposed method. The values obtained by the proposed and reference methods for formulations were compared statistically by the t and F- tests and found not to differ significantly.

(vii) Recovery studies

Recovery studies were conducted by analyzing each pharmaceutical formulation in the first instance for the active ingredient by the proposed methods. Known amount of pure drug was added to each previously analysed formulation and the total amount of the drug was once again determined by all proposed methods after bringing the active ingredient concentration within the Beer’s law limits. The results are reported in table 3.14, p.208-209.

(viii) Interferences

The effect of wide range of inactive ingredients usually present in the formulations for the assay of DSP under optimum conditions were investigated separately. In all the methods proposed, none of them interfered in the methods proposed even when they are present in excess fold than anticipated in pharmaceutical formulations.
Variations of the pH < 6 and > 11 resulted in low absorbance values. 1.0 ml of buffer is preferable for maintenance of pH 9.8. 5 ml of dye was necessary for covering the broad range of Beer's law limits.

The other water immiscible solvents tested for the extraction of the coloured complex into organic phase include chlorobenzene, dichloromethane, carbon tetrachloride, n-butanol, benzene, CHCl₃ was preferred for it's selectivity extraction of the complex from the aqueous phase. The extraction of the coloured species into chloroform layer was incomplete when the ratio of aqueous to CHCl₃ phase was more than the specified ratio in each case.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum range</th>
<th>Conditions in procedure</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>640-660 (M₃)</td>
<td>650 for M₃</td>
<td></td>
</tr>
<tr>
<td></td>
<td>510-530 (M₄)</td>
<td>520 for M₄</td>
<td></td>
</tr>
<tr>
<td>Effect of buffer pH on colour development</td>
<td>9.0-10.0 (M₃, M₄) (NH₃ buffer)</td>
<td>pH-9.8 (M₃, M₄)</td>
<td>Variations of the pH &lt; 6 and &gt; 11 resulted in low absorbance values.</td>
</tr>
<tr>
<td>Vol. of buffer required for maximum intensity of colour</td>
<td>0.5-1.5 ml buffer</td>
<td>1.0 ml of buffer (M₃, M₄)</td>
<td>1.0 ml of buffer is preferable for maintenance of pH 9.8.</td>
</tr>
<tr>
<td>Effect of vol. of 3.12×10⁻⁴ M MB 2.85×10⁻⁴ M SFN-O</td>
<td>4.0-6.0 ml (for M₃, M₄)</td>
<td>5.0 ml (for M₃, M₄)</td>
<td>5 ml of dye was necessary for covering the broad range of Beer's law limits.</td>
</tr>
<tr>
<td>Choice of organic solvent for the extraction of the coloured species</td>
<td>Chloroform</td>
<td>Chloroform</td>
<td></td>
</tr>
<tr>
<td>Effect of the ratio of aqueous to organic phase on extraction</td>
<td>1:1 (for M₃, M₄)</td>
<td>1:1 (for M₃, M₄)</td>
<td>The extraction of the coloured species into chloroform phase was more than the specified ratio in each case.</td>
</tr>
</tbody>
</table>
Constant absorbance values were obtained for the shaking period 3-8 min.

At low temp. (<20°C) the extraction of the coloured species was found to be improper. At high temp. (>35°C) the stability of the coloured species was found to be less. The coloured species after separation from organic layer was stable for 60 min, afterwards the absorbance gradually decreased.

<table>
<thead>
<tr>
<th>Effect of shaking time</th>
<th>3-8 min.</th>
<th>5 min.</th>
<th>Constant absorbance values were obtained for the shaking period 3-8 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of temp. on the coloured species.</td>
<td>Laboratory temp. (28±2°C)</td>
<td>Laboratory Temp.</td>
<td>At low temp. (&lt;20°C) the extraction of the coloured species was found to be improper. At high temp. (&gt;35°C) the stability of the coloured species was found to be less.</td>
</tr>
<tr>
<td>Stability of the coloured species.</td>
<td>1-60 min.</td>
<td>5 min.</td>
<td>The coloured species after separation from organic layer was stable for 60 min, afterwards the absorbance gradually decreased.</td>
</tr>
</tbody>
</table>
Variation in volume of brucine beyond the upper and lower limits resulted in low absorbance values. 

2.0 ml of 2.3 M H₂SO₄ was necessary to attain maximum colour and stability.

It was found that heating for 15 min. on boiling water bath is necessary for uniform temperature and maximum colour development. Below this temperature and time (10 min.) the intensity of colour is less.

If the order of addition was changed, the absorbance of coloured species decreased.

The absorbance was found to decrease when water miscible solvents like MeOH, ethanol, IPA, and acetonitrile were used.

After that stability period the intensity of coloured species was found to decrease with time.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum range</th>
<th>Conditions in procedure</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>λₘₐₓ (nm)</td>
<td>520 – 530</td>
<td>520</td>
<td>---------</td>
</tr>
<tr>
<td>Effect of volume of (5.0x10⁻³ M) brucine solution on colour development.</td>
<td>2.5 – 3.5 ml</td>
<td>3.0 ml</td>
<td>Variation in volume of brucine beyond the upper and lower limits resulted in low absorbance values.</td>
</tr>
<tr>
<td>Effect of volume of (9.35x10⁻³ M) NaIO₄ solution on colour development.</td>
<td>1.0 – 2.0 ml</td>
<td>1.5 ml</td>
<td>&lt;1.0 ml of NaIO₄ results in decrease of absorbance and &gt;2.0 ml results in development of intense rose colour in the blank.</td>
</tr>
<tr>
<td>Effect of vol. of 2.3 M H₂SO₄ on colour development.</td>
<td>1.5 – 2.5 ml</td>
<td>2.0 ml</td>
<td>2.0 ml of 2.3 M H₂SO₄ was necessary to attain maximum colour and stability.</td>
</tr>
<tr>
<td>Nature of oxidant.</td>
<td>NaIO₄</td>
<td>NaIO₄</td>
<td>Other oxidants tried such as Fe(III), Cr(VI), IO₃⁻ and S₂O₅²⁻ were found to be inferior over IO₄⁻.</td>
</tr>
<tr>
<td>Effect of time and temperature.</td>
<td>10 – 20 min. on boiling water bath.</td>
<td>15 min. on boiling water bath.</td>
<td>It was found that heating for 15 min. on boiling water bath is necessary for uniform temperature and maximum colour development. Below this temperature and time (10 min.) the intensity of colour is less.</td>
</tr>
<tr>
<td>Order of addition of reagents on colour development.</td>
<td>Drug, brucine, oxidant.</td>
<td>Drug, brucine, oxidant.</td>
<td>If the order of addition was changed, the absorbance of coloured species decreased.</td>
</tr>
<tr>
<td>Solvent for final dilution.</td>
<td>Water</td>
<td>Water</td>
<td>The absorbance was found to decrease when water miscible solvents like MeOH, ethanol, IPA, and acetonitrile were used.</td>
</tr>
<tr>
<td>Stability period after final volume.</td>
<td>Immediate – 40 min.</td>
<td>5 min.</td>
<td>After that stability period the intensity of coloured species was found to decrease with time.</td>
</tr>
</tbody>
</table>
TABLE 3.07
OPTIMUM CONDITIONS ESTABLISHED FOR METHOD M₁₅

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum range</th>
<th>Conditions in procedure</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>610 – 630</td>
<td>620</td>
<td>--</td>
</tr>
<tr>
<td>Effect of volume of 1.90x10⁻³ M DCQC in IPA</td>
<td>1.5 – 2.5 ml</td>
<td>2.0 ml</td>
<td>Two ml of the DCQC solution in isopropanol was found necessary for maximum colour development and to cover broad range of Beer’s law limits. Increasing the volume of DCQC further has no added advantage.</td>
</tr>
<tr>
<td>pH</td>
<td>9.0-10.0</td>
<td>pH-9.4</td>
<td>Variations of the pH&lt;6 and &gt;11 resulted in low absorbance values.</td>
</tr>
<tr>
<td>Vol. of buffer required for maximum intensity</td>
<td>5.0-7.0 ml buffer</td>
<td>5.0 ml of buffer</td>
<td>5.0 ml. of buffer is preferable for maintenance of pH 9.4.</td>
</tr>
<tr>
<td>of colour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>Lab temp. (28±5°C)</td>
<td>Lab temp. (28±5°C)</td>
<td>At low temp. (&lt;25°C) the reaction time was found to be more. At high temp. (&lt;32°C) no added advantage was observed.</td>
</tr>
<tr>
<td>Solvent for final dilution</td>
<td>Distilled water</td>
<td>Distilled water</td>
<td>The absorbance of the coloured species has been found less.</td>
</tr>
<tr>
<td>Stability of the coloured species after final</td>
<td>5 – 60 min</td>
<td>15 min.</td>
<td>The absorbance of the coloured product decreased slowly with time after 60 min.</td>
</tr>
<tr>
<td>dilution</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 3.08
OPTIMUM CONDITIONS ESTABLISHED FOR METHOD M24

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum range</th>
<th>Conditions in procedure</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>550 - 570</td>
<td>560</td>
<td>---------</td>
</tr>
<tr>
<td>Volume of 2.63x10^{-2}M Vanillin for colour development</td>
<td>1.5 - 3.0 ml</td>
<td>2.0 ml</td>
<td>Two ml of 2.63x10^{-2}M Vanillin was necessary for covering broad range of Beer’s law limits.</td>
</tr>
<tr>
<td>Effect of volume of conc. H$_2$SO$_4$ on colour development</td>
<td>2.0 - 4.0 ml</td>
<td>3.0 ml</td>
<td>&lt; 1.5 ml Conc. H$_2$SO$_4$ results in low absorbance values and &gt;4.0 ml results in instability of the coloured product.</td>
</tr>
<tr>
<td>Effect of the order of addition of reagents on colour development</td>
<td>DSP, Vanillin, Conc. H$_2$SO$_4$</td>
<td>DSP, Vanillin, Conc. H$_2$SO$_4$</td>
<td>If the order of addition is changed, low absorbance values resulted.</td>
</tr>
<tr>
<td>Effect of temperature and time</td>
<td>40 - 50°C 10 - 20 min.</td>
<td>50°C 15 min.</td>
<td>Above 50°C methanol evaporates.</td>
</tr>
<tr>
<td>Solvent for final dilution</td>
<td>Methanol</td>
<td>Methanol</td>
<td>The absorbance of the test solution decreased when water was used instead of Methanol for final dilution.</td>
</tr>
<tr>
<td>Stability period after final dilution</td>
<td>Immediate - 30 min.</td>
<td>5 min.</td>
<td>---------</td>
</tr>
</tbody>
</table>
### TABLE 3.09
OPTIMUM CONDITIONS ESTABLISHED FOR METHOD M\textsubscript{25}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum range</th>
<th>Conditions in procedure</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>460 – 500</td>
<td>480</td>
<td></td>
</tr>
<tr>
<td>Volume of 2.68x10$^{-2}$ M PDAB for colour development</td>
<td>1.5 – 3.0 ml</td>
<td>2.0 ml</td>
<td>Two ml of PDAB was found necessary for covering broad range of Beer’s law limits.</td>
</tr>
<tr>
<td>Effect of volume of conc. H$_2$SO$_4$ on colour development.</td>
<td>2.5 – 3.5 ml</td>
<td>3.0 ml</td>
<td>&lt; 2.5 ml Conc. H$_2$SO$_4$ results in low absorbance values and &gt;3.5 ml results in instability of the coloured species.</td>
</tr>
<tr>
<td>Effect of the order of addition of reagents on colour development.</td>
<td>DSP, PDAB, Conc. H$_2$SO$_4$</td>
<td>DSP, PDAB, Conc. H$_2$SO$_4$</td>
<td>If the order of addition is changed the absorbance values are less.</td>
</tr>
<tr>
<td>Effect of temperature and time</td>
<td>40 – 50°C</td>
<td>50°C</td>
<td>Above 50°C methanol evaporates.</td>
</tr>
<tr>
<td>Solvent for final dilution</td>
<td>Methanol</td>
<td>Methanol</td>
<td>The absorbance of the test solution decreased when water was used instead of Methanol for dilution.</td>
</tr>
<tr>
<td>Stability period after final dilution.</td>
<td>Immediate – 30 min.</td>
<td>5 min.</td>
<td></td>
</tr>
</tbody>
</table>
Remarks

One ml of NH was found to be necessary for colour product formation and to cover broad range of Beer's law limits. No added advantage was observed even when excess ninhydrin was used.

Four ml of pH 5.0 buffer was found to be optimum for maximum colour development.

Among the two reducing agents tried (AA, SnCl₂), AA was found to be more efficient with respect to stability attainment. Half ml of AA was found to be adequate for maximum colour development.

Order of addition has no significant effect.

Heating in boiling water bath for 15 min. was required to obtain better results (sensitivity and reproducibility).

No advantage was observed with usage of other water miscible solvents (acetone, MeOH) instead of water.

The absorbance of the coloured product decreased slowly with time after 1 hr.

### TABLE 3.10

**OPTIMUM CONDITIONS ESTABLISHED FOR METHOD M₂₇**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum range</th>
<th>Conditions in procedure</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</td>
<td>550 - 570</td>
<td>560</td>
<td></td>
</tr>
<tr>
<td>Volume of 5.60x10⁻²M ninhydrin in acetone required</td>
<td>0.8 - 1.2 ml</td>
<td>1.0 ml</td>
<td>One ml of NH was found to be necessary for colour product formation and to cover broad range of Beer's law limits. No added advantage was observed even when excess ninhydrin was used.</td>
</tr>
<tr>
<td>Volume and pH of the buffer</td>
<td>3.5 - 4.5 ml</td>
<td>4.0 ml</td>
<td>Four ml of pH 5.0 buffer was found to be optimum for maximum colour development.</td>
</tr>
<tr>
<td>pH 4.8 - 5.2</td>
<td>pH 5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nature of the reducing agent</td>
<td>AA</td>
<td>AA</td>
<td>Among the two reducing agents tried (AA, SnCl₂), AA was found to be more efficient with respect to stability attainment.</td>
</tr>
<tr>
<td>Volume of 5.678x10⁻³M AA solution</td>
<td>0.4 - 0.6 ml</td>
<td>0.5 ml</td>
<td>Half ml of AA was found to be adequate for maximum colour development.</td>
</tr>
<tr>
<td>Order of addition</td>
<td>Buffer - NH</td>
<td>Buffer - NH</td>
<td>Order of addition has no significant effect.</td>
</tr>
<tr>
<td>- AA</td>
<td>- AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time and temperature for maximum colour development</td>
<td>10 - 20 min boiling water bath</td>
<td>15 min boiling water bath</td>
<td>Heating in boiling water bath for 15 min. was required to obtain better results (sensitivity and reproducibility)</td>
</tr>
<tr>
<td>Solvent for final dilution</td>
<td>Water</td>
<td>Water</td>
<td>No advantage was observed with usage of other water miscible solvents (acetone, MeOH) instead of water.</td>
</tr>
<tr>
<td>Stability of the coloured species after final dilution</td>
<td>5 - 60 min.</td>
<td>10 min.</td>
<td>The absorbance of the coloured product decreased slowly with time after 1 hr.</td>
</tr>
</tbody>
</table>
### TABLE 3.11

**OPTIMUM CONDITIONS ESTABLISHED FOR METHOD M$_{28}$**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum range</th>
<th>Conditions in procedure</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>480 – 490</td>
<td>480</td>
<td>-------</td>
</tr>
<tr>
<td>Effect of NQS on colour development (1.92x10^{-2}M)</td>
<td>0.25 – 0.75 ml</td>
<td>0.5 ml</td>
<td>The use of &lt;0.25 ml NQS resulted in a decrease in absorbance at 70°&gt;0.75 ml resulted in cloudiness.</td>
</tr>
<tr>
<td>Effect of NaOH, 5M on the absorbance of the final coloured species</td>
<td>1.4 – 2.8 ml</td>
<td>2.0 ml</td>
<td>&lt; 1.4 ml and &gt; 2.8 ml was found to disturb Beer's law obeyance in a broad range.</td>
</tr>
<tr>
<td>Solvent for final dilution</td>
<td>Water</td>
<td>Water</td>
<td>Other water miscible solvent did not enhance the colour of final coloured solution.</td>
</tr>
<tr>
<td>Stability period of the coloured species after final dilution.</td>
<td>8.0 – 45 min.</td>
<td>10 min.</td>
<td>Eight minutes was necessary for the attainment of maximum colour. It remained stable for a further period of 45 minutes.</td>
</tr>
</tbody>
</table>
### TABLE 3.12

**OPTIMUM CONDITIONS ESTABLISHED FOR METHOD M<sub>32</sub>**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum conditions</th>
<th>Conditions in Procedure</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{\text{max}} )</td>
<td>545-555</td>
<td>550</td>
<td>——</td>
</tr>
<tr>
<td>Effect of volume of acetone on the coloured product</td>
<td>0.5-0.75 ml</td>
<td>0.5 ml</td>
<td>&lt;0.5 ml of acetone colour production is less. &gt;0.75 ml absorbance of the blank will be increased.</td>
</tr>
<tr>
<td>Effect of volume of 3.38x10&lt;sup&gt;-1&lt;/sup&gt; M SNP solution on the coloured species.</td>
<td>0.15-0.65 ml</td>
<td>0.3 ml</td>
<td>Addition of SNP (3.38x10&lt;sup&gt;-1&lt;/sup&gt;M) beyond the optimum range resulted in non-linear colour production</td>
</tr>
<tr>
<td>Effect of volume of saturated solution of sodium tetrahydroborate decahydrate</td>
<td>1-3 ml</td>
<td>2 ml</td>
<td>Addition of 2 ml of the sodium tetrahydroborate decahydrate is enough to colour development</td>
</tr>
<tr>
<td>Reaction time</td>
<td>45-75 min.</td>
<td>60 min.</td>
<td>——</td>
</tr>
<tr>
<td>Solvent for final dilution</td>
<td>Distilled water</td>
<td>Distilled water</td>
<td>——</td>
</tr>
<tr>
<td>Stability of the coloured species after final dilution</td>
<td>5-60 min.</td>
<td>15 min.</td>
<td>The intensity of coloured product begins to decrease slowly after 60 min.</td>
</tr>
</tbody>
</table>
Fig. 3.12: Beer's law plot of DSP with MB system (M₉).

- [MB] = 1.56 x 10⁻⁴ M
- pH = 9.8

Fig. 3.13: Beer's law plot of DSP with SFN-O system (M₉).

- [SFN-O] = 1.43 x 10⁻⁷ M
- pH = 9.8

Fig. 3.14: Beer's law plot of DSP with Brucine - IO⁻₇ system (M₉).

- [Brucine] = 6.00 x 10⁻⁴ M
- [H₂SO₄] = 1.84 x 10⁻⁷ M

Fig. 3.15: Beer's law plot of DSP with DCQC system (M₉).

- [DCQC] = 1.52 x 10⁻⁷ M
- pH = 9.4
Fig. 3.16: Beer's law plot of DSP with VN system ($M_N$)

Fig. 3.17: Beer's law plot of DSP with PDAB system ($M_{pd}$)

Fig. 3.18: Beer's law plot of DSP with NH/AA system ($M_{nh}$)

Fig. 3.19: Beer's law plot of DSP with NQS system ($M_{qs}$)
Fig. 3.20: Beer's law plot of DSP with SNP/CH₃COCH₃ system (M₃₂)

Concentration (μg/ml)

Absorbance

[SNP] = 1.01 x 10⁻² M
Fig 3.21: Ringbom plot of DSP with MB system (M).  

Fig 3.22: Ringbom plot of DSP with SFN-O system (M).  

Fig 3.23: Ringbom plot of DSP with Brucine-IO system (M).  

Fig 3.24: Ringbom plot of DSP with DCQC system (M).
Fig 3.25: Ringbom plot of DSP with VN system (M

Fig 3.26: Ringbom plot of DSP with PDAB system (M

Fig 3.27: Ringbom plot of DSP with NH/AA system (M

Fig 3.28: Ringbom plot of DSP with NQS system (M

204
Fig 3.29: Ringbom plot of DSP with SNP-Acetone system (M32).
TABLE 3.13
OPTICAL CHARACTERISTICS, PRECISION, ACCURACY OF THE METHODS PROPOSED IN THE DETERMINATION OF DISODIUMPAMIDRONATE

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Optical characteristics</th>
<th>M₃</th>
<th>M₄</th>
<th>M₁₃</th>
<th>M₁₅</th>
<th>M₂₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MB</td>
<td>SFN-O</td>
<td>Brucine-IO₄⁻</td>
<td>DCQC</td>
<td>VN</td>
</tr>
<tr>
<td>1</td>
<td>λ_max (nm)</td>
<td>650</td>
<td>520</td>
<td>520</td>
<td>620</td>
<td>560</td>
</tr>
<tr>
<td>2</td>
<td>Beer's law limits (µg ml⁻¹)</td>
<td>2-10</td>
<td>2-10</td>
<td>5-25</td>
<td>2-10</td>
<td>2-10</td>
</tr>
<tr>
<td>3</td>
<td>Molar absorptivity (1 mole⁻¹ cm⁻¹)</td>
<td>1.43x10⁴</td>
<td>1.39x10⁴</td>
<td>5.90x10⁻³</td>
<td>1.08x10⁴</td>
<td>1.37x10⁴</td>
</tr>
<tr>
<td>4</td>
<td>Correlation coefficient (r)</td>
<td>0.9999</td>
<td>0.9998</td>
<td>0.9998</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
<tr>
<td>5</td>
<td>Sandell's sensitivity (µg/cm²/0.001 absorbance unit)</td>
<td>0.0163</td>
<td>0.0168</td>
<td>0.0398</td>
<td>0.0216</td>
<td>0.0171</td>
</tr>
<tr>
<td>6</td>
<td>Regression equation (Y = a+bc)</td>
<td>0.061</td>
<td>0.059</td>
<td>0.025</td>
<td>0.046</td>
<td>0.0058</td>
</tr>
<tr>
<td></td>
<td>(i) Slope (b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ii) Standard deviation on slope (Sb)</td>
<td>3.57x10⁻⁴</td>
<td>6.21x10⁻⁴</td>
<td>1.65x10⁻⁴</td>
<td>2.20x10⁻⁴</td>
<td>4.90x10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>(iii) Intercept (a)</td>
<td>0.001</td>
<td>0.00003</td>
<td>0.00001</td>
<td>0.00005</td>
<td>0.00005</td>
</tr>
<tr>
<td></td>
<td>(iv) Standard deviation on intercept(Sa)</td>
<td>2.37x10⁻³</td>
<td>4.12x10⁻³</td>
<td>2.75x10⁻³</td>
<td>1.45x10⁻³</td>
<td>3.25x10⁻³</td>
</tr>
<tr>
<td></td>
<td>(v) Standard error of estimation (Se)</td>
<td>2.26x10⁻³</td>
<td>3.93x10⁻³</td>
<td>2.62x10⁻³</td>
<td>1.39x10⁻³</td>
<td>3.10x10⁻³</td>
</tr>
<tr>
<td>7</td>
<td>Optimum photometric range (µg/ml)</td>
<td>4.16-12.58</td>
<td>4.46-10</td>
<td>10-28.18</td>
<td>4.46-12.58</td>
<td>4.16-13.18</td>
</tr>
<tr>
<td>8</td>
<td>Relative standard deviation *</td>
<td>0.438</td>
<td>0.463</td>
<td>0.44</td>
<td>0.447</td>
<td>0.52</td>
</tr>
<tr>
<td>9</td>
<td>% Range of error (Confidence limits)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(i) 0.05 level</td>
<td>0.46</td>
<td>0.486</td>
<td>0.466</td>
<td>0.469</td>
<td>0.545</td>
</tr>
<tr>
<td></td>
<td>(ii) 0.01 level</td>
<td>0.722</td>
<td>0.762</td>
<td>0.731</td>
<td>0.736</td>
<td>0.856</td>
</tr>
<tr>
<td>10</td>
<td>% Error in bulk samples**</td>
<td>0.268</td>
<td>0.197</td>
<td>0.208</td>
<td>0.185</td>
<td>0.179</td>
</tr>
</tbody>
</table>
## Optical Characteristics

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Optical characteristics</th>
<th>M_{25}</th>
<th>M_{27}</th>
<th>M_{28}</th>
<th>M_{32}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PDAB</td>
<td>NH/AA</td>
<td>NQS</td>
<td>SNP-CH₃COCH₃</td>
</tr>
<tr>
<td>1</td>
<td>λ_{max} (nm)</td>
<td>480</td>
<td>560</td>
<td>480</td>
<td>550</td>
</tr>
<tr>
<td>2</td>
<td>Beer's law limits (µg ml⁻¹)</td>
<td>4-20</td>
<td>5-25</td>
<td>4-20</td>
<td>4-20</td>
</tr>
<tr>
<td>3</td>
<td>Molar absorptivity (1 mole⁻¹ cm⁻¹)</td>
<td>6.18x10⁻²</td>
<td>4.64x10⁻²</td>
<td>6.19x10⁻²</td>
<td>7.57x10⁻³</td>
</tr>
<tr>
<td>4</td>
<td>Correlation coefficient (r)</td>
<td>0.9998</td>
<td>0.9999</td>
<td>0.9998</td>
<td>0.9999</td>
</tr>
<tr>
<td>5</td>
<td>Sandell's sensitivity (µg/cm²/0.001 absorbance unit)</td>
<td>0.0379</td>
<td>0.0505</td>
<td>0.0379</td>
<td>0.0310</td>
</tr>
<tr>
<td>6</td>
<td>Regression equation (Y= a+bc)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(i) Slope (b)</td>
<td>0.026</td>
<td>0.019</td>
<td>0.026</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>(ii) Standard deviation on slope (S_b)</td>
<td>4.49x10⁻⁴</td>
<td>10.64x10⁻⁴</td>
<td>4.80x10⁻⁴</td>
<td>3.13x10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>(iii) Intercept (a)</td>
<td>0.00004</td>
<td>0.00007</td>
<td>0.00003</td>
<td>0.00002</td>
</tr>
<tr>
<td></td>
<td>(iv) Standard deviation on intercept (S_a)</td>
<td>5.96x10⁻³</td>
<td>17.64x10⁻³</td>
<td>6.36x10⁻³</td>
<td>4.15x10⁻³</td>
</tr>
<tr>
<td></td>
<td>(v) Standard error of estimation (S_e)</td>
<td>5.68x10⁻³</td>
<td>16.82x10⁻³</td>
<td>6.07x10⁻³</td>
<td>3.96x10⁻³</td>
</tr>
<tr>
<td>7</td>
<td>Optimum photometric range (µg/ml)</td>
<td>7.94-25.11</td>
<td>10-28.84</td>
<td>8.91-25.11</td>
<td>7.94-23.98</td>
</tr>
<tr>
<td>8</td>
<td>Relative standard deviation *</td>
<td>0.528</td>
<td>0.441</td>
<td>0.582</td>
<td>0.473</td>
</tr>
<tr>
<td>9</td>
<td>% Range of error (Confidence limits)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(i)0.05 level</td>
<td>0.554</td>
<td>0.463</td>
<td>0.611</td>
<td>0.496</td>
</tr>
<tr>
<td></td>
<td>(ii)0.01 level</td>
<td>0.869</td>
<td>0.727</td>
<td>0.958</td>
<td>0.778</td>
</tr>
<tr>
<td>10</td>
<td>% Error in bulk samples**</td>
<td>0.213</td>
<td>0.224</td>
<td>0.264</td>
<td>0.225</td>
</tr>
</tbody>
</table>

*Average of six determinations considered. ** Average of three determinations.
### TABLE 3.14
DETERMINATION OF DSP IN PHARMACEUTICAL FORMULATIONS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Labeled amount (mg)</th>
<th>Amount found by proposed Methods*</th>
<th>Ref. method</th>
<th>% Recovery by proposed Methods**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M₃</td>
<td>M₄</td>
<td>M₁₃</td>
</tr>
<tr>
<td>INJ I</td>
<td>30</td>
<td>29.11±</td>
<td>29.82±</td>
<td>29.67±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.31</td>
<td>±0.37</td>
<td>±0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=1.06</td>
<td>F=1.52</td>
<td>F=1.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t=0.99</td>
<td>t=1.37</td>
<td>t=1.73</td>
</tr>
<tr>
<td>INJ II</td>
<td>60</td>
<td>59.78±</td>
<td>59.92±</td>
<td>59.83±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.22</td>
<td>±0.17</td>
<td>±0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=1.49</td>
<td>F=1.12</td>
<td>F=1.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t=1.00</td>
<td>t=1.16</td>
<td>t=1.05</td>
</tr>
<tr>
<td>INJ III</td>
<td>90</td>
<td>89.72±</td>
<td>90.19±</td>
<td>89.62±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.40</td>
<td>±0.31</td>
<td>±0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=2.36</td>
<td>F=1.42</td>
<td>F=1.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t=1.11</td>
<td>t=0.84</td>
<td>t=0.56</td>
</tr>
<tr>
<td>INJ IV</td>
<td>90</td>
<td>89.17±</td>
<td>89.93±</td>
<td>90.18±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.20</td>
<td>±0.17</td>
<td>±0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=1.38</td>
<td>F=1.00</td>
<td>F=1.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t=0.83</td>
<td>t=0.90</td>
<td>t=1.00</td>
</tr>
</tbody>
</table>

Labeled amount (mg)
Table 3.14 (Contd..)

% Recovery by proposed Methods**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Labeled amount (mg)</th>
<th>Amount found by proposed Methods*</th>
<th>Ref. Method</th>
<th>% Recovery by proposed Methods**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$M_{25}$</td>
<td>$M_{27}$</td>
<td>$M_{28}$</td>
</tr>
<tr>
<td>INJ I</td>
<td>30</td>
<td>29.67 ± 0.30</td>
<td>29.66 ± 0.36</td>
<td>29.93 ± 0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=1.00</td>
<td>F=1.44</td>
<td>F=1.33</td>
</tr>
<tr>
<td>INJ II</td>
<td>60</td>
<td>59.02 ± 0.13</td>
<td>59.08 ± 0.23</td>
<td>60.18 ± 0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=1.91</td>
<td>F=1.63</td>
<td>F=1.49</td>
</tr>
<tr>
<td>INJ III</td>
<td>90</td>
<td>89.61 ± 0.30</td>
<td>90.16 ± 0.33</td>
<td>89.74 ± 0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=1.33</td>
<td>F=1.61</td>
<td>F=1.08</td>
</tr>
<tr>
<td>INJ IV</td>
<td>90</td>
<td>89.37 ± 0.22</td>
<td>89.82 ± 0.23</td>
<td>89.17 ± 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F=1.67</td>
<td>F=1.83</td>
<td>F=1.12</td>
</tr>
</tbody>
</table>

*Average ± standard deviation of six determinations; the t- and F- values refer to comparison of the proposed method with the reference method. Theoretical values at 95% confidence limit, $t = 2.57$, $F = 5.05$.

**After adding 3 different amounts of the pure labeled to the pharmaceutical formulation, each value is an average of 3 determinations.
Nature of the coloured species

As Disodium pamidronate (DSP) possesses functional groups such as bis phosphonate, aliphatic primary amine and hydroxyl moieties, new methods have been developed for its determination (Chapter I, Table 1.03, p.15-17). The reviews concerning the reagent used for colour development by exploiting appropriate functional moiety of Disodium pamidronate are presented in chapter I. An attempt has been made to indicate the nature of coloured species in each proposed method for DSP tentatively based on analogy (reactive functional moiety in drug, reagent nature) and probability (relative reactivities and impact of functional moieties one over the other).

Methods: M₃, M₄

DSP possesses carboxylic acid in 4 - oxo - 3 - quinoline carboxylic acid portion and it involves in ion association complex formation with a basic dye (MB, M₃; SFN-O, M₄) which is extractable into chloroform from the aqueous phase. The anion form of carboxylic acid (negative charge) of DSP molecule in alkaline pH medium is expected to attract the oppositely charged part (positive charge) of the dye and behave as a single unit being held together by electrostatic forces of attraction. The slope ratio study revealed that the drug to dye mole ratio is 1:2. Based on analogy the structure of ion association complexes are shown in scheme 3.01, p.211.
Method: M_{13}

The dimethoxy benzene nucleus of brucine is attacked by IO_{4}^{-} with the formation of o-quinone (bruciquinone), which in turn undergoes nucleophillic attack on the most electron rich portion of the coupler (i.e imino substituent in DSP), to give 1-mono substituted bruciquinone derivative. The reaction of DSP with brucine in the presence of IO_{4}^{-} is described in scheme 3.02, p.212.
Scheme 3.02
Method: $M_{15}$

The colour formation by DCQC with DSP (which possesses amino group) may be explained in scheme 3.03.

\[
\begin{align*}
\text{ CHO} & \quad \text{OCH}_3 \\
\text{ OH} & \quad + \quad \text{ H}_2\text{N} \\
\end{align*}
\]

\[
\begin{align*}
\text{ HC= N} & \quad \text{R} \\
\text{ OH} & \quad + \quad \text{ H}_2\text{N} \\
\end{align*}
\]

$R$ : same as given under scheme 3.02, p. 212.

Scheme 3.03

Method $M_{24}$:

In the present work, aliphatic primary amine group in DSP condenses with vanillin. The coloured species may be postulated as follows. The scheme of reaction is shown below.

\[
\begin{align*}
\text{ CHO} & \quad \text{OCH}_3 \\
\text{ OH} & \quad + \quad \text{ H}_2\text{N} \\
\end{align*}
\]

\[
\begin{align*}
\text{ HC= N} & \quad \text{R} \\
\text{ OH} & \quad + \quad \text{ H}_2\text{N} \\
\end{align*}
\]

$R$ : same as given under scheme 3.02, p. 212.

Scheme 3.04
**Method: M_{25}**

The primary amino group in DSP involves in condensation reaction with PDAB giving coloured Schiff's base.

\[ \text{CHO} \quad + \quad \text{H}_2\text{N} \quad \overset{\text{R}}{\longrightarrow} \quad \text{HC} = \text{N} \quad \overset{\text{R}}{\longrightarrow} \]

\[ \text{N(CH}_3\text{)}_2 \quad \text{N(CH}_3\text{)}_2 \]

\[ \text{R} : \text{same as given under scheme 3.02, p. 212.} \]

**Scheme 3.05**

**Method: M_{27}**

In the present investigation, the drug DSP, which possess primary amino group, when heated with ninhydrin in presence of ascorbic acid afforded a blue colour product. The reaction pathway can be represented in scheme 3.06.

\[ 2 \quad \text{CO} \quad \text{C} \quad \text{OH} \quad + \quad \text{H}_2\text{N} \quad \overset{\text{R}}{\longrightarrow} \quad \text{COCOOH} \quad + \]

\[ \text{COOH} \]

\[ \text{O}^- \]

\[ \text{C} \quad \text{C} \quad \overset{\text{R}}{\longrightarrow} \quad \text{N} \quad \overset{\text{R}}{\longrightarrow} \quad \text{CHNH} \quad \overset{\text{R}}{\longrightarrow} \quad \text{H}_2\text{O} \]

\[ \text{CO} \quad \text{OC} \]

\[ \text{R} : \text{same as given under scheme 3.02, p. 212.} \]

**Scheme 3.06**

214
Method: M$_{28}$

In this method, the presence of amino group of DSP permits the development of new spectrophotometric method for its determination (Method M$_{28}$) through the nucleophilic substitution reaction with NQS (scheme 3.07).

\[
\begin{align*}
\text{SO}_3\text{Na} & \quad + \quad \text{H}_2\text{N} & \rightarrow & \quad \text{HN} \\
\text{R} & \quad \text{H} & \quad \text{R} & \quad \text{H} \\
\end{align*}
\]

$\text{R}$ : same as given under scheme 3.02, p. 212.

Scheme 3.07

Methods: M$_{32}$

Certain derivatives of ammonia which contain the primary amino group (aliphatic primary amine, hydroxyl amine, phenyl hydrazine or semicarbazide) add to the carbonyl group of ketone (acetone to form unstable intermediates) loose a molecule of water to yield respective condensation product giving colour with SNP. In the present investigation, the coloured product formed with drug on treatment with SNP - CH$_3$COCH$_3$ appears to be inner complex formation involving SNP and the condensation product in scheme 3.08.
\[
\text{Scheme 3.08}
\]

\[
[\text{Fe(CN)}_5 \text{NO}]^{2+} (\text{Na}^+) \text{$_2$} \xrightarrow{\text{Alkali}} [\text{Fe(CN)}_5 \text{H}_2\text{O}]^{3-}
\]

\[
[\text{Fe(CN)}_5 \text{H}_2\text{O}]^{3-} + \text{R} \xrightarrow{\text{C = N}} \left[ \begin{array}{c}
\text{R} \\
\text{C = N} \\
\text{Fe(CN)}_5
\end{array} \right]^{3-}
\]

\(\text{R} : \text{same as given under scheme 3.02, p. 212.}\)
PART B
HPLC METHOD FOR THE DETERMINATION OF DISODIUM PAMIDRONATE

Introduction:

The purpose of a HPLC method is to enable quantification of a drug under a variety of physical, chemical and photochemical stress conditions. It is required that the method is stability specific for the drug, i.e., the drug peak is separated from all its degradation products. Also, the linearity and the minimum level of quantification of the method should be established. The method should be as simple to perform as possible. The method should be a rugged one i.e., it should tolerate minor variations in experimental conditions and can be run easily by an average chromatographer. Isocratic methods are preferable to gradient methods. Room temperature is the first choice, HPLC columns are a key component in quality control procedures for pharmaceutical products, so columns must be able to reproducibly separate the components of the typically complex samples.

Even though four HPLC assay procedures have been reported for the determination of DSP, many of them concern with biological fluid samples and very few in pharmaceutical formulations. This part of the investigation reports a sensitive and precise HPLC method for the determination of DSP in bulk samples and pharmaceutical formulations by using reversed-phase chromatography (with Hypersil BDS phenyl), mobile phase (Methanol: Buffer) and no internal standard was used.

EXPERIMENTAL

Instrumentation:

Quantitative HPLC was performed on a gradient High Pressure Liquid Chromatograph (Shimadzu HPLC Class VP series) with LC-10AD VP Liquid
Chromatographic pump, DGU 14A Mobile phase degasser, FCV 10AL VP Valve selector, SUS Mixer, SIL 10AD VP Auto injector, CTO 10 AS VP Column oven, SPD 10 AVP UV-Vis detector and SCL 10AVP System Controller. The HPLC system was equipped with the software “Class-VP 6.13 SPI series”.

**Preparation of standard drug solution:**

Stock solution of DSP was prepared by dissolving 100 mg in 100 ml volumetric flask containing 25 ml of HPLC grade methanol, sonicated for about 30 min and then made up to volume with methanol to get 1 mg/ml solution. It was further diluted with respective mobile phase in each procedure to prepare standard solution of 100 µg/ml.

**Preparation of Sample drug solution for Pharmaceutical formulations:**

An accurate amount of tablet powder equivalent to 100 mg was weighed, extracted with HPLC grade methanol (3 x 15 ml) and filtered through a 0.45 µm membrane filter, followed by adding methanol upto 100 ml to get the stock solution of 1 mg/ml. This solution was further diluted stepwise with mobile phase as under preparation of standard solution to get the concentration required.

**Reagents used:**

- Water HPLC grade (Qualigens)
- Potassium dihydrogen phosphate AR grade (Qualigens)
- Methanol HPLC grade (E. Merck)
- Disodium hydrogen phosphate AR grade (Qualigens)

After systematic and detailed study of the various parameters involved, as described under results and discussion in this chapter, the following procedure (M34) was recommended for the determination of DSP in bulk samples and pharmaceutical formulations.
ANALYTICAL METHODOLOGY

Method M₃₄:

Preparation of Mobile Phase:

Mobile phase was prepared from filtered and degassed mixture of methanol and buffer (272 mg of potassium dihydrogen phosphate and 2.554 gm of disodium hydrogen phosphate) in the ratio of 50:50.

Chromatographic conditions:

<table>
<thead>
<tr>
<th>Column</th>
<th>Hypersil BDS Phenyl {250cm×4.6mm with 5μ}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Run time</td>
<td>30 min</td>
</tr>
<tr>
<td>Column temperature</td>
<td>35°C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>10μl</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>254 nm</td>
</tr>
<tr>
<td>Retention time</td>
<td>9.297 min</td>
</tr>
</tbody>
</table>

iii. Recommended Procedure:

After systematic and detailed study of the various parameters involved, as described under results and discussion in this chapter, the following procedure (M₃₄) was recommended for the determination of DSP in bulk samples and pharmaceutical formulations.

Method M₃₄:

Working standard solution:

Into a series of 10ml volumetric flasks, 0.1 ml to 0.3 ml was transferred and the volume in each flask was made up to 10 ml with the mobile phase and filtered through 0.45μ membrane filter.
Procedure:

Initially the mobile phase was pumped for about 30 min to saturate the column thereby to get the baseline corrected. Then ten micro liters of (DSP) standard and sample solutions was injected for six times. A quantitative determination of the active ingredient was made by comparison of the peak area from a sample injection to the corresponding peak area from a standard injection. The amount of DSP present in a sample was calculated through the standard calibration curve (Fig.3.31, p.221).

RESULTS AND DISCUSSION

The appropriate wavelength in UV region was selected for the measurement of active ingredient in the proposed method. The method was validated by linear fit curve and all other parameters were calculated just like a visible spectrophotometric method and were discussed in the following pages.

Chromatogram:

The chromatogram indicating the separation of DSP with Anion exchange column and mobile phase has been given in fig 3.30, p.221. Blank samples tested by the same procedure showed no interfering peaks.

Parameters fixation:

In developing this method, systematic study of the effects of various parameters was undertaken by varying one parameter at a time and controlling all other parameters. The following studies were conducted for this purpose.

Mobile phase characteristics:

In order to get sharp peaks and baseline separation of the components, the author has carried out number of experiments by varying different components like percentage of organic phase in the mobile phase, pH of the aqueous phase,
total pH of the selected mobile phase, modifiers and flow rate by changing one at a time and keeping all other parameters constant. The optimum condition obtained by proposed method was included in the procedure proposed.

**Fig. 3.30 MODEL CHROMATOGRAM OF DSP FOR M₃₄**

**Fig. 3.31: Standard Calibration graph of DSP for M₃₄**
Detection characteristics:

To test whether the DSP has been linearly eluted from the column in the method (M₃₄) different amounts of DSP were taken and all the solutions were analyzed by following the procedure. Quantitative determinations were made by comparison of the peak area from a sample injection to the corresponding peak area from standard injection. The linear fit of each system was illustrated graphically (Fig 3.31, p.221). Least square regression analysis for the method was carried out for the slope, intercept and correlation coefficient. The results are presented in Table 3.15, p.224.

Performance calculations:

To ascertain the system suitability for the proposed method, a number of statistical values such as relative retention, theoretical plates, HETP, resolution and peak asymmetry have been calculated with the observed readings and the results are recorded in Table 3.15, p.224.

Method validation:

The UV absorption maximum of DSP was fixed as 242 nm (reference method, Fig. 3.01, p.173). As the final detection was made by the UV absorption spectrum, each method was validated by linear fit curve and all other parameters of the method were calculated just like a visible spectrophotometric method and were discussed.

Precision:

The precision of the method (M₃₄) was ascertained separately from the peak area obtained by actual determination of eight replicates of a fixed amount of drug. The percent relative standard deviation and percent range of errors (at 0.05 and 0.01 confidence limits) calculated for DSP are presented in Table 3.15, p.224.
Accuracy:

To determine the accuracy of the proposed method, different amounts of bulk sample of DSP within the linearity limits were taken and analyzed by the proposed method. The results \((M_{34})\) of method are recorded in Table 3.15, p.224.

Interference studies:

The effect of wide range of excipients and other additives usually present in the formulations of DSP in the determination under optimum conditions were investigated. In the method proposed, none of the inactive ingredients even when they are present in excess fold than anticipated in pharmaceutical formulations. They have not disturbed the elution or quantification of DSP \((M_{34})\). In fact many have no absorption at this UV maximum.

Analysis of formulations:

To find out the suitability of the proposed method for the assay of pharmaceutical formulations (tablets) containing DSP were analyzed by the proposed and reference methods. The results obtained from the proposed and reference methods were compared statistically by the t and F- tests and were found that this proposed method do not differ significantly in precision and accuracy from reference method. The results are recorded in Table 3.16, p.225.
TABLE 3.15
PERFORMANCE CALCULATIONS, DETECTION CHARACTERISTICS, PRECISION AND ACCURACY OF THE PROPOSED METHODS FOR DSP

<table>
<thead>
<tr>
<th>METHOD</th>
<th>$M_{34}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSP</td>
<td></td>
</tr>
<tr>
<td>Parameter</td>
<td>M34</td>
</tr>
<tr>
<td>Retention time (t) (min)</td>
<td>9.297</td>
</tr>
<tr>
<td>Theoretical plates (n)</td>
<td>11077</td>
</tr>
<tr>
<td>Plates per meter (N)</td>
<td>73850</td>
</tr>
<tr>
<td>Height equivalent to theoretical plate (HETP) (mm)</td>
<td>13.541</td>
</tr>
<tr>
<td>Peak asymmetry (T)</td>
<td>1.136</td>
</tr>
<tr>
<td>Linearity range ($\mu g/10\mu l$)</td>
<td>10-30</td>
</tr>
<tr>
<td>Detection limits ($\mu g/10\mu l$)</td>
<td>0.00169</td>
</tr>
<tr>
<td>Regression equation ($Y = a + bC$)</td>
<td></td>
</tr>
<tr>
<td>Slope (b)</td>
<td>2.097</td>
</tr>
<tr>
<td>Standard deviation of slope ($S_b$)</td>
<td>$5.48 \times 10^{-4}$</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>$-3 \times 10^{-7}$</td>
</tr>
<tr>
<td>Standard deviation of intercept ($S_a$)</td>
<td>$1.184 \times 10^{-3}$</td>
</tr>
<tr>
<td>Standard error of estimation ($S_e$)</td>
<td>$7.75 \times 10^{-4}$</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9999</td>
</tr>
<tr>
<td>Relative standard deviation (%)*</td>
<td>0.398</td>
</tr>
<tr>
<td>% Range of error (Confidence limits)*</td>
<td></td>
</tr>
<tr>
<td>0.05 level</td>
<td>0.361</td>
</tr>
<tr>
<td>0.01 level</td>
<td>0.567</td>
</tr>
<tr>
<td>% Error in bulk samples**</td>
<td>0.018</td>
</tr>
</tbody>
</table>

*Average of six determinations considered. ** Average of three determinations.
TABLE 3.16

ASSAY AND PERCENT RECOVERY OF DSP IN DOSAGE FORMS

<table>
<thead>
<tr>
<th>Pharmaceutical Formulations</th>
<th>Labelled Amount (mg)</th>
<th>Proposed methods</th>
<th>Found by Reference Method ± S.D.</th>
<th>% Recovery By proposed Methods** ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amount Found*(mg) ± S.D.</td>
<td>t</td>
<td>F</td>
</tr>
<tr>
<td>Injection I</td>
<td>30</td>
<td>29.9±0.31</td>
<td>1.51</td>
<td>1.27</td>
</tr>
<tr>
<td>Injection II</td>
<td>60</td>
<td>59.8±0.22</td>
<td>1.86</td>
<td>1.61</td>
</tr>
<tr>
<td>Injection III</td>
<td>90</td>
<td>89.9±0.24</td>
<td>0.78</td>
<td>1.46</td>
</tr>
<tr>
<td>Injection IV</td>
<td>30</td>
<td>29.8±0.29</td>
<td>0.63</td>
<td>1.34</td>
</tr>
</tbody>
</table>

*Average ± standard deviation of six determinations; the t- and F- values refer to comparison of the proposed method with the reference method. Theoretical values at 95% confidence limit, t = 2.57, F = 5.05.

**After adding 3 different amounts of the pure labeled to the pharmaceutical formulation, each value is an average of 3 determinations.

Recovery studies:

Recovery studies were conducted by analyzing each pharmaceutical formulations in the first instance for the active ingredient by the proposed method. Known amounts of pure drug was once again determined by the proposed method after bringing the active ingredient concentration within the linearity limits. The results are recorded in Table 3.16, p.225.
Conclusion:

So far no visible spectrophotometric methods have been reported for the determination of DSP either in bulk form or pharmaceutical formulations. Hence the nine visible spectrophotometric (part A) methods developed for the assay are valuable. The visible spectrophotometric methods are based on the characteristic properties of different functional groups such as (bisphosphonate : $M_3$, $M_4$; Aliphatic primary amine : $M_{13}$, $M_{15}$, $M_{24}$, $M_{25}$, $M_{27}$, $M_{28}$ & $M_{32}$). Each method uses a specific reagent and the $\lambda_{\text{max}}$ and $\epsilon_{\text{max}}$ values of each method are different. Statistical analysis of the results (Table 3.13, p.206-207) shows that the proposed procedures have good precision and accuracy. Results of the analysis of the pharmaceutical formulations revealed that the proposed methods are suitable for their analysis with virtually no inference of the usual additives. The decreased order of sensitivity among the nine proposed visible spectrophotometric methods is $M_3>M_4>M_{24}>M_{15}>M_{32}>M_{28}>M_{25}>M_{13}>M_{27}$. The $\lambda_{\text{max}}$ order of the visible spectrophotometric methods is $M_3>M_{15}>M_{24}=M_{27}>M_{32}=M_4=M_{13}>M_{25}=M_{28}$.

Though there are few HPLC (part B) methods reported for the determination of DSP in the literature (Table 1.11, p.79-81) prior to the commencement of this work, many of the methods concerned with biological fluids and very few in pharmaceutical formulations hence the HPLC method ($M_{34}$) developed for its assay is valuable.

All the proposed methods (visible spectrophotometry or HPLC) are simple, sensitive, and reliable and can be used for the routine determination of DSP in bulk samples and pharmaceutical formulations depending upon the needs of the situation.