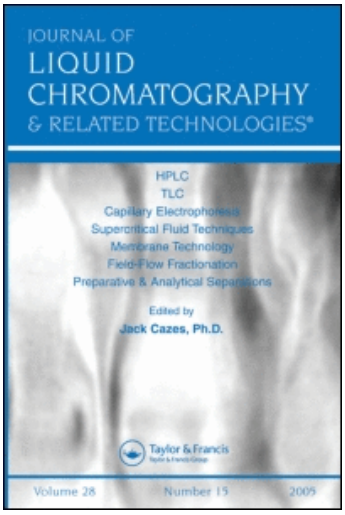

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A Study of Adrenaline-Dansyl Derivatives for Separation by Liquid Chromatography. Application to Blood Plasma Analysis

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A STUDY OF ADRENALINE-DANSYL DERIVATIVES FOR SEPARATION BY LIQUID CHROMATOGRAPHY. APPLICATION TO BLOOD PLASMA ANALYSIS

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ABSTRACT

A plausible mechanism and explanation for the instability of the tri-dans adrenaline has been given. The formation of dimeres accompanied by the loss of one dansyl moiety per adrenaline molecule seems to take place upon U.V. irradiation. This is followed by an increase in fluorescence due to release of strain (steric hindrance) on the molecule.

The feasibility of the pre-column dansylation technique for the analysis of adrenaline and noradrenaline in plasma samples has been demonstrated. Taking necessary precautions, quantitation in the low nonagram region is possible with a reproducibility

of below 5% rel. S.D. inspite of the inherent instability of the derivatives.

INTRODUCTION

The HPLC separation of catecholamines, adrenaline and noradrenaline in particular, has been discussed in several papers recently and a comparative table for the various techniques has been published by Schwedt[1]. Thus it seems that the separation and determination of these components at least in artificial mixtures does not pose any great problems.

The determination of these two components in blood samples or, to a lesser degree in urine samples, is still less than satisfactory resolved. For routine clinical analysis a fluorimetric approach[2] is frequently used, forming a trihydroxyindole derivative. This technique is sensitive and selective enough to permit quantitation of adrenalines in a complex biological matrix but is not ideal for an individual assessment of adrenaline and noradrenaline. In such a situation the separation should be effected chromatographically while still maintaining the needed low detection limits.

The coupling of HPLC and post-column derivatization has been discussed for this purpose[1,3]. The use of a phtalaldehyde (OPT)-reaction detector[3] following reversed-phase or ion-exchange separations, works well for catecholamines with primary amino groups but does not permit determination of adrenaline. Adoption of the trihydroxy indole technique[2] seems to be an elegant approach to routine determination of adrenaline and noradrenaline as separate entities; unfortunately its application to actual samples has not been demonstrated[1].

The above approach, while excellent for large series of samples, is rather complex from the apparative point of view and therefore less suited for the occasional analysis. In such a case pre-column derivatization techniques with fluorigenic reagents such as described in a recent review for catecholamines[4] offer an alternative while still maintaining the needed selectivity and sensitivity.

The pre-chromatographic derivatization of catecholamines with Fluram^{R} has been discussed [5,6] but like with OPT, adrenaline can not be handled since it has no primary amino group.

The most promising approach seems therefore the dansylation procedure discussed for these types of compounds allready in 1965[7]. A first systematic study for the dansylation of adrenaline was published by Nachtmann et al.[8] with the purpose of developing a fluorescence densitometric TLC method. Schwedt and Bussemas[9,10] have shown later, that these derivatives are separated advantageously with HPLC rather than TLC due to their strong sensitivity toward light. It was thereby possible to completely separate the dansyl derivatives of noradrenaline and adrenaline even in ratios of 5:1 (NA:A), as expected in natural plasma samples. Unfortunately again, a practical application has not been shown and it was the purpose of this work to investigate the inherent problems of such an approach and to test the feasibility of blood plasma analysis via dansyl derivatives of these compounds despite the high instability of the derivatives and the high danger of artefact formation.

EXPERIMENTAL

Reagents

L-adrenaline } C.H. Boeringer Sohn, Ingelheim am
L-noradrenaline Rhein (GFR)
I-dimethylamino-naphtaline-5-sulfochloride puriss. (dans-Cl). p.a. Fluka, Buchs, Switzerland.
acetone, benzene, methanol, anhydrous sodium sulphate, sodium carbonate, hydrochloric acid; analytical grade, Merck Darmstadt, GFR.

Apparatus

Zeis PMQ II spectrophotometer equiped with ZFM4 fluorescence attachment (Zeiss, Oberkochen, GFR). Perkin-Elmer MPF-4 spectrofluorimeter equiped with TLC scanner and SIP-1 integrator (Perkin-Elmer Inc. Norwalk, Conn. U.S.A.).

Standard TLC equipment; Merck silica gel plates Cat. No. 5721. HPLC equipment: Pump Waters M 6000, (Waters Inc. Mass. U.S.A.). Detector Aminco - Fluoro Monitor (Amer. Instr. Co., Silver Springs, U.S.A.). Column: Lichrosorb SI 100 5 µm, length 10 cm, i.d. 0.46 cm packed according to a dynamic slurry procedure[11].

Preparation of Derivatives for Structural Confirmation

27 mg adrenaline (0,15 mmoles) are dissolved in 12 ml 0.01 N HCl and 10 ml H_2O are added. To this solution one successively adds a solution of 280 mg dans-Cl in 150 ml acetone and then 100 ml of a 0.1% sodium carbonate solution. The pH is adjusted to 8.5 by adding a few drops of 2 N HCl solution. The reaction takes place at 45° C for 20 minutes. After cooling the reaction mixture, 10 ml saturated NaCl solution is

added and extraction of the derivatives carried out with 2 x 200 ml benzene. The benzene fractions are dryed over anhydrous sodium sulphate, evaporated to dryness and redissolved in 25 ml benzene.

This solution is treated with 2 x 25 ml and 1 x 10 ml 2 N HCl and by 10 ml H_2O. The combined aqueous fractions are neutralized with a 5% solution of sodium carbonate and adjusted to pH 11.5 with solid sodium carbonate. A yellow noncrystalline precipitate appears which is extracted with 2 x 25 ml benzene. The benzene fractions are again dryed over anhydrous sodium sulphate evaporated to dryness and checked for purity by TLC. About 110 mg of the completely substituted adrenaline are available for further studies.

Derivatization for Chromatography

The derivatization of components in a simple matrix can occur according to procedures described earlier. For plasma samples these have to be modified somewhat according to the following procedure:

To 1 ml plasma sample 1,2 ml 0.01 N HCl and 18 ml dans-Cl solution (1.87 mg dans-Cl/ml acetone) are added. To neutralize, 10 ml of a 0.1% sodium carbonate solution are used and the pH adjusted to 8.5 with 0.5 ml of a 5% sodium carbonate solution. The reaction is carried out at 40° C for 20 minutes. Following this, the acetone of the reaction mixture is evaporated off with a rotary evaporator; otherwise separation into two clear layers during extraction becomes difficult. The excess reagent is hydrolyzed by adding 2 drops of a 2 N NaOH and 1 ml NaCl solution. Extraction is carried out with benzene 2 x 30 ml, evaporated to dryness and taken up in 1 ml benzene for chromatography. The first extraction step has to occur without excessive shaking; otherwise layers

are difficult to separate. For HPLC, the residue can also be taken up in the mobile phase.

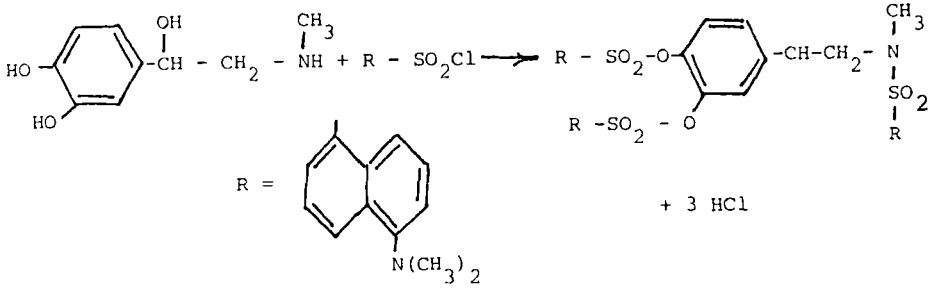
Chromatography

The chromatographic conditions are given in the legends to the figures and in the following text.

RESULTS AND DISCUSSION

Fluorescence and Structure of the Dans-adrenaline

Nachtmann et al.[8] have allready shown via titrimetric techniques that, under suitable reaction conditions, a threefold substituted derivative can be obtained.



This was verified by NMR and IR studies of the derivative.

The fluorescence spectrum recorded with the MPF-4 is shown in Fig.1 and is typical of dans-derivatives the fluorescence maxima being only slightly shifted by the parent molecule. In earlier studies[8] the relative fluorescence yields of the dans-derivatives of adrenaline and some alkaloids have been compared. The adrenaline derivative, even though being the highest substituted (3:1), showed the lowest fluorescence yield, less than 50% of the 2:1 cephaeline derivative and still considerably lower than the 1:1 morphine and

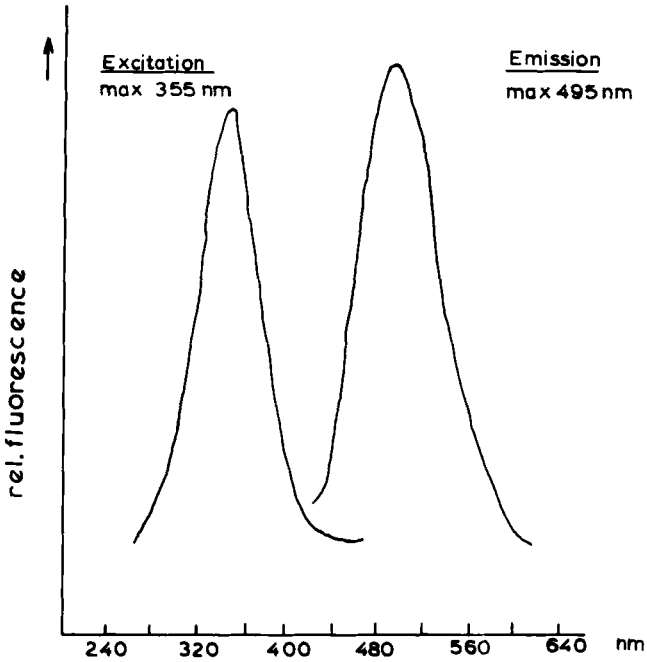


Fig.1. Fluorescence spectra of the tri-dans adrenaline in benzene. Recorded with a Perkin-Elmer Model MPF-4 spectrofluorimeter.

emetine derivatives. A satisfactory explanation was not possible at this time.

Stability of the Adrenaline Derivative

Generally, experience with dansylated species has shown that, upon UV irradiation, the fluorescence decreases probably due to decomposition of the derivative and the formation of dansyl-OH which fluoresces in a different wavelength region[13]. The dansylated adrenaline behaves differently in that the fluorescence first increases and then decreases[8]. This has been checked again and the decomposition studied by TLC

(see Fig.2). Both at 254 nm and 366 nm UV irradiation of the tridans adrenaline different products are formed. One was positively identified as the hydrolyzed dansyl moiety (dans-OH) split off from the relatively unstable tri-dans derivative. Product A was isolated and further investigated. From the NMR spectrum it is evident that

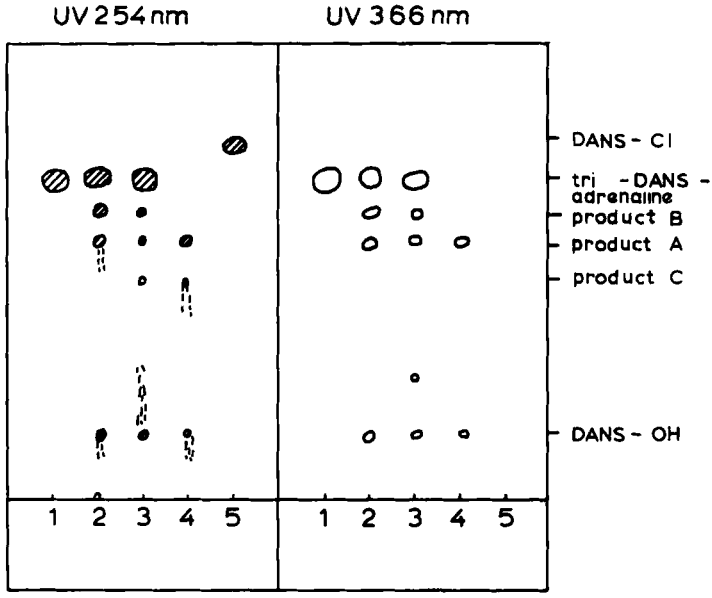
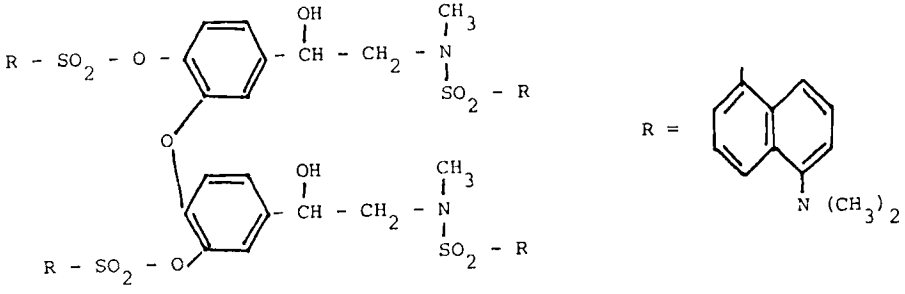


Fig.2. Thin-layer chromatogram of tri-dans adrenaline and its photodegradation products.

1) Tri-dans adrenaline
2) Tri-dans adrenaline irradiated in benzene solution
3) Tri-dans adrenaline irradiated on the plate
4) Product A irradiated in benzene solution
5) Dans-Cl

Chromatography carried out on Merck silica gel plates SI 60 F254.
Mobile phase: benzene : toluene 3 : 1 (v/v)

two dansyl groups remain per adrenaline molecule one at an OH-position and one in the amine position. Since no free OH-groups were detected we suspect the formation of the following asymetric dimere to have taken place:



It is conceivable that product B, based on its polarity (position on the chromatogram), corresponds to the symmetric dimere.
These dimeres are also rather unstable as can be seen from Figure 3 where upon further irradiation at 254 nm in benzene a further drastic increase in fluorescence is observed and additional decomposition takes place (Fig.2). Upon longer irradation the fluorescence decreases again.

An explanation of these facts seems to be rather straight forward. Due to the steric hindrance of the bulky dansyl groups on the neighbouring OH-positions the dans-adrenaline becomes rather strained and non-polar. This could be shown clearly on a model and accounts for the relative instability and the poor fluorescence yield of the molecule. Upon irradiation one dansyl group is lost (formation of dans-OH) and some strain released due the formation of dimeres which, inspite of the loss of one fluorescent group, results in a somewhat higher net fluorescence. This

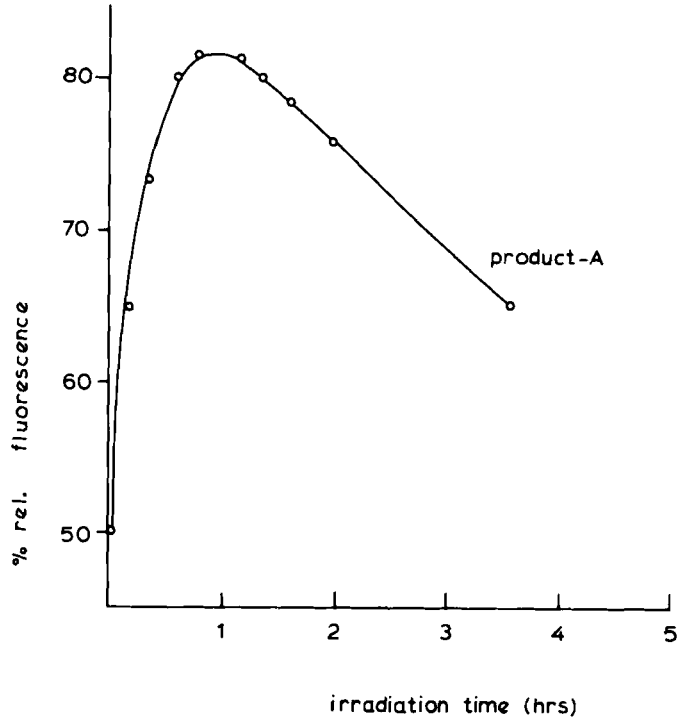


Fig.3. Change of fluorescence yield for the product A as a function of irradiation (λ irradiation 254 nm).

situation is further improved when forming the monomere (disubstituted adrenaline) which is conceivably the more polar product in Fig.2c. Upon prolonged irradiation the disubstituted adrenaline decomposes further and responds like a normal derivative with a net loss in fluorescence.

Application to Plasma Analysis

It has been shown earlier to be possible to use the dansylation technique for analytical purpose[8-10]. The application of the HPLC technique to the deter-

mination of adrenaline and noradrenaline in plasma samples has been attempted here. Although according to Schwedt and Bussemas[10] reaction times of 5-10 min. (at 40° C) should be sufficient, we used a 20 min. reaction time for the plasma samples; exclusion of light was strictly observed. No real advantage was seen by using reversed phase chromatography since an extraction clean-up was necessary in any case for such a complicated matrix. Ethylacetate-cyclohexane mobile phase as recommended[10] was found suitable for the plasma work.

A chromatogram of a plasma sample containing adrenaline and noradrenaline is shown in Figure 4. Adrenaline was added. The separation can be done in seven minutes. The reproducibility of k'-values is good (range k'4.6-4.9 for adrenaline and k'7.4-7.9 for noradrenaline; 4 determinations). The detection limits (< 10 ng/injection) permit good quantitation of common levels of these catecholamines and the reproducibility of quantitative data can be kept below 5% rel. S.D.

CONCLUSIONS

Inspite of the relatively poor stability of the dans-adrenaline one can adopt this technique reliably for the quantitation of the two catecholamines in plasma. With good care (5 extraction steps) a nearly complete recovery of the derivatives from plasma samples is possible and the interferences are well separated within a reasonably short time. For a large scale routine monitoring of samples, the method seems to be less advantageous. It is also obvious from the study, that with adrenaline we have a borderline case as to feasibility of pre-column derivatization techniques, due to the steric circumstances. In this

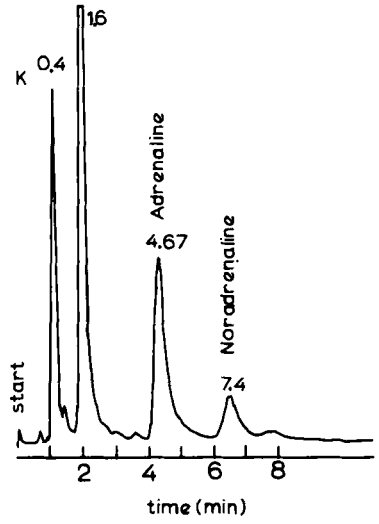


Fig.4. High performance liquid chromatogram of adrenaline and noradrenaline in a blood plasma sample. Conditions: Column Lichrosorb SI 100, 5 µm L 10 cm; i.d. 0,46 cm; injection stop-flow 20 µl; mobile phase: ethylacetate : cyclohexane 3 : 7 (v/v) flow 2.5 ml/min; fluorescence detection.

context the study should draw attention to the general danger of artefact formation with this approach. It should also demonstrate the usefulnes of using models in doubtful cases and of carrying through this type of investigations in order to fully master the reaction. Only then will a reliable interpretation of data be possible.

REFERENCES

1. G. Schwedt, J. Chromatogr., 143, 463 (1977).
2. H. Weil-Malherbe, Methods Biochem. Anal., 16, 194 (1968).
3. G. Schwedt, Anal. Chim. Acta, 92, 337 (1977).
4. N. Seiler, J. Chromatogr., 143, 221 (1977).
5. K. Imai, J. Chromatogr., 105, 135 (1975).
6. G. Schwedt, J. Chromatogr., 118, 429 (1976).
7. N. Seiler and M. Wiechmann, Experientia, 21, 203 (1965).
8. F. Nachtmann, H. Spitzy and R.W. Frei, Anal. Chim. Acta, 76, 57 (1975).
9. G. Schwedt and H. Bussemas, Chromatographia, 9, 17 (1976).
10. G. Schwedt and H. Bussemas, Z. Anal. Chem., 283, 23 (1977).
11. H.R. Linder, H.P. Keller and R.W. Frei, J. Chromatogr., 14, 234 (1976).
12. R.W. Frei, W. Santi and M. Thomas, J. Chromatogr., 116, 365 (1976).
13. J.F. Lawrence and R.W. Frei, "Chemical Derivatization in Liquid Chromatography", Elsevier, Amsterdam, 1976.