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ORIGINAL PAPER

Highly Sensitive Synchronous Fluorescence Measurement of Danofloxacin in Pharmaceutical and Milk Samples Using Aluminium (III) Enhanced Fluorescence

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Abstract A simple, rapid and sensitive constant wavelength synchronous fluorescence method is developed for the determination of danofloxacin (DAN) in pharmaceutical formulations and its residue in milk based on Al(III) enhanced fluorescence. The synchronous fluorescence intensity of the system is measured at 435 nm using $\Delta\lambda$=80 nm and an excitation wavelength of 280 nm. A good linear relationship between enhanced fluorescence intensity and DAN concentration is obtained in the range of 3–100 ng mL^{-1}(r^2=0.9991). The limit of detection (LOD, S/N=3) of the present method is 0.9 ng mL^{-1}. The proposed method can be successfully applied to the determination of DAN in pharmaceutical formulations and in milk without serious interferences from common excipients, metal ions and other co-existing substances. The method can be used as a rapid screening to judge whether the DAN residues in milk exceed Maximum Residue Limits (MRLs) or not.

Keywords DAN · Synchronous fluorescence · Fluoroquinolones · Determination

Introduction

Fluoroquinolones are an important group of drugs used for prevention of diseases in both humans and animals due to their high activity against a broad spectrum of gram negative and gram positive bacteria [1]. Fluoroquinolones are widely used in the treatment of systematic infections including urinary tract, respiratory, gastrointestinal and skin infections. DAN {(1-cyclopropyl-6-fluoro-1,4 dihydro-7-[(1s,4s)-5-methyl-2,5-diazabicyclo[2.2.1]hept-2-yl]-4-oxo-3 quinolinecarboxylic acid) (Fig. 1) is a member of fluoroquinolone antibiotics developed specifically for veterinary use [2]. It is used in the treatment of respiratory disease in chicken, pigs and cattle. The drug has a wide spectrum of antimicrobial activity, a large volume of distribution and is active even at low concentrations. Use of DAN in lactating cows may result in higher concentrations of this drug in milk. Use of fluoroquinolone antibiotics in animals used for food has generated concern because the presence of these residues in food may lead to increased antibiotic resistance in humans [3, 4]. It is important to have efficient methods for monitoring levels of these residues in the food supply to ensure that they do not exceed their maximum residue limits (MRLs). MRLs have been established by the European Union legislation for all veterinary medicines, residues of which may be present in animal products intended for human consumption. For DAN, MRL in bovine milk has been fixed at 30 μg L^{-1} [5]. All this has led to an increased interest in the development of new and sensitive methods for the determination of antibiotics in food.

For determination of fluoroquinolones in food, the most commonly employed techniques are chromatographic techniques. Fluoroquinolones have been determined in pig muscle [6–9], poultry tissue [10] fish tissue and sea food [11] using liquid chromatography or HPLC with various detection techniques like UV-visible, fluorescence, mass spectrometric or tandem mass spectrometric detection. For determination of fluoroquinolones in milk, the methods reported include mainly LC or HPLC methods involving prior sample clean up by simple precipitation or solid phase extraction [12–16] Recently a CE-MS method has also been reported for quantification

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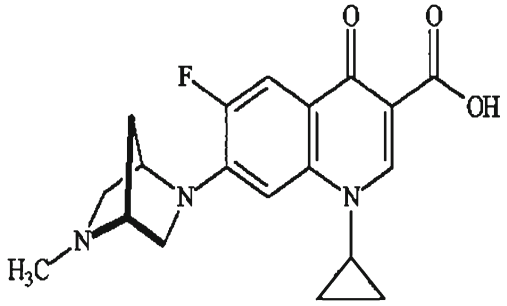

Fig. 1 Structure of DAN

of quinolones in milk [17]. However these methods suffer from some limitations such as column clogging, involving complex procedures, need personal expertise and require expensive equipment.

The spectrofluorimetric techniques are highly sensitive and selective due to which they have been widely used to estimate pharmaceuticals and their metabolites [18–20]. Conventional spectrofluorimetry consists of measuring fluorescence emission intensity using suitable excitation wavelength. The conventional spectrofluorimetric methods can be successfully used for determination of fluoroquinolones in pharmaceuticals, however these are not very successful in determining the drug in fluorescent matrices like urine and milk because the proteins present in these matrices cause interferences as they give an overlapping spectra. Synchronous fluorescence spectrometry (SFS) involves simultaneous scanning of both the excitation and emission monochromators while maintaining a constant wavelength interval ($\Delta\ \lambda$) between them. It has become an important tool for determination of drugs [21, 22]. This is because SFS gives a sharp, narrow spectrum leading to reduction of band width and interferences like Rayleigh and Raman scattering and causing simplification of the spectrum. The synchronous scanning fluorimetric technique is better than conventional spectrofluorimetry as regards sensitivity [23].

For determination of DAN in milk, only one spectrofluorimetric method based on chemometrics has been reported [24]. In the present work it was found that addition of Al(III) results in enhancement of fluorescence intensity of the drug. Based on this, a simple and rapid method was developed for determination of DAN in pharmaceutical and milk samples. The method is more rapid and convenient as compared to the standard HPLC or LC-MS methods employed for determination of this drug. The proposed method is based on constant wavelength synchronous spectrofluorimetry which reduces the interferences from residual proteins and other substances. So the method does not require complicated treatment procedures like SPE and can be successfully applied for determination of DAN in trace amounts in milk samples to judge whether the values exceed MRLs or not.

Experimental

Apparatus

Fluorescence spectra were recorded and intensity measurements were made on RF-5301 PC spectrofluorophotometer (Shimadzu, Japan). A UV-1800 Pharmaspec UV-Visible spectrophotometer (Shimadzu, Japan) was used to record absorption spectra. An ATC pH meter model 132-E (Electronics, India) was used for all pH measurements.

Reagents

A stock standard solution of DAN, (100 μg mL^{-1}) was prepared by dissolving the corresponding amount of DAN (Sigma Aldrich, Steinheim, Germany) in pure water. The mixture was sonicated in an ultrasonic bath for 15 min and stored at 4 °C. The working solutions of the drug were prepared by diluting the stock solution with triply distilled water daily. A stock solution of Al(III) (1×10^{-2} M) was prepared by dissolving 0.4742 $KAl(SO_4)_2.12H_2O$ in a beaker and diluting with double distilled water to 100 mL. Working Al(III) solution was freshly prepared by dilution of the stock solution with distilled water. Sodium acetate-acetic acid buffer solutions (0.1 M) of various pH values were prepared by addition of acetic acid to 0.1 M sodium acetate. All reagents used were of analytical reagent grade and purchased from Merck India unless stated otherwise. Triply distilled water was used throughout.

Sample Preparations

Pharmaceutical Preparations

The contents of ten sachets were weighed and thoroughly mixed. The powder equivalent to 10 mg of DAN was dissolved in distilled water and sonicated in an ultrasonic bath for 15 min. The solution was filtered into a 100 mL calibrated flask, the residue was washed several times with water and the solution was diluted to the mark with water to obtain a solution of 100 μg mL^{-1}

For determination of drug in injectable form, the mass of the drug per ml was determined. An amount of liquid equivalent to 10 mg of DAN was transferred into a 100 ml calibrated flask and diluted to the mark with distilled water.

All solutions were then appropriately diluted with water so that final concentration was in the working range.

Milk Samples Preparation

The milk samples for this study were collected from local groceries. The extraction procedure was as follows: In a 10 mL centrifuge tube, 1 ml of milk was taken and 2 ml

of acetonitrile was added to precipitate proteins. The mixture was then centrifuged at 5000 rpm for 10 min. Then the clear supernatant was transferred to a 10 mL volumetric flask for determination of DAN residue.

General Procedures

To a 10 mL volumetric flask, the solutions were added in the following sequence: a suitable aliquot of DAN standard solution or milk sample, 2 mL of sodium acetate-acetic acid buffer solution of pH 3.5, 1 mL of 1×10^{-3} M Al(III) solution and 2 mL of acetonitrile. The mixture was then made upto the mark with distilled water, shaken and allowed to stand for 15 min. The synchronous fluorescence measurements were then made at 435 nm using $\Delta\lambda$=80 nm and an excitation wavelength of 280 nm. The slit band widths for excitation and emission monochromators were fixed at 5 nm. All measurements were performed in 10 mm quartz cell at room temperature. The enhanced fluorescence intensity was represented as $\Delta F = F\text{-}F_0$, where F and F_0 were the fluorescence intensities of the system with and without the drug, respectively.

Results and Discussion

Fluorescence Spectra

Aqueous solutions of DAN show native fluorescence. The excitation and emission wavelengths of the native fluorescence of DAN are 280 and 444 nm respectively. The fluorescence peak of DAN appeared at 435 nm in the presence of Al (III), which showed a blue shift of 9 nm and its fluorescence intensity was significantly enhanced (Fig. 2). Based on the fluorescence enhancement effect of this new system, a new method could be developed for the determination of DAN. But this method based on conventional emission spectrum suffers serious interferences from fluorescent substances. It cannot be applied successfully to the determination of drug in milk samples. This is because the fluorescence peaks of residual proteins and other organic substances in the milk matrices overlap with the drug peak and interfere in the determination. In order to reduce these interferences and apply this method to determination of DAN in milk, synchronous fluorescence measurement technique was used.

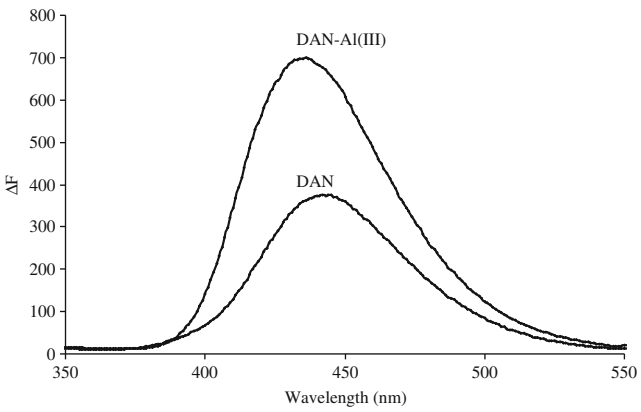

Fig. 2 Fluorescence emission spectra of (a) DAN and (b) DAN-Al(III) system. Conditions: DAN: 30 ng mL^{-1}; Al(III): 1×10^{-4} mol L^{-1}; acetate buffer: 1×10^{-2} mol L^{-1}; pH 3.5; λexc 280 nm

Constant Wavelength Synchronous Spectrofluorimetry

This technique enhances sensitivity of the determination while maintaining simplicity at the same time. According to this technique, the difference between two wavelength monochromators($\Delta\lambda$) can extensively modify the spectrum shape. The band width of the emission spectrum of DAN-Al (III) system measured at half the height of the peak decreases considerably in synchronous fluorescence spectra as compared to conventional spectrum thus giving rise to a comparatively sharp peak.

The synchronous fluorescence peak of DAN was observed at 435 nm. Upon addition of Al(III) to the DAN solution, the fluorescence intensity of the drug was significantly enhanced which indicated an interaction between DAN and Al(III) (Fig. 3). For selection of the appropriate value of $\Delta\lambda$, various synchronous spectra at different values were recorded. At $\Delta\lambda$=80 nm most intense synchronous signal with the narrowest spectral band was obtained. Thus, $\Delta\lambda$=80 nm was selected for further study.

Optimum Reaction Conditions

Effect of pH

It is well known that fluorescence intensity of fluoroquinolones is pH dependent. The pH sensitivity of fluoroquinolones

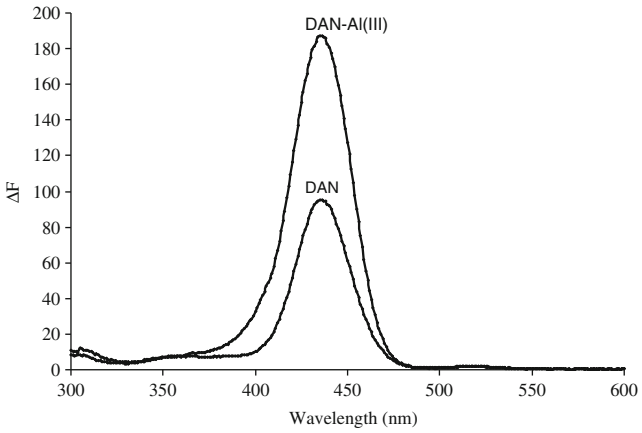

Fig. 3 Synchronous fluorescence spectra of (a) DAN and (b) DAN-Al (III) system. Conditions: DAN: 30 ng mL^{-1}; Al(III): 1×10^{-4} mol L^{-1}; acetate buffer: 1×10^{-2} mol L^{-1}; pH 3.5; $\Delta\lambda$=80 nm; λexc 280 nm

can be explained as due to presence of carboxylic group. So the effect of pH value on the fluorescence intensity of the system was investigated over the range 2–8 by using acetic acid-sodium acetate buffer solutions (0.1 M). The fluorescence intensity of DAN-Al(III) system was also found to be pH dependent. This shows that the interaction between DAN and Al(III) occurs through carboxylic group. The results are shown in Fig. 4. The fluorescence intensity was found to be maximum and nearly constant for the pH interval 3–4 but decreased outside this range. So an acetate buffer of pH 3.5 was used for all subsequent measurements.

Effect of Al(III) Ion Concentration

The effect of Al(III) concentration on the fluorescence intensity of the system was studied in the range 1×10^{-5}–1×10^{-4} M. When the concentration of DAN fixed at 50 ng mL^{-1}, the concentration of Al(III) was increased. As shown in Fig. 5, it was observed that fluorescence intensity increased with increase in concentration of Al(III) upto 5×10^{-5} M but levelled off at higher concentrations. An Al(III) concentration of 1×10^{-4} M was therefore chosen for further study.

Effect of Acetonitrile

Acetonitrile was used for deproteinization of the milk sample in sample preparation step. Therefore it was necessary to investigate the effect of acetonitrile on the fluorescence intensity of the system. It was observed that the concentration of acetonitrile used in the extraction procedure (nearly 20 %*v/v*) could enhance the fluorescence intensity of the system. Therefore in analytical procedures, 2 mL of acetonitrile was also added to standard solutions in order to reduce the influence of acetonitrile on fluorescence intensity.

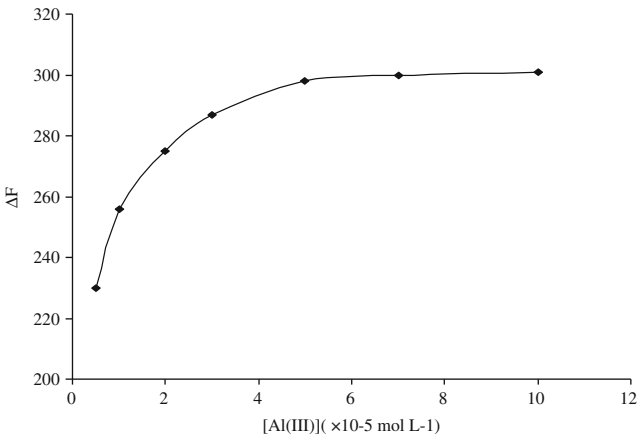

Fig. 4 Effect of Al(III) concentrations on the fluorescence intensity of DAN-Al(III) system. Conditions: DAN, 50 ng mL^{-1}; acetate buffer: 1×10^{-2} mol L^{-1}; pH 3.5

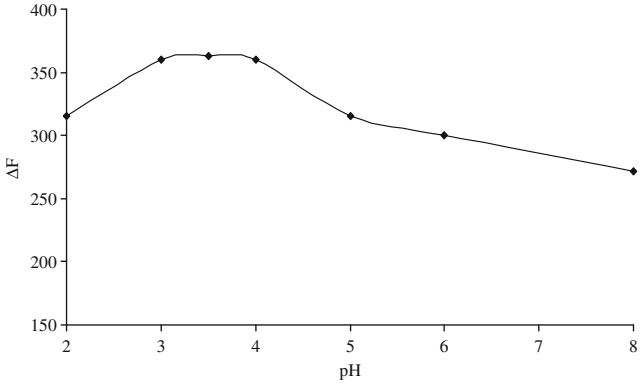

Fig. 5 Effect of pH on the fluorescence intensity of DAN-Al(III) system. Conditions: DAN: 60 ng mL^{-1}; Al(III): 1×10^{-4} mol L^{-1}; acetate buffer: 1×10^{-2} mol L^{-1}

Effect of Addition Order of Reagents

A series of solutions with different addition order of reagents but same concentration of various reagents were prepared and their synchronous fluorescence spectra were recorded at $\Delta\lambda=80$ nm. It was found that adding the reagents in different order had a negligible influence on F, F_0 and ΔF values. The following order of addition of various reagents was followed: DAN, buffer, Al(III), acetonitrile.

Stability Test

It was observed that fluorescence intensity of the system reached a stable value in 15 min and remained constant for about 2 h. Hence all the measurements were made about15 min after all the reagents had been added.

Interference of Foreign Substances

Under the optimal conditions, the influence of foreign substances that commonly accompany DAN in real samples, were examined for interferences. At a DAN concentration of 1.39×10^{-7} M, the highest permissible molar excesses of other substances causing a ±10 % relative error in the fluorescence intensity were investigated and the results are listed in Table 1.

It was observed that when the concentrations of metal ions and other organic substances were close to the concentration of DAN, they did not interfere in the determination. Other fluoroquinolone drugs were also studied for interferences. It was observed that when concentration ratio of DAN to the fluoroquinolone drug was 1:1, they caused less than ±10 % relative error in the determination thus eliminating serious interferences except enrofloxacin which would interfere in the determination of DAN if present at same concentration. As enrofloxacin and DAN are both used for treatment of respiratory diseases in cattle, they are very less likely to be used together thus eliminating this drawback.

Table 1 Influence of co-existing substances on the fluorescence intensity of DAN-Al(III) system

Species	Concentration (mol L^{-1})	ΔF(%)
Fe^{2+}	5.0×10^{-4}	+2.9
Cu^{2+}	5.0×10^{-5}	−5.6
Ni^{2+}	1.0×10^{-4}	+5.5
Zn^{2+}	2.0×10^{-3}	−3.8
Ca^{2+}	1.0×10^{-2}	+4.5
Mg^{2+}	1.0×10^{-3}	+5.8
Mn2+	2.0×10^{-4}	+3.2
EDTA	5.0×10^{-4}	−8.0
Glucose	1.0×10^{-4}	+4.2
Ofloxacin	1.0×10^{-7}	+3.4
Norfloxacin	1.0×10^{-7}	+8.5
Enrofloxacin	5.0×10^{-8}	+8.5
Difloxacin	1.0×10^{-7}	+9.0
Enoxacin	1.0×10^{-7}	+6.8

Analytical Parameters

Under optimum conditions, calibration graphs for the determination of DAN were constructed. The enhanced fluorescence intensity of the system showed a good linear relationship with the concentration of DAN in the range of 3–100 ng mL^{-1} (r^2=0.9991). The regression equation was $\Delta F = F\text{-}F_0 = \Delta F = (6.09\pm0.19)c+(0.11\pm0.10)$ (n=9, r^2= 0.9991) where ΔF represents enhanced fluorescence intensity, F and F_0 represent fluorescence intensity in the presence and absence of drug respectively and c = concentration of DAN in ng mL^{-1}. The limit of detection (LOD,S/N=3) was estimated to be 0.9 ng mL^{-1} and limit of quantification (LOQ, S/N=10) was determined to be 2.97 ng mL^{-1} The accuracy and precision of the method was established by determining DAN in spiked samples at different concentrations. The recovery values and RSD values were calculated.

Applications

The above described methods were used for the determination of DAN in real samples.

Table 2 Results of the determination of DAN in pharmaceutical samples

Sample[a]	Amount per sachet or ml Labelled	Found[b] ± RSD
Advocip powder (sachet)	12.5 g	12.2±2.1
Advocip injection	25.0 mg	24.5±2.5

[a] From Cipla pharmaceuticals Ltd, India

[b] Average value of five determinations

Table 3 Recoveries of DAN added to real pharmaceutical preparations

Sample	Amount added (ng mL^{-1})	Amount found[a] (ng mL^{-1})	Recovery[a] (%) ± RSD
Advocip powder (sachet)	20.0	20.5	102.5±1.9
	50.0	49.0	98.0±2.3
	70.0	70.8	101.1±2.0
Advocip injection	20.0	19.6	98.0±2.1
	50.0	48.3	96.6±2.4
	70.0	70.9	101.3±2.3

[a] Average value of five determinations

Analysis of Pharmaceutical Preparations

The pharmaceutical samples containing DAN were analysed by synchronous fluorescence measurement following the procedure described under experimental section. The results obtained are given in Tables 2 and 3. There were found to be no significant differences between labelled contents and those obtained by the proposed method. The recoveries were determined by adding various amounts of DAN to each pharmaceutical preparation by subtracting the results obtained for pharmaceuticals prepared in a similar manner but to which no DAN has been added. In all cases quantitative recoveries between 96.6 and 102.5 % were obtained with RSD less than 3 %.

Analysis of Milk Samples

Milk is a fluorescent matrix because of which it provides high background fluorescence. So it was not feasible to determine DAN by conventional spectrofluorimetry. In order to determine DAN in milk, synchronous constant wavelength spectrofluorimetry was used. For recovery tests, the milk samples were spiked with certain concentrations of DAN so that final concentration of the drug in milk were in the linear range of the method and extraction procedure was followed as described above. Table 4 shows the analytical recoveries of DAN added to milk samples. In all cases, quantitative recoveries between 83.3 and 116.0 % were obtained with RSD less than 8 %.

Table 4 Analytical recoveries of DAN added to milk

Sample	Amount added (ng mL^{-1})	Amount found[a] (ng mL^{-1})	Recovery[a] (%) ± RSD
Milk 1	15	13.0	86.6±4.5
	20	18.6	93.0±5.1
	30	25.0	83.3±5.6
Milk 2	40	35.0	87.5±7.2
	50	58.0	116.0±5.9
	60	66.0	110.0±6.0

[a] Average value of five determinations

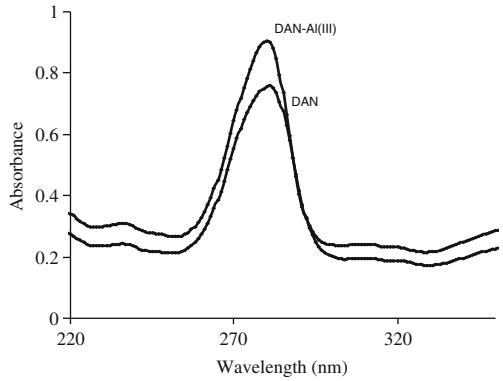

Fig. 6 Absorption spectra of (a) DAN (b) DAN-Al(III) system. Conditions: DAN: 5 μg mL^{-1}; Al(III): 1×10^{-4} M; acetate buffer: 1×10^{-2} mol L^{-1} pH=3.5

This method can also be used as a simple qualitative method for rapid screening DAN residues in milk in order to judge whether its levels exceed MRLs or not. This can be done by just comparing the fluorescence intensities of the milk samples with a standard milk sample (having DAN residues equal to 30 ng mL^{-1}) after subjecting the milk samples to sample preparation process. If fluorescence intensity of certain milk sample is stronger than that of the standard milk sample, it can be primarily concluded that this milk sample might exceed MRLs. Otherwise the result is contrary.

Luminescence Mechanism

The fluorescence intensity of DAN is significantly enhanced in the presence of Al(III)ions as shown in Fig. 2. Also the emission spectra suffer a blue shift of about 9 nm which indicates interaction between DAN and Al(III). The absorption spectrum of 5 μg mL^{-1} solution of DAN in water shows an absorption maximum at 280 nm. The absorption at 280 nm increases when Al(III) is added to the solution as shown in Fig. 6. This increase in absorption resulted in fluorescence enhancement of the system. So it was reasonably assumed that Al(III) forms a complex with DAN which resulted in fluorescence enhancement of the system. Further it is well established that fluoroquinolones form complexes with metal ions in which the drug coordinates to the metal through carbonyl and carboxyl groups of fluoroquinolone drug. So it might also be supposed that Al(III) binds to carbonyl and carboxylic groups of DAN resulting in the formation of complex which leads to increase in absorption and enhancement of fluorescence intensity of the system on addition of Al(III).

Conclusions

Molecular fluorescence techniques combine sensitivity with simplicity and can be successfully used for the determination of residues in real matrices as shown in the present work. The constant wavelength synchronous fluorescence spectrometry as used in the present work provides greater sensitivity as compared to conventional fluorescence technique because the synchronous spectrum of DAN is more straightforward and the band is narrower. The fluorescent complex formed between DAN and Al(III) was used for determination of the drug in pharmaceutical preparations using synchronous spectrofluorimetry eliminating interferences from the excipients in case of pharmaceutical samples and reducing background fluorescence in milk. The conventional spectrofluorimetric method in case of milk sample is not very successful because of background fluorescence which is due to some residual proteins and other organic substances existing in the prepared sample solutions even after deproteinization. The method can be used to judge whether the DAN residues in milk exceed MRLs or not.

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