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Journal of Chromatography A, 694 (1995) 407–413

JOURNAL OF
CHROMATOGRAPHY A

Sensitive fluorescence detection of robenidine by derivatization with dansyl chloride and high-performance liquid chromatography

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First received 17 June 1994; revised manuscript received 7 November 1994; accepted 8 November 1994

Abstract

A normal-phase high-performance liquid chromatographic procedure is presented for the determination of robenidine following derivatization with dansyl chloride. The derivatization, separation and detection conditions were optimized. The dansyl derivative is first purified on a silica gel cartridge followed by HPLC analysis on a silica gel column. The eluate is monitored by fluorescence detection at 320 nm excitation and 485 nm emission wavelength. The lower limit of detection of robenidine is 0.4 $\mu\text{g/ml}$.

1. Introduction

Medicated animal feedstuffs play an important role in intensive livestock production systems throughout the world. In the European community the use of these substances in feedstuffs is controlled by Council Directive [1] which has been amended many times over the last few years. The latest consolidated version of the annexes in this directive was published as a Commission Directive [2] and contains a list of 21 coccidiostats including robenidine. Robenidine hydrochloride, 1,3-bis(*p*-chlorobenzylideneamino)guanidine hydrochloride, is the active ingredient in American Cyanamid's Robenz, used in chicken feeds and supplements [3]. In Canada, robenidine is used as an aid in prevention of coccidiosis in chickens, turkeys

and rabbits at doses of 33 or 50 mg/kg for poultry and rabbit feeds respectively. The absorption, excretion, and metabolism of ^{14}C -labelled robenidine hydrochloride was studied in rats [4] to assess the safety of robenidine in poultry products [5,6].

Very few analytical methods have been developed for detection of robenidine. The thin-layer chromatography method [7] uses a colorimetric sodium hydroxide in dimethyl formamide, and absorbance measured at 373 nm, whereas a HPLC method with detection at 280 nm shows a poor sensitivity (52.68 $\mu\text{g/ml}$) [8,9].

Robenidine does not have native fluorescence, however this difficulty has been overcome in the present work by introducing a highly fluorescent moiety into the robenidine. Such an approach has found application in the field of amino acid and peptide chemistry and also in pesticide residue analysis [10–13]. The reagent most often

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utilized in such labelling techniques is 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride, Dns-Cl) which reacts with primary and secondary amino groups and phenolic hydroxy groups to form highly fluorescent derivatives.

The present work details the use of Dns-Cl as fluorogenic labelling reagent for determination of robenidine. The technique can detect robenidine at 0.4 $\mu\text{g/ml}$.

2. Experimental

2.1. Chemicals and materials

Dns-Cl and 4-dimethylaminopyridine were obtained from Aldrich (Milwaukee, WI, USA). Robenidine was donated by Cyanamid (Gosport, Hants, UK). Water was purified using a Millipore Milli-Q system. All other solvents were purchased from J.T. Baker (Phillipsburg, NJ, USA) and were HPLC grade. Chloroform did not contain alcohol, but was stabilized with a non-polar hydrocarbon. Tetrahydrofuran was not stabilized against peroxide formation. Sep-Pak VAC/3cc silica cartridges were obtained from Waters/Millipore (Milford, MA, USA). Silica gel 60 was 70–230 mesh, from EM Science (Gibbstown, NJ, USA).

2.2. Apparatus

Reactions were carried out in Pyrex (100 \times 13 mm I.D.) test tubes closed with PTFE-lined screw caps. An aluminum block heater with wells was used to heat the tubes. Silica cartridges were eluted using a vacuum manifold equipped with Luer-Loc adapters (Supelco, Oakville, Canada), set to maintain a slight pressure.

The HPLC system consisted of a Beckman 110A pump, a Waters U6K injector, a Perkin-Elmer LS-4 fluorescence detector and a Spectra-Physics 4270 integrator. A mobile phase of chloroform–hexane–tetrahydrofuran–methanol (50:50:2:1) was pumped at 2 ml/min through a Zorbax Sil (5 μm silica, 25 cm \times 4.6 mm I.D.) column protected by a Brownlee (7 μm silica, 15

cm \times 3.2 mm I.D.) guard column. The detector was set for excitation at 320 nm and emission at 485 nm.

^1H and ^{13}C NMR spectra were recorded on a Bruker AM 500 MHz NMR spectrometer at 500 and 125.28 MHz, respectively. Chemical shifts are referenced with respect to [$^2\text{H}_6$]dimethyl sulfoxide (DMSO- d_6) at 2.49 ppm (^1H) and 39.5 ppm (^{13}C) and are reported relative to tetramethylsilane.

Chemical shift assignments were made via $^1\text{H}/^{13}\text{C}$ heteronuclear correlation and homonuclear decoupling experiments and by consideration of substituents effect.

Mass spectrometry was performed on a Finnigan-MAT-90. Samples were introduced by direct probe heated from 30 to 350°C and held at 350°C. Electron impact (EI) spectra were recorded from 200 to 600 u at 1 scan/s.

2.3. Preparation of solutions

Robenidine (20 mg) was weighed into a volumetric flask (100 ml) and dissolved with a solution of dichloromethane–methanol (90:10) to obtain a concentration of 0.2 mg/ml. The flask was shaken and then left overnight or until dissolution was complete.

Dns-Cl and dimethylaminopyridine (DMAP) were dissolved in dichloromethane to give solutions of 2 and 4 mg/ml, respectively. Solutions were stored in low actinic glass at 4°C.

2.4. Derivatization conditions

Robenidine solution was evaporated to dryness under a slow stream of nitrogen by heating on a water bath at 60°C to remove traces of methanol that could react with Dns-Cl. The residue was dissolved in 1.0 ml DMAP solution and 1.0 ml Dns-Cl solution was added. The tube was capped tightly, and heated for 60 min at 80°C. The mixture was then cooled on ice. During derivatization the reaction tube was wrapped in aluminium foil since dansyl derivatives are known to be photosensitive.

2.5. Purification of the reaction mixture for HPLC analysis

The cooled reaction mixture was transferred from the reaction tube to a silica gel cartridge. The tube was washed with 5.0 ml of dichloromethane, and this was applied to the silica gel cartridge. The dichloromethane eluate was discarded. The cartridge was then eluted with 5 ml of ethyl acetate to collect the reaction product. Ethyl acetate was evaporated to dryness under a gentle stream of nitrogen in a water bath at 60°C. The residue was taken up in 2.0 ml of HPLC mobile phase mixture, and 20 μ l were injected into the HPLC system. Chromatograms are shown in Fig 2: (A) without a clean-up and (B) after clean-up.

2.6. Preparation of a quantity of the derivative for NMR and MS studies

A solution of 750 mg robenidine, 1500 mg Dns-Cl and 1500 mg DMAP in 500 ml dichloromethane was heated at 60°C for 2 h in a 2-l round-bottom flask equipped with a condenser. The reaction was protected from atmospheric moisture with a calcium chloride filled tube fitted on the condenser.

Preliminary clean-up of the reaction mixture was done using a 15 cm chromatography column (5 cm I.D.) filled to 5 cm with silica gel 60 slurried in dichloromethane. Half the reaction mixture (250 ml) was added and the column was washed with 2 \times 100 ml of dichloromethane. The product was eluted in 200 ml ethyl acetate, and solvent removed on a rotary evaporator to dryness and the residues were taken up in few ml of dichloromethane.

Further purification was done on a 20 cm column of 2% water-deactivated silica gel 60 packed in dichloromethane in a 3 cm I.D. chromatography column. The column was pre-washed with 100 ml 2% ethyl acetate in dichloromethane and the product of the previous column was added. The column was then washed with 600 ml of 2% ethyl acetate in dichloromethane, while the progress of the green fluorescent product band was followed by illumination with a 366

nm UV lamp. The band was eluted with 250 ml of 5% ethyl acetate in dichloromethane and evaporated to dryness on a rotary evaporator using methanol to drive off water. The residue was taken up in dichloromethane and recrystallized from methanol–dichloromethane (1:1) as yellow-green needles. These were dried in a vacuum drying pistol at 60°C for 16 h. The crystalline product showed a sharp melting point at 221°C.

2.7. Reaction conditions studies

Temperature

Time series run at 60 and 80°C with solvent and reagent concentrations as described under *Derivatization conditions* show that the reaction proceeds more quickly at 80°C than 60°C. A temperature of 80°C is the practical limit for the solvent, tubes and heater used.

Dns-Cl concentration

Reactions were run with 0.1 mg/ml robenidine in dichloromethane and Dns-Cl concentrations were varied from 0.03 to 1 mg/ml. The concentration of DMAP was twice that of Dns-Cl in each case. Increasing the Dns-Cl concentration was found to increase product yield. The limiting factor is the solubility of Dns-Cl in dichloromethane. The highest practical concentration for a stock solution is 2 mg/ml, equivalent to 1 mg/ml in the reaction mix.

Catalyst concentration

A series of reactions run at various concentration ratios (DMAP/Dns-Cl) with robenidine at 1.0 mg and 0.1 mg/ml show that the optimum concentration of DMAP varies with the concentration of Dns-Cl but not with the level of robenidine. The optimum was found at a concentration ratio of 2 mg/ml DMAP/1 mg/ml Dns-Cl.

Time

Time studies were carried out to a maximum of 3 h. The reactions were run at 80°C with a concentration of robenidine of 0.1 mg/ml, Dns-Cl 1 mg/ml and DMAP 2 mg/ml. The study

Table 1

Solvents	Bases, at a concentration of 1 mg/ml					
	DMAP	DMAP- K ₂ CO ₃	DMAP- NaOH	K ₂ CO ₃	K ₂ CO ₃ - 18-crown-6 ether	NaOH
Acetone	++	++	++	+	-	-
Acetone-acetonitrile (1:1)				+	+	
Acetone-tetrahydrofuran (1:1)	++					
Acetone-water (1:1)				-		++
Acetonitrile	+	-		+	-	-
Acetonitrile-water (1:1)				+		
Chlorobenzene	+					
Chloroform	++			-		
Dichloromethane	++-	+++	-	-		

Fluorescence of product on TLC: - = none; + = weak; ++ = medium; +++ = strong.

shows the optimum time is 60 min with the product amount decreasing afterward.

Thin-layer chromatography (TLC)

Normal- and reversed-phase systems were used.

On silica gel plate (Whatman) LK6 (normal phase), the migration solvent used is ethyl acetate-heptane (90:10). The R_F of the product is 0.5. Using a reversed-phase system of acetonitrile-water (80:20) on silica gel LKC₁₈ (C₁₈ bonded to silica) the R_F of the product is 0.3.

Solvents/bases

The reaction was tested in a series of solvents with different bases in order to find the best system. Robenidine was present at 0.2 mg/ml and Dns-Cl and bases were each added at 1.0 mg/ml. The mixture was heated at 80°C for 1 h. The amount of the product was determined visually on TLC plates. Results are recorded in Table 1.

3. Results and discussion

Because robenidine has no native fluorescence, the technique of spectrofluorometry has not been previously applied to its determination. This difficulty has been overcome in the present work by introducing a highly fluorescent moiety

into the molecule of robenidine. The reagent most often used in such labelling techniques is Dns-Cl which reacts with primary and secondary amino groups and also phenolic hydroxy groups to form highly fluorescent derivatives. The robenidine molecule contains three possible reaction sites for dansylation, all on the nitrogens attached to C₈ (Fig. 1). The possibility of obtain-

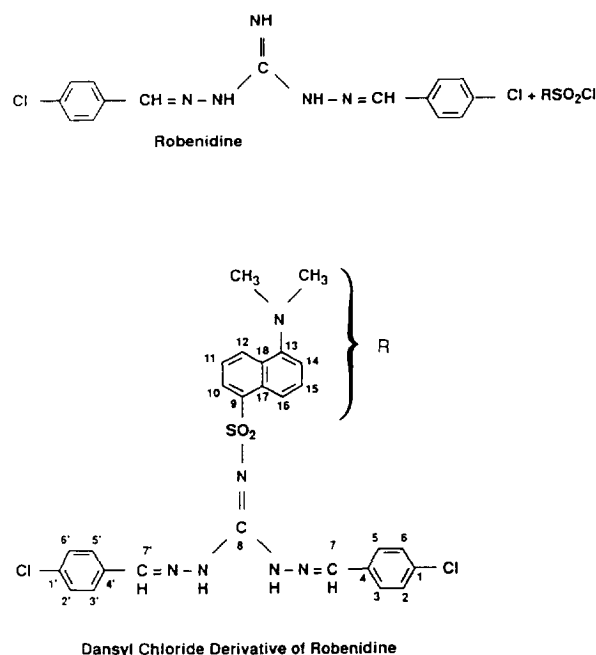


Fig. 1. Structures of robenidine and the Dns-Cl derivative of robenidine.

ing a mixture of isomers was eliminated by preparing the derivative and analyzing the structure by MS and NMR. The results from spectral data have clearly indicated that only one derivative is obtained.

The derivative, yellow green needles, exhibited a molecular ion at m/z 566 (10%) and a two chlorine isotopic peak at 568 (7%) with fragmentation ions at m/z 414 (25%), 412 (75%), 301 (100%, the base peak), 237 (55%) and 210 (24%). The ^1H and ^{13}C NMR shifts of the derivative are recorded in Table 2. The data indicate that robenidine's symmetry with respect to C_8 is retained in the derivative since a single resonance is obtained for both C_7 and C_7' (Table 2). In the absence of symmetry, these two carbons should have exhibited two different signals. This retention of plan of symmetry shows that Dns-Cl reacts only with the imine at C_8 . The mass spectrum of the derivative shows a mass of 566^+ , which corresponds to the

robenidine basic structure that has a symmetrical plan in relation to the $\text{C}_8\text{-NH}$.

In all applications using Dns-Cl as reagent for amino acids, peptides and proteins the derivatization occurs in aqueous media and at alkaline pH. In our case even though robenidine is soluble mainly in water (the standard solution), the water is evaporated with methanol to dryness, and the derivatization reaction proceeds in dichloromethane, with DMAP (an organic base). In this regard this is a new way to use this derivatization reaction in anhydrous conditions.

The parameters investigated included the yield of reaction product as a function of time. Reactions were performed at 80°C in capped tubes with the same concentrations of robenidine, Dns-Cl and DMAP in all. Tubes were withdrawn at different time intervals, cooled and the reaction mixture analyzed by HPLC.

Several systems of base/solvent were studied (Table 1), the best mixture was chosen for

Table 2
 ^1H NMR and ^{13}C NMR signal for dansylated robenidine derivative

Position	^{13}C (δ ppm) ^a	^1H (δ ppm) ^b	J (Hz) ^c
1,1'	132.7 (132.2)		
2,2'	128.8 (128.7)	7.50 (7.95)	d, 12.5 (d, 8.5)
3,3'	129.0 (129.5)	7.78 (7.49)	d, 7.8 (d, 8.4)
4,4'	134.8 (135.2)		
5,5'	129.0 (129.5)	7.78 (7.49)	d, 7.8 (d, 8.4)
6,6'	128.8 (128.7)	7.50 (7.95)	d, 12.5 (d, 8.5)
7,7'	146.6 (147.6)	8.40 (8.45)	
8	151.0 (152.8)		
9	140.1		
10	125.8	8.24	d, 7.9
11	123.4	7.57	dd, 8.5, 7.9
12	128.4	8.38	d, 8.5
13	149.2		
14	114.9	7.23	d, 7.5
15	127.1	7.60	dd, 8.6, 7.5
16	120.9	8.59	d, 8.6
17	129.4		
18	129.1		
$\text{N}(\text{CH}_3)_2$	45.1	2.80	
-NH		8.59	

^a Chemical shift ^{13}C (in ppm).

^b J values in Hz and chemical shifts in δ units (downfield of tetramethylsilane).

^c J of robenidine displacement/coupling; italic numbers between parentheses are for underivatized robenidine.

further optimization and the conditions were as follows: robenidine was 0.2 mg/ml, Dns-Cl was 1 mg/ml, base at concentration 1 mg/ml and the reaction time 1 h at 80°C. The use of DMAP and dichloromethane was found to be the best system.

A series of five concentration of robenidine (6.4, 3.2, 1.6, 0.8 and 0.4 $\mu\text{g/ml}$) were taken and derivatized as described above and purified with silica gel cartridge prior to injection to HPLC.

Application of the formula $y = a + bx$ using peak areas gave a very good linearity with correlation coefficient of 0.9990 with intercept $a = -1136$ and slope $b = 62539$.

The procedure reported clearly established that the minimum level of detection of robenidine was 0.4 $\mu\text{g/ml}$ or 8 ng in a 20- μl injection at S/N of 3.

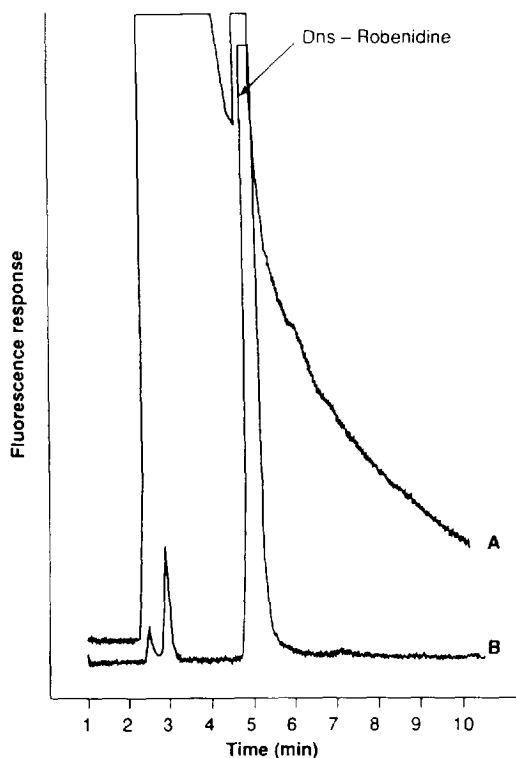


Fig. 2. Chromatograms of robenidine: A = without cleanup, 12.8 $\mu\text{g/ml}$; B = after silica gel cartridge cleanup, 12.8 $\mu\text{g/ml}$. HPLC conditions given in the text.

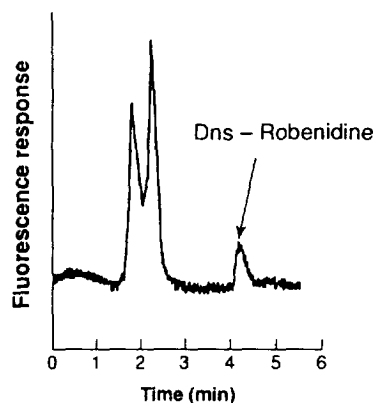


Fig. 3. HPLC of robenidine 0.4 $\mu\text{g/ml}$. Conditions given in the text.

A normal-phase chromatogram (Fig. 2) shows the separation of Dns-robenidine prior to and after silica column clean up and Fig. 3 shows the minimum detectable amount of robenidine.

In the catalyst concentration studies (0.1 and 1.0 mg/ml), the latter concentration of 1 mg/ml was selected because of the poor solubility of robenidine at higher concentration in the reaction solvent (dichloromethane-methanol, 90:10). Data showed that at these levels the maximum yield of the derivative was obtained when the ratio of Dns-Cl and DMAP was 1 to 2. DMAP acted both as a catalyst and a scavenger of H^+ (HCl). It was observed that cleanup improved the quality of the chromatogram as seen in Fig. 2 which records the chromatograms of the reaction mixture before (A) and after (B) cleanup on a silica gel cartridge. The recovery of the derivative was done through the Sep-Pak clean up step. Using peak areas the recovery was at 96.6% and the relative standard deviation 1.26% ($n = 4$).

4. Conclusions

The detection method has a detection limit superior to others reported using HPLC. The detection limit is improved by two orders of magnitude. The specific solubility of the derivative in dichloromethane allows its purification with a silica gel column. We are currently inves-

tigating the technique to determine robenidine in both spiked and actual samples.

Acknowledgements

The authors thank J. Nikiforuk (Research Branch, Agriculture Canada) for the NMR spectra and analysis and also N. Snider for MS spectra (Laboratory Services Division, Agriculture Canada).

References

[1] *Off. J. European Communities, Special Edition*, 8 (1973) 4.

- [2] *Off. J. European Communities*, L245 (1985).
- [3] H.D. Chapman, *Parasitol. Today*, 9 (1993) 159.
- [4] J. Zulalian and P.E. Gatterman, *J. Agr. Food Chem.*, 21 (1973) 794.
- [5] K. Al-Shawabkeh, *Disarat Ser. B, Pure Appl. Sci.*, 17 (1990) 86.
- [6] M.Q. Xie, T. Fukata, J.M. Gilbert and L.R. MacDougald, *Parasitol. Res.*, 77 (1991).
- [7] G.F. Bories, *Analyst*, 100 (1975) 567.
- [8] M.K. Cody, G.B. Clark, B.O.B. Conway and N.T. Crosby, *Analyst*, 115 (1990) 1.
- [9] J.B. Zagar, P.O. Ascione and G.P. Chrekian, *J. Assoc. Off. Anal. Chem.*, 58 (1975) 822.
- [10] F. Garcia Sanchez and C. Cruse Blanco, *Analyst*, 116 (1991) 851.
- [11] D.W. Fink, *Trends Anal. Chem.*, 1 (1982) 254.
- [12] W.R. Seitz, *CRC Crit. Rev. Anal. Chem.*, 8 (1980) 367.
- [13] N.P.J. Price, J.L. Firmin and D.O. Gray, *J. Chromatogr.*, 598 (1992) 51.