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LIQUID CHROMATOGRAPHY OF DANSYL DERIVATIVES OF SOME ALKALOIDS AND THE APPLICATION TO THE ANALYSIS OF PHARMACEUTICALS

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SUMMARY

Derivatization of the alkaloids cephaeline, codeine, emetine, ephedrine, morphine, narcotine and others with dansyl chloride has been studied with the aim of developing a sensitive and specific liquid chromatographic method for these substances in complex pharmaceutical dosage forms. While codeine and narcotine do not react, the other compounds form completely substituted derivatives which possess maxima in their fluorescence emission spectra between 470 and 500 nm. The structure of the derivatives has been confirmed by nuclear magnetic resonance spectroscopy. The dansylated compounds have been separated by thin-layer chromatography and high-pressure liquid chromatography. The improved selectivity and sensitivity have permitted an analysis of these substances present in low concentrations in 10–100-fold excesses of other drugs. Direct derivatization of syrups and aqueous slurries of capsules having a complex excipient and drug composition is feasible and time saving and serves as a pre-clean-up step. Detection limits are in the 1–10-ng range or better, depending on the efficiency of the detection device. The reproducibility of the method is limited by the derivatization step, but a relative standard deviation of less than 2% can be obtained. The analysis time for these pharmaceuticals may be reduced by at least one fifth of that required by conventional techniques.

INTRODUCTION

The advantages of derivatization in liquid chromatography of trace amounts of substances in a complex matrix are evident and have been discussed earlier[1,2]. While much of the work has been done with thin-layer chromatography (TLC)[1], high-pressure liquid chromatography (HPLC) has also been used to separate the derivatives (pre-column derivatization)[2–6].

Carbamate pesticides in soil and water samples have been analyzed as dansyl derivatives without necessitating pre-clean-up[3]. In the analysis of drugs, Siggia and Dishman[4] combined the techniques of UV derivatization and HPLC for steroids. Dünges *et al.*[5] demonstrated the feasibility of analyzing barbiturates in serum via a dansylation procedure. Several alkaloids containing amino or phenolic groups were

successfully derivatized with dansyl chloride by Nachtmann *et al.*[6] in an attempt to develop a sensitive and selective method for these compounds in pharmaceutical preparations. The chromatographic behaviour of these derivatives has now been investigated by TLC and HPLC in order to enhance the specificity of this approach.

EXPERIMENTAL

Reagents

5-Dimethylamino-1-naphthalenesulphonyl chloride (Dns–Cl) was obtained from Fluka, Buchs, Switzerland. Analytical grade solvents and reagents were used for the derivatization and chromatographic procedures. The alkaloids (Sandoz, Basle, Switzerland) were dried at 110° for 5 h before use. TLC plates and HPLC adsorbents were obtained from E. Merck, Darmstadt, G.F.R.

Preparation of derivatives for spectral and chromatographic studies

A three-fold excess of Dns–Cl in 100 ml of acetone was added to *ca.* 10–20 ml of a 0.1 *M* aqueous alkaloid solution. 75 ml of a 0.1 % (w/v) sodium carbonate solution were then added and the mixture was allowed to react for 30 min at 45 ± 2° in a water-bath. After cooling, the derivatives were extracted with 2 × 100 ml of benzene and dried over anhydrous sodium sulphate. After filtration and evaporation to dryness, the residue was redissolved in 10 ml of benzene and cleaned by filtration through a column of neutral aluminium oxide (Alox, Woelm, Eschwege, G.F.R.) with an activity of 2. Dns–emetine crystallized as a greenish yellow solid, Dns–cephaeline as a yellow solid and Dns–ephedrine gave a viscous dark yellow mass. The purity of these products was checked by TLC. Fluorescence spectra were recorded with a Perkin-Elmer MPF-3 spectrofluorimeter. NMR investigations were carried out with a Varian Model A60 spectrometer.

Preparation of derivatives for analysis

The optimum conditions for the derivatization step have already been studied by Nachtmann *et al.*[6]. *Ca.* 10 µl of aqueous alkaloid solution containing not more than 30 nmoles of reactive groups were mixed with 50 µl of Dns–Cl (0.1 % in acetone) and 50 µl of 0.1 *M* sodium carbonate. All of the solutions were pipetted with Oxford samplers in to 10-ml centrifuge-tubes. The centrifuge-tube was stoppered tightly, shaken and dipped into a water-bath with exclusion of light. The contents were allowed to react for 20 min at 45 ± 2°. After cooling to room temperature, 0.5 ml of benzene was added and the tube was shaken mechanically for 3 min. The phases were usually well separated, or they were separated by centrifugation. The benzene fraction was utilized for chromatography.

A syrup can be derivatized directly without prior extraction of the active substances. The syrup was diluted with an equal volume of distilled water and 2 ml of the resulting solution were mixed with 2 ml of a 1 % solution of Dns–Cl in acetone in a 10-ml test-tube or centrifuge-tube. 200 µl of a 1.5 *M* aqueous solution of sodium carbonate were then added. The mixture was warmed at 45 ± 2° for 20 min in the dark. After cooling, 3 ml of distilled water and 0.5 ml of benzene were added. The benzene supernatant was used directly for TLC. For HPLC, the benzene was evaporated, the residue was dissolved in the mobile phase and an aliquot portion was injected.

For capsules, the derivatization was carried out with three capsules in 20 ml of an aqueous slurry containing the content of one capsule. This slurry was treated in an ultrasonic field for 10 min and centrifuged for 10 min at 2000 *g*. 2 ml of this solution were mixed with 200 μl of a 1.5 *M* sodium carbonate solution and 2 ml of a 1% solution of Dns–Cl in acetone. Further treatment was identical to that of the samples of syrup.

TLC and evaluation

Separations were carried out after spotting with 2-μl Microcaps. The solvent system toluene–methanol–acetone (9:1:1) was used for separations on Merck silica gel no. 5721 plates without a fluorescent indicator. The chromatograms were developed by the ascending technique for 15 cm and dried in a cold stream of air for 45 min. All these operations were carried out with the exclusion of light. The plates were examined under a Camag UV lamp at 254 nm and measured on a Perkin-Elmer MPF-3 spectrofluorimeter equipped with a TLC scanning attachment. Excitation wavelength, λ_{ex}, 360 nm; emission wavelength, λ_{em}, 500–510 nm with UV filter No. 39; excitation slit-width, 20 nm; emission slit-width, 14 nm; low scan speed.

Electronic integration was made with a Perkin-Elmer SIP-1 integrator. For quantitative evaluation, standards were treated separately (external standardization) according to the above procedure or an internal-addition method was used.

High-pressure liquid chromatography

Pneumatic Haskel pumps were used in a home-made chromatograph[7]. The detection was carried out simultaneously with a LDC fluorescence detector and a Perkin-Elmer fixed-wavelength (254 nm) UV detector. 10-μl samples were injected via a septum or stop-flow procedure[8]. Separations were carried out on columns (25 cm × 2.8 mm I.D.) filled by a slurry-packing procedure[9] with Merck silica gel SI 100 (particle size, 10 μm). Diisopropyl ether–isopropanol–conc. ammonia (48:2:0.3) was used as mobile phase for the syrup formulations and for the determination of emetine in capsules. For less polar components such as ephedrine diisopropyl ether saturated with conc. ammonia–isopropanol (99:1) is recommended.

RESULTS AND DISCUSSION

Spectroscopic investigation of derivatives

The NMR spectra for the derivatives of cephaeline (Fig. 1) and emetine (Fig. 2) confirm the predicted reactions as shown in Fig. 3. The reactions were stipulated as the basis of the titration data of Nachtmann *et al.*[6]. Mono-Dns derivatives were found for ephedrine and morphine[6].

The following maxima were found in the fluorescence study carried out in benzene using the Perkin-Elmer MPF-3 spectrofluorimeter:

Dns–cephaeline: λ_{ex} = 358 nm, λ_{em} = 492 nm; Dns–emetine: λ_{ex} = 356 nm, λ_{em} = 481 nm; Dns–ephedrine: λ_{ex} = 354 nm; λ_{em} = 476 nm; Dns–morphine: λ_{ex} = 354 nm; λ_{em} = 476 nm.

These maxima differ somewhat those given by Nachtmann *et al.*[6] since the spectra were not corrected and a Zeiss instrument (filter, λ_{ex} 365 nm) was used in the earlier study. The relative fluorescence intensity for equimolar concentrations of the deriv-

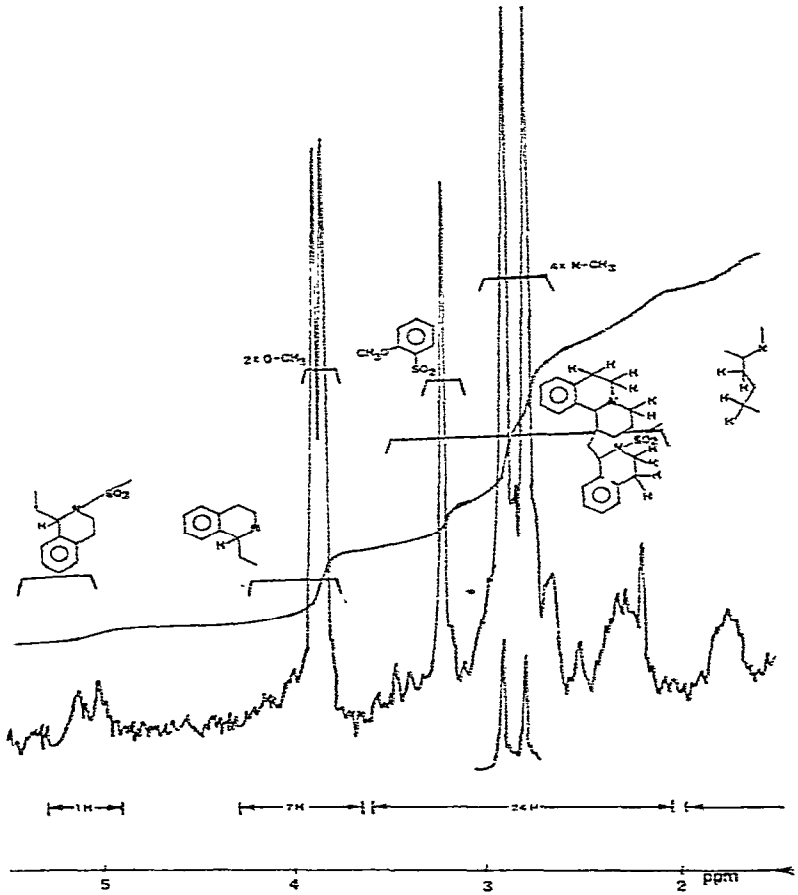

Fig. 1. 100-MHz NMR spectrum of dansylated cephaeline. Solvent, deuterochloroform.

atives was highest for the disubstituted cephaeline, as expected, but not double that of the monosubstituted derivatives[6] since structural factors are important. Spectra were also recorded with the MPF-3 instrument equipped with the TLC attachment. The spectrum of cephaeline taken directly from a dry chromatographic spot on silica gel is shown in Fig. 4. A small bathochromic shift of λ_{em} was observed for the adsorbed derivative. The difference between λ_{ex} and λ_{em} is 140 nm which should permit a relatively simple evaluation of the chromatographed zones with a minimum of interference.

Separation by TLC

With the solvent system toluene–methanol–acetone (9:1:1) a satisfactory separation was obtained with R_F values of 0.50 for ephedrine, 0.32 for cephaeline, 0.22 for emetine and 0.1 for morphine. The reagent Dns–Cl had an R_F value of 0.65. The chromatogram of a cough syrup containing ephedrine chlorohydrate and the bromohydrates of emetine and cephaeline is shown in Fig. 5. The concentrations of

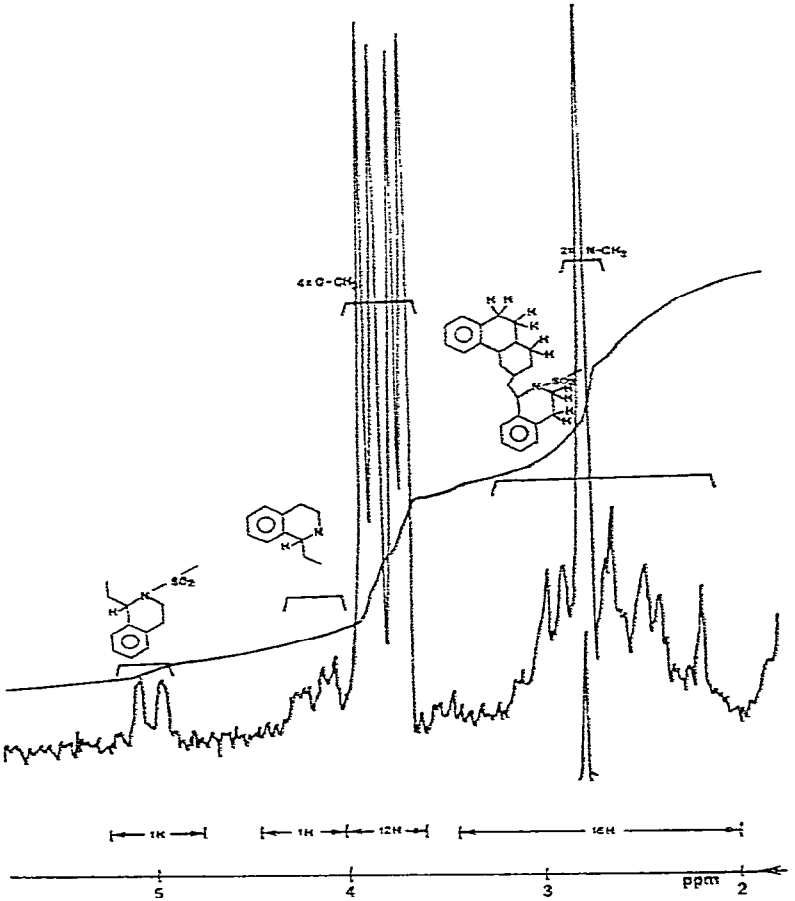

Fig. 2. 100-MHz NMR spectrum of dansylated emetine. Solvent, deuterochloroform.

cephaeline, emetine and ephedrine were 6, 16, and 160 ng, respectively. Other fluorescent spots, which could contain by-products of Dns–Cl and other undesirable components ($R_F < 0.2$), were well separated. Narcotine (400 ng per spot) and codeine (500 ng per spot) do not form derivatives with Dns–Cl and are shown as dark spots. They do not interfere with the other active substances and can be measured by UV densitometry after modification of the chromatographic system. The problem with this pharmaceutical product is the quantitation of cephaeline and emetine which are present in very low concentration. The problem can now be solved with the dansylation procedure. A fluorescence scan of the chromatogram in Fig. 6 is shown in Fig. 7. The reproducibility of such scans and the detection limit were improved considerably by choice of an appropriate cut-off filter, as can be seen in Fig. 8. Filter no. 39, which has no spectral band pass below 390 nm, was the most suitable. The limits of detection under the present conditions were better than 1 ng of alkaloid per spot.

Another application of the technique is shown in Fig. 9, which gives the chromatogram for dansyl derivatives of a syrup containing other opium alkaloids in ad-

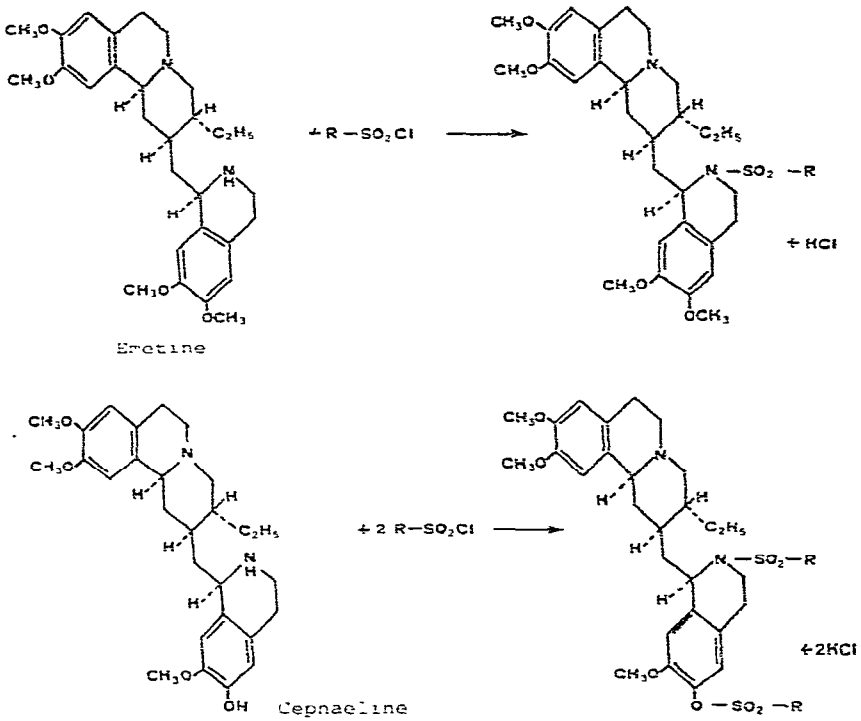

Fig. 3. Scheme for the reaction of dansyl chloride (R–SO_2Cl) with emetine and cephaeline.

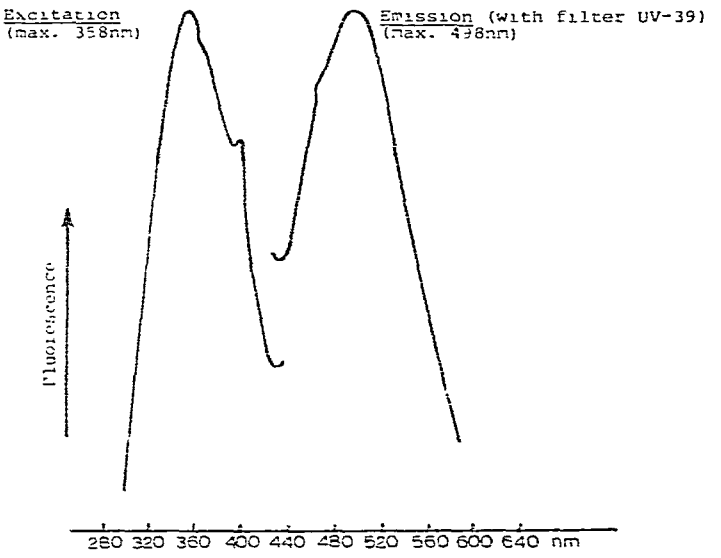

Fig. 4. Fluorescence spectrum of dansylated cephaeline measured directly from a spot on a thin-layer plate.

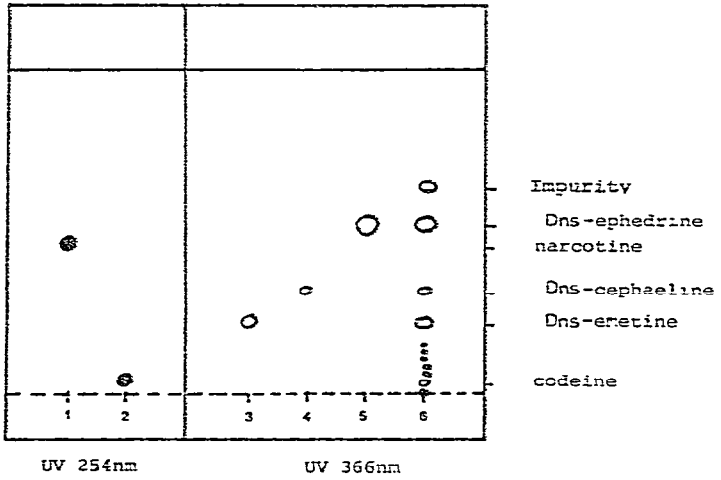

Fig. 5. Chromatogram (TLC) of a cough syrup. The shaded spots were visible by fluorescence quenching and the unshaded spots by fluorescence.

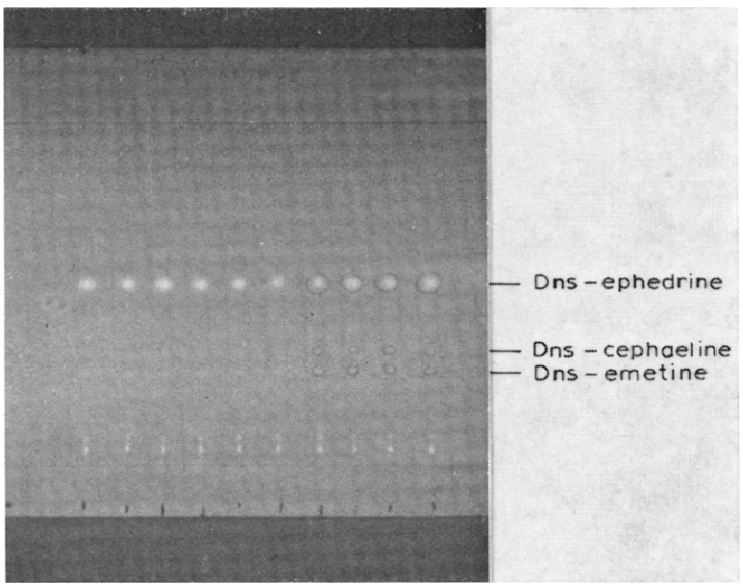

Fig. 6. Chromatogram (TLC) of a series of cough syrup assays after derivatization with Dns–Cl.

dition to those mentioned above. Only morphine was identified, but at least three other spots were formed which could be narceine, thebaine and papaverine all of which form dansyl derivatives.

The feasibility of dansylation for solid pharmaceutical forms was demonstrated with capsules containing β-hydroxypropyltheophylline, caffeine, allobarbital, ephedrine and emetine together with excipients. Ephedrine, emetine and allobarbital form derivatives with Dns–Cl. The chromatogram for such a separation is shown in

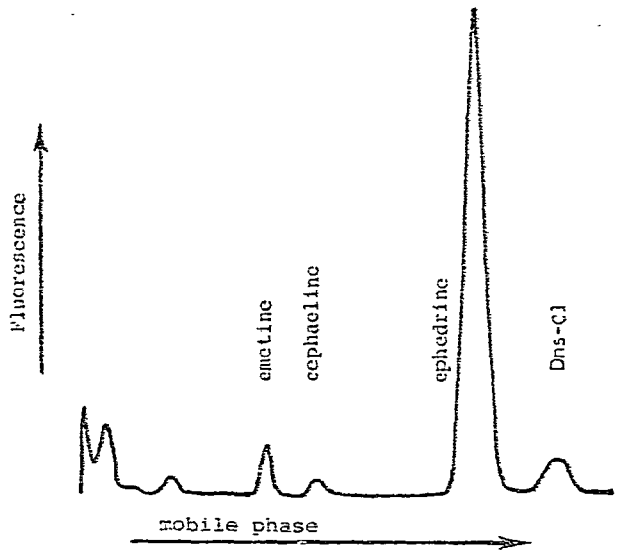

Fig. 7. Typical fluorescence scan of the chromatogram shown in Fig. 6.

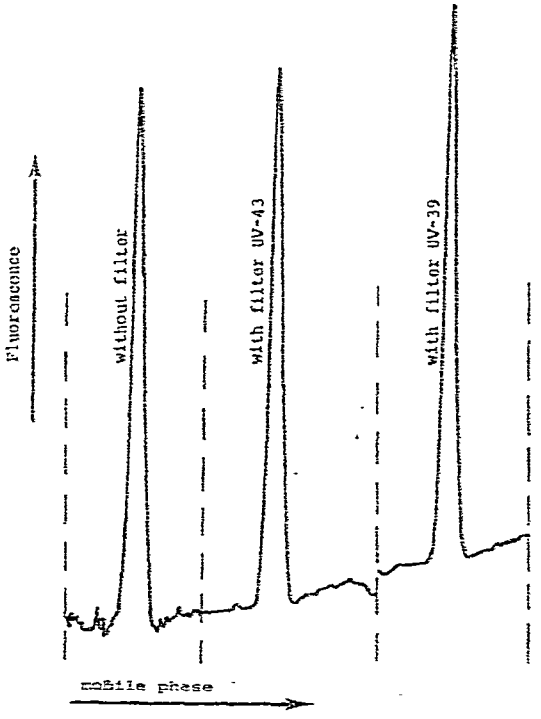

Fig. 8. Influence of the cut-off filter on baseline noise.

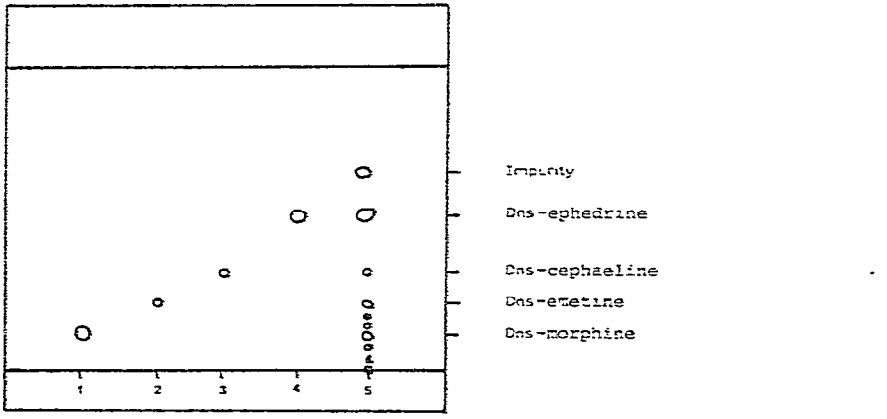

Fig. 9. Chromatogram (TLC) of a more complex cough syrup.

Fig. 10. It was possible to determine emetine, present in nanogram amounts, with little interference and difficulty. The dansylation procedure was not optimized for the barbiturate allobarbital and formation of more than one derivative is possible; hence, some unidentified spots were observed. Interference from excipients also occurred.

Separation by HPLC

With combined UV–fluorescence detection, the TLC solvent system could not be used for HPLC. Diisopropyl ether–isopropanol–conc. ammonia (48:2:0.3) was suitable for separation of the compounds of interest (Fig. 11). Dodecylbenzene was used for t_0, but is only applicable for UV detection; narcotine and codeine do not form derivatives with Dns–Cl and can be detected only by the UV method. For good reproducibility of the separation, it is recommended that, after packing, the column be washed with methanol and then with dichloromethane to ensure that the silica gel attains a constant activity. The mobile phase should be in a closed system in order

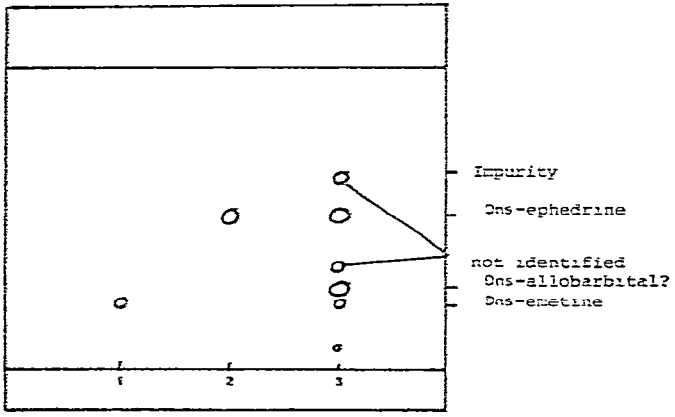

Fig. 10. Chromatogram (TLC) of derivatized active ingredients contained in capsules.

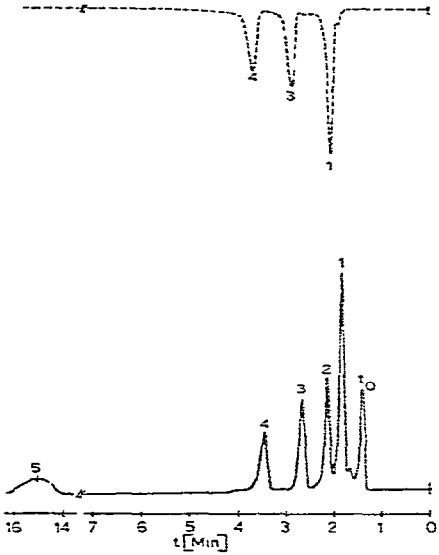

Fig. 11. Chromatogram (HPLC) of a cough syrup after derivatization. ———, UV detection; — — —, fluorescence detection. Peaks: 1 = Dns–ephedrine; 2 = narcotine; Dns–cephaeline; 4 = Dns–emetine; 5 = codeine; and t_0 = dodecylbenzene.

to avoid evaporation of diisopropyl ether and hence a change in polarity. The concentration of the ammonia solution was checked periodically by titration.

Separation of the derivatives of the cough syrup using a solvent system of slightly higher polarity is shown in Fig. 12. This condition was necessary to determine conveniently codeine (peak 5). The chromatogram obtained by UV detection

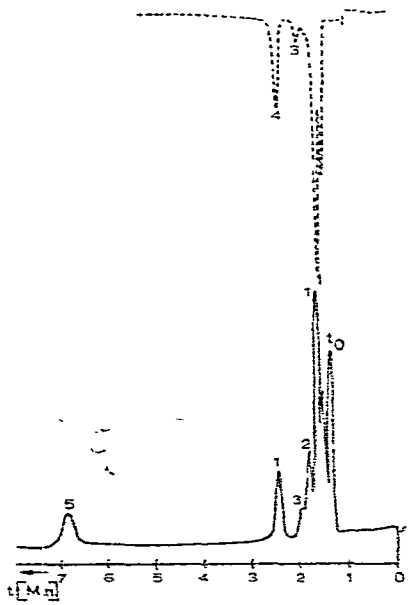

Fig. 12. Chromatogram (HPLC) of the syrup shown in Fig. 11 with a more polar eluent (see text).

(solid line) shows the strong interference of narcotine (peak 2) with cephaeline (peak 3) and ephedrine (peak 1), which would definitely prevent quantitation of the small amount of cephaeline. This interference was eliminated in the fluorescence detection mode (broken line). The peak preceding ephedrine is probably due to a by-product of Dns–Cl and can be avoided with the use of a smaller excess of reagent.

The analysis of the capsules studied by TLC was also attempted by HPLC. Fig. 13 shows the UV and fluorescence chromatograms for the separation of 0.4 μg of emetine in the presence of 30 μg of allobarbital and 15 μg of ephedrine. The mobile phase was identical to the one used for the syrup. Both UV and fluorescence detection would be suitable for the determination of the emetine derivative (peak 3). The neighbouring peak (peak 2) was not definitely identified, but is most likely an artefact of allobarbital (monosubstituted allobarbital) which would require more vigorous conditions for disubstitution[5]. The less polar components were not resolved under these conditions. A separation was attempted with a less polar solvent, diisopropyl ether saturated with conc. ammonia–isopropanol (99:1). UV detection (Fig. 14, solid line) showed dodecylbenzene (peak 1) followed by an unidentified non-fluorescent component. Peak 3 is due to allobarbital (probably the disubstituted derivative) and may contain a trace of Dns–Cl. The ephedrine peak (4) was well separated in both detection modes and can be analyzed quantitatively. Emetine was retained strongly ($k' > 5$) under these conditions.

Detection limits for HPLC (UV and fluorescence detection) were about the same as those for TLC fluorescence densitometry, even though the fluorescence conditions were far from optimal.

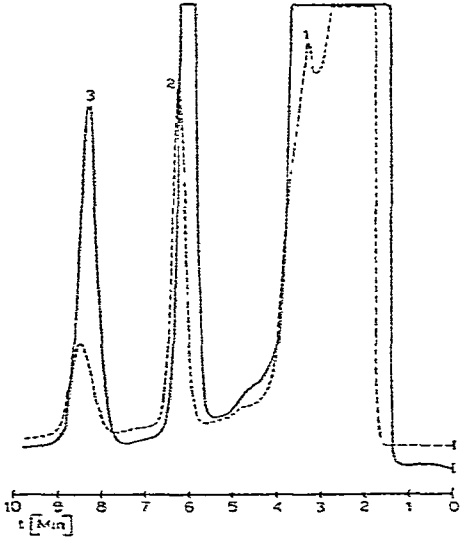

Fig. 13. Chromatogram (HPLC) of derivatized active ingredients contained in capsules. ———, UV detection; — — —, fluorescence detection. Peaks: 1 = not identified; 2 = artefact of allobarbital (monosubstituted allobarbital); and 3 = Dns–emetine.

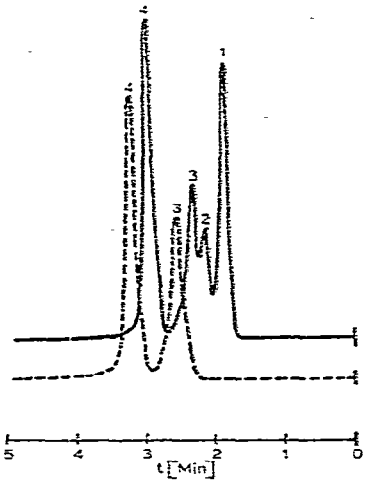

Fig. 14. Chromatogram (HPLC) of derivatized active ingredients contained in capsules with a less polar eluent than used in Fig. 13 (see text). Peaks: 1 = dodecylbenzene (t_0); 2 = not identified; 3 = derivative of allobarbital (probably the disubstituted derivative); and 4 = Dns–ephedrine.

Quantitation

The limiting factor in the quantitation of these alkaloids is the reproducibility of the derivatization step. With care it is possible to obtain a relative standard deviation (S.D.) of *ca.* 3% and occasionally below 2% (ref. 1).

With direct dansylation in the syrup, for example, a S.D. of $\pm$ 1.9% was obtained for emetine (10 assays derivatized and chromatographed simultaneously) and $\pm$1.6% for cephaeline. For ephedrine the best result was $\pm$5.0%, but ephedrine is known[6] to react more slowly with Dns–Cl and this could be overcome by modifying the reaction conditions.

The linearity of calibration graphs was checked both for solutions[6] and *in situ* on TLC and was excellent (correlation coefficient, < 0.990) over concentration ranges of two to three orders of magnitude. The quantitative analysis of samples of syrup yielded reasonable results within the expected error range using internal or external standardization. Five analyses can be carried out in one day.

CONCLUSIONS

Derivatization liquid chromatography can contribute significantly to the solution of complex pharmaceutical analysis problems. In the present case, the analysis time for complicated formulations has been reduced by *ca.* one fifth for the TLC method, compared to those required by previous techniques (*i.e.*, TLC with elution of zones and spectrophotometry). HPLC may offer an even better efficiency and some automation possibilities for large series of analyses. From our preliminary results, it can be concluded that many opium alkaloids could be analyzed by this technique and the range of application could be extended to many other groups of compounds and types of matrix.

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