Journal of Chromatography, 172 (1979) 141–151

CHROM. 11,638

DETERMINATION OF AMINES USING THE FLUORIMETRIC ION-PAIR TECHNIQUE AS A POST-COLUMN REACTOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J. C. GFELLER, G. FREY, J. M. HUEN and J. P. THEVENIN

Analytical Research and Development, Pharmaceutical Department, Sandoz Ltd., CH-4002 Basle (Switzerland)

(Received November 30th, 1978)

SUMMARY

A post-column derivatization system using the fluorimetric ion-pair technique is described. The separated amines are mixed in an air-segmented flow with 9,10-dimethoxyanthracene-2-sulphonate and the ion pairs formed are extracted and detected by their fluorescence (383/446 nm).

The mobile phase will affect the sensitivity by either increasing the blank or reducing the extractability of the ion pairs. This problem was solved by using a LiChrosorb DIOL stationary phase with an exclusively aqueous mobile phase. The reaction system was tested with hyoscyamine, which was moderately retained ($k' = 0.8$) under the chosen conditions. The repeatability of the system was checked by multiple injections ($n = 10$) of a standard solution (200 ng per injection) and a relative standard deviation of 1.7% was found. The regression was linear over the concentration range of 40–600 ng per injection ($r = 0.9998$). The limit of detection was about 200 pg (signal-to-noise ratio = 3:1), the improvement therefore being at least 200-fold in comparison with UV detection.

The peak width increased by about 40%, probably owing to the dead volume of the reactor. Miniaturization of the system using a smaller phase separator and polypropylene mixing coils of 1 mm I.D. did not provide any improvement. The ion pairs may be adsorbed on the surface of the polypropylene, and glass coils as utilized in the newer generation of AutoAnalyzers will therefore solve that problem.

INTRODUCTION

The ion-pair technique has proved of value for the assay of amines. It is based on the property of these compounds to form ion pairs with ions of opposite charge. The ion pairs are extracted with a suitable organic solvent and, as a rule, are determined by spectrophotometry[1]. Greater sensivity may be achieved by using a fluorescent counter ion. Sodium-9,10-dimethoxyanthracene-2-sulphonate has proved to be a suitable reagent, as it has a strong fluorescence and forms ion pairs with good extraction properties[2]. An automated analysis system was developed, which makes it

possible to determine amines in pharmaceutical products in the parts per billion (10^{-9}) range with high reproducibility[3]. This system has now been adapted as a post-column reactor as previously described[4], and first reported for the assay of hyoscyamine in low doses[5]. In this paper, the usefulness of this method for other drugs and the problems associated with it are considered.

EXPERIMENTAL

Drugs and reagents

The drugs investigated were emetine, ephedrine, hyoscyamine, dihydroergotamine (DHE), bromocriptine, pindolol, pizotifen, ketotifen, guanfacin and clemastine (all supplied by Sandoz, Basle, Switzerland). Their structural formulae are shown in Fig. 1.

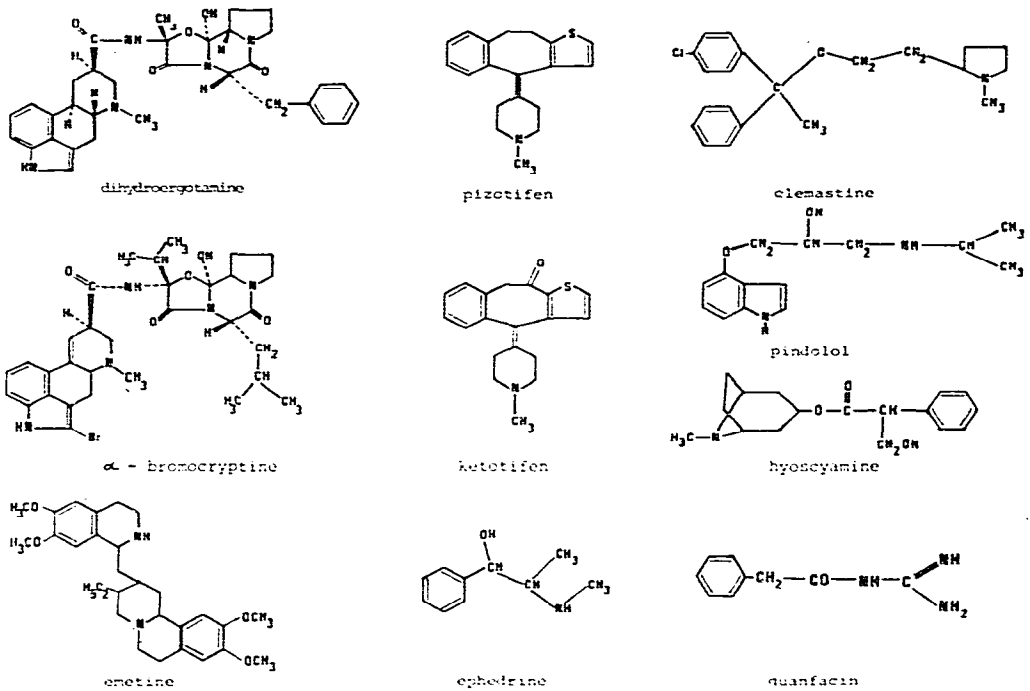

Fig. 1. Structural formulae of the drugs tested.

The phosphate buffer solutions were prepared as described in ref. 6. Sodium 9,10-dimethoxyanthracene-2-sulphonate (Na-DAS) was synthesized as described in the literature[7]. Unless otherwise stated, a reagent concentration of 200 mg/l of Na-DAS in phosphate buffer at pH 2.0 was used. The ion pairs were extracted from the aqueous phase with either chloroform or 1,2-dichloroethane (analytical-reagent grade, Merck, Darmstadt, G.F.R.). The mobile phases, chromatographic conditions and column materials (LiChrosorb DIOL and LiChrosorb RP-8, 10 μm; Merck) are indicated in the figure legends or in the text.

Apparatus

The chromatographic system consisted of an Altex 100 high-pressure pump (Altex Scientific, Berkeley, Calif., U.S.A.), a LiChrosorb DIOL column (25 × 0.46 cm; Knauer, Oberursel, G.F.R., No. 103.07.16) or a LiChrosorb RP-8 column (10 × 0.46 cm; Merck) and an LC-55 spectrophotometer (Perkin-Elmer, Norwalk, Conn., U.S.A.). The injection volume was 20 μl in all instances (7010 loop; Rheodyne, Berkeley, Calif., U.S.A.).

Post-column derivatization was carried out as shown in Fig. 2. The peristaltic pump, pump tubings, fittings and mixing coils were supplied by Technicon (Tarrytown, N.Y., U.S.A.). A Perkin-Elmer 204 fluorimeter equipped with a high-performance liquid chromatographic (HPLC) flow-through cell of 30-μl volume and a 150-W xenon high-pressure lamp was used. The system shown in Fig. 2a was designed for an eluent flow-rate of 2 ml/min.

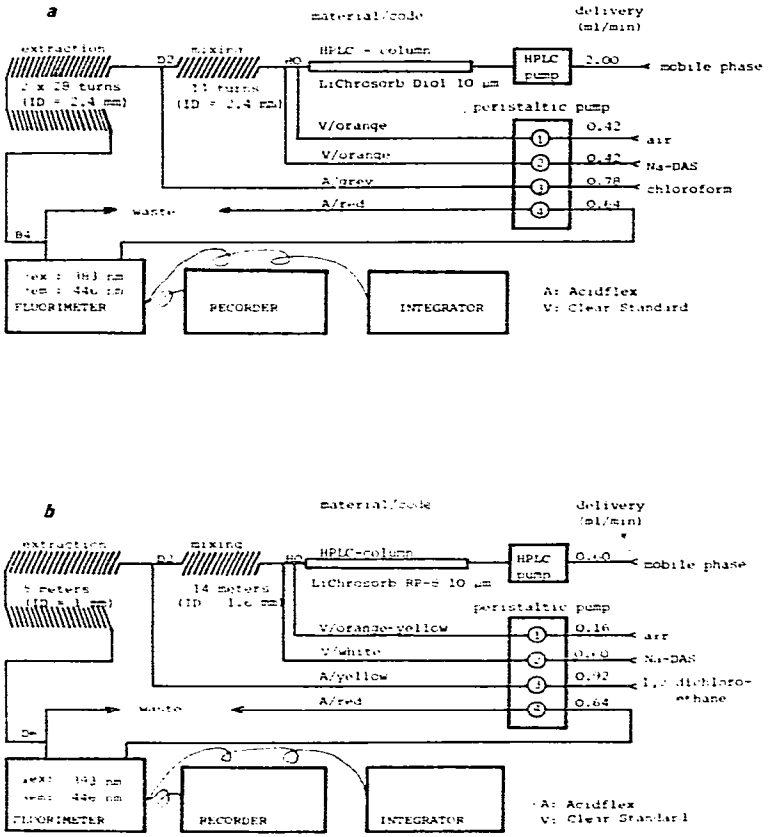

Fig. 2. Flow chart of the post-column fluorescence reactor for eluent flow-rates of (a) 1–3 and (b) up to 1 ml/min.

For lower flow-rates the derivatization system must be miniaturized (Fig. 2b). For the mixing coils, polypropylene tubes of I.D. 1 mm (Portex Ltd., Hythe, Great Britain) and a smaller phase separator were used. The conditions specified are optimal for an eluent flow-rate of 0.6 ml/min.

RESULTS AND DISCUSSION

Optimization of ion-pair extraction

It is known from previous studies[2,3] that the optimal pH for ion-pair formation is in the range 1–4. The same studies showed that about a 25-fold excess of reagent is usually necessary for complete extraction. When the ion pair is markedly hydrophilic, it is incompletely extracted despite a great excess of reagent. In such a case, extraction can be improved by choosing an appropriate organic solvent. Thus it can be seen in Table I that the hyoscyamine ion pair yields maximal fluorescence when methylene chloride is employed for extraction. Nevertheless, the values do not even approach those obtained with pizotifen, which yielded roughly the same fluorescence intensity with all three extraction solvents tested (chloroform, methylene chloride and dichloroethane). In order to eliminate the solvent effect, which is frequently observed in fluorimetry, the organic phases were diluted three-fold with ethanol before the measurements.

TABLE I

EXTRACTABILITY OF PIZOTIFEN AND HYOSCYAMINE ION PAIRS WITH DIFFERENT ORGANIC SOLVENTS

Extraction medium	*Intensity of fluorescence (% of full-scale deflection)*	
	Pizotifen	*Hyoscyamine*
Chloroform	64.4	11.2
Methylene chloride	77.2	15.6
1,2-Dichloroethane	72	6.0

Effect of the mobile phase

In order to study the effect of the mobile phase on post-column derivatization, solutions of reagent in various mobile phases were extracted, using the organic phases mentioned above, and the blank values measured. The lowest blank values were obtained with the pure aqueous phase, followed by methanolic solutions extracted with 1,2-dichloroethane.

It should be noted, however, that the extractability of the ion pairs with 1,2-dichloroethane may be affected by the polarity of the aqueous phase, *e.g.*, the percentage of methanol in this phase, and therefore this effect had to be examined. As can be seen in Table II, the fluorescence intensity with the pizotifen ion pair does not decrease appreciably until the concentration of methanol in the mobile phase reaches 80%. On the other hand, only a fraction of the hyoscyamine ion pair is extracted into the organic phase from a solution containing as little as 40% of methanol. The use of this fluorescence reactor with mobile phases with a high content of organic solvent is one of the problems still to be elucidated.

Choice of chromatographic system

As the studies showed before, the mobile phase can be a limiting factor, as it may increase the blank value and result in poorer extraction. For this reason, a stationary phase was sought that would allow the use of an exclusively aqueous

TABLE II

INFLUENCE OF THE MOBILE PHASE ON THE EXTRACTABILITY OF THE PIZOTIFEN AND HYOSCYAMINE ION-PAIRS WITH 1,2-DICHLOROETHANE

Drug ($2 \cdot 10^{-6}$ *M*)	*Reagent* (10^{-3} *M Na-DAS*)	*Intensity of fluorescence (% of full-scale deflection)*				
		Methanol in the mobile phase (%)				
		0	*20*	*40*	*60*	*80*
Pizotifen	0.6	41	41	37	35	28
	1.2	42	41	37	35	32
	1.8	43	43	37	35	33
	2.4	43	43	39	39	—
Hyoscyamine	0.6	11	9	4	2	<1
	1.2	15	14	8	4	1
	1.8	18	18	10	5	1
	2.4	22	21	12	8	—

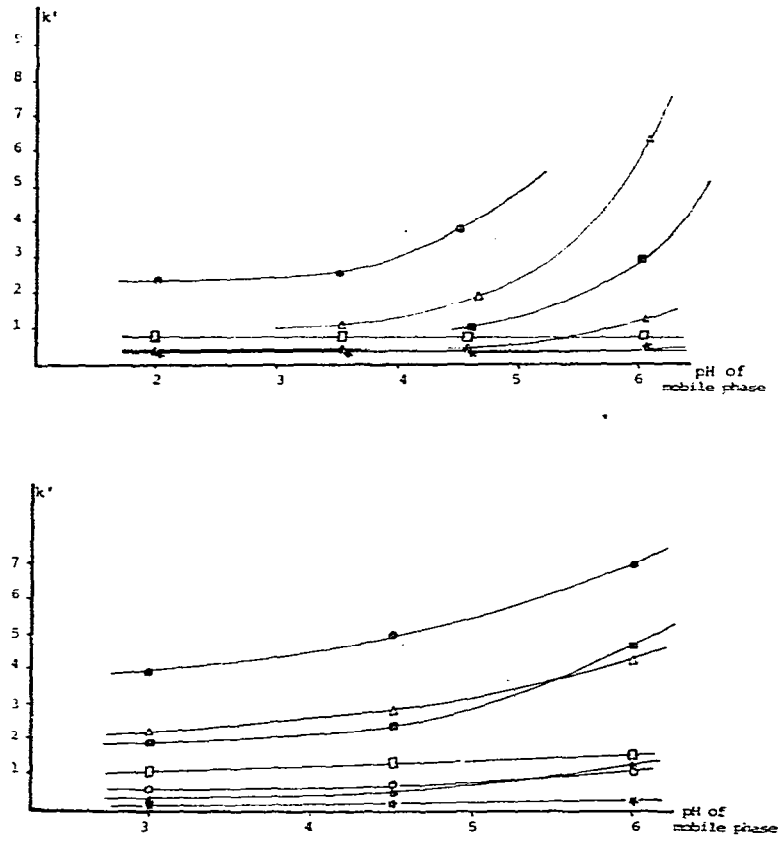

Fig. 3. Variation in the capacity factors of drugs with the LiChrosorb DIOL system. Column, 25 × 0.46 cm; eluent flow-rate, 2.0 ml/min; mobile phase, phosphate buffer (see text). △, DHE; ▲, emetine; ★, ephedrine; □, guanfacin; ○, pindolol; ⊜, pizotifen; ■, ketotifen.

mobile phase. The LiChrosorb DIOL system was found to meet this requirement and, further, the capacity factors of the drugs investigated can be varied over a wide range by changing the pH and the buffer capacity (Fig. 3).

The less polar reversed phase has also been compared with the DIOL phase. The materials supplied with carbon chains of different lengths (C_2, C_8 and C_{18}) can afford greater flexibility and, under certain conditions, a greater selectivity can be expected. LiChrosorb RP-8 was chosen as a typical reversed phase, as it had been found to be highly effective in the analysis of pharmaceuticals. For post-column derivatization, an eluent mixture containing 60% (v/v) of methanol was used, and the same tests were carried out as with the DIOL system. As can be seen from Fig. 4, the capacity factors are increased by either increasing the pH or decreasing the buffer capacity.

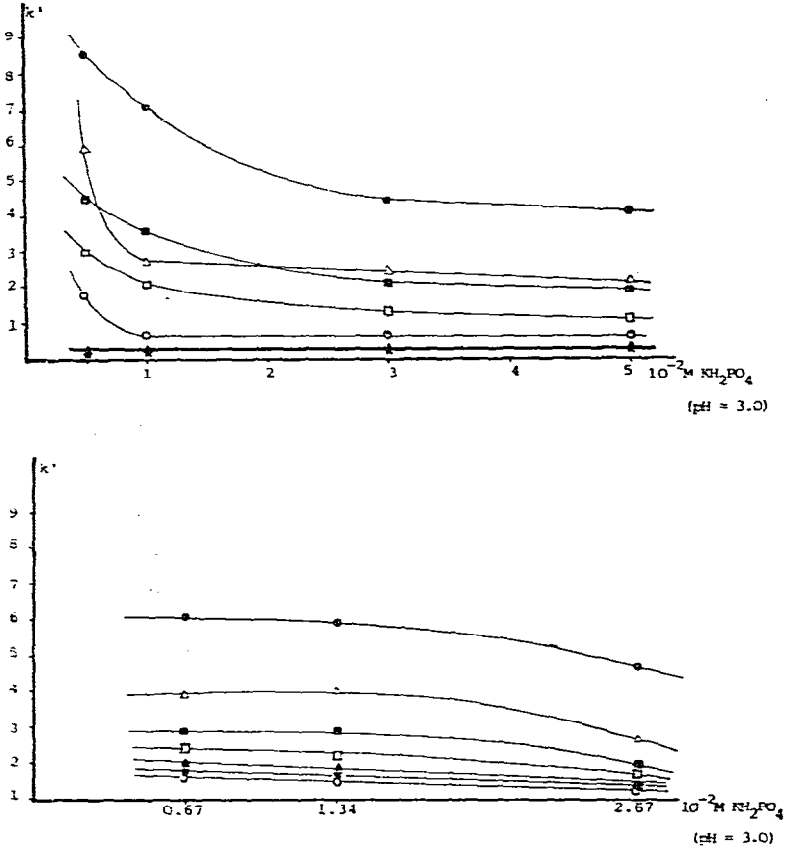

Fig. 4. Variation in the capacity factors of drugs with the LiChrosorb RP-8 system. Column, 10 × 0.46 cm, eluent flow-rate, 0.6 ml/min; mobile phase, phosphate buffer–methanol (2:3) (see text). △, DHE; ▲, emetine; ★, ephedrine; □, guanfacin; ○, pindolol; ●, pizotifen; ■, ketotifen.

Coupling of fluorescence reactor with HPLC

LiChrosorb DIOL system

The first experiments were carried out with a buffer system (0.1 *M*, pH 3.0) and with hyoscyamine as the test substance. Under these conditions, hyoscyamine is

weakly retained ($k' = 0.8$); therefore, it is suitable for evaluating band broadening and for determining sensitivity and reproducibility.

Band broadening. In view of the eluent flow-rate of 2.0 ml/min, reaction spirals of I.D. 2.4 mm and a separator for the organic phase with a dead volume of 300 μl were used (Fig. 2a). A UV detector was inserted in front of the reaction system to measure band broadening. Band broadening, determined from the peak width at half-height between UV and fluorescence detection, was 40% for hyoscyamine and can be regarded as highly satisfactory, despite the relatively large separator employed.

Reproducibility. The overall standard deviation in the post-column derivatization technique consists of the standard deviations of the chromatographic and the reaction system. Repeated injections ($n = 10$) of a standard solution (200 ng of hyoscyamine per injection) yielded a relative standard deviation of 1.7%. The regression is linear over the concentration range 40–600 ng of hyoscyamine per injection ($r = 0.9998$). Accordingly, even a drug that cannot be completely extracted (Table II) can still be assayed if a large excess of reagent is used.

Sensitivity. The detection limit for hyoscyamine was 200 pg (signal-to-noise ratio = 3:1). Thus the sensitivity is 200 times greater than with UV detection (Fig. 5). By using a chromatographic column with a smaller inner diameter (*e.g.*, 3 mm), it might be possible to lower the detection limit.

LiChrosorb RP-8 system

As these results show, the technique of derivatization using the fluorimetric ion-pair technique affords a remarkable increase in sensitivity, especially for amines with weak chromophores. The extent to which the addition of methanol to the eluent affects post-column derivatization is considered below.

The chromatographic system used was a LiChrosorb RP-8 column (10 × 0.46 cm, particle size 10 μm) with an eluent flow-rate of 0.6 ml/min [ratio of phosphate buffer (0.02 *M*, pH 3.0) to methanol = 2:3]. The test substance used was DHE which was preferred to pizotifen for chromatographic reasons.

Miniaturization of the fluorescence reactor. The eluent flow-rate of 0.6 ml/min made it necessary to miniaturize the derivatization system, as shown in Fig. 2b. The spiral used to mix the reagent with the eluate was of 1.6 mm I.D. (Technicon System II), and that used for extraction was of 1 mm I.D., with a view to increase the flow-rate and thus improve the extraction. The dead volume of the separator was reduced to 150 μl by using a D6 fitting.

Band broadening was determined by inserting a UV detector, (as in the LiChrosorb DIOL system) and was found to be 50%. In spite of miniaturization, therefore, no improvement in band broadening was achieved. The probable reason for this is the use of polypropylene mixing spirals, as the hydrophobic ion pairs may be adsorbed on the surface of the polypropylene during the extraction process. This could possibly be improved by using glass capillary coils (*e.g.*, AutoAnalyzer Generation III).

Sensitivity. As already described, the addition of methanol to the mobile phase reduces the sensitivity (Table II). To determine the extent to which the peaks were influenced, various drugs were analysed according to both chromatographic systems with the fluorescence reactors shown in Fig. 2. As can be seen in Fig. 6. the detection signals[3] were reduced by a factor of about 3–5 at a methanol concentration of 60%.

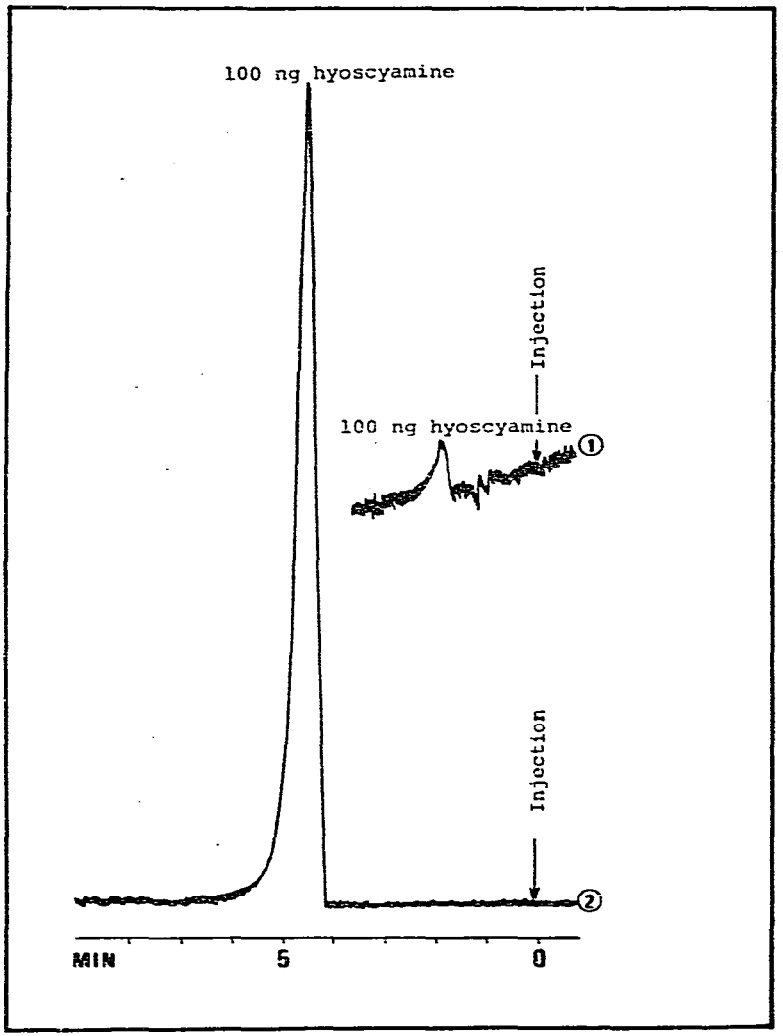

Fig. 5. Comparison of (1) UV detection (208 nm) and fluorescence detection (after post-column derivatization) of hyoscyamine. Chromatographic conditions as in Fig. 3 and in the text.

For some drugs, therefore, this derivatization technique does not bring any appreciable gain in sensitivity in comparison with UV detection, but as the next example will show, it can improve the selectivity.

Example of application

Detection problems may arise in the assay of pharmaceuticals in complex mixtures (*e.g.*, urine), and in such instances post-column derivatization has much to commend it. DHE was added to a sample of urine, which was then neutralized. Proteins were precipitated out and separated as described by Graffeo and Karger[9]. The aqueous phase was extracted with chloroform and the extract was evaporated to dryness. The residue was taken up in the mobile phase and chromatographed. As the chromatogram (Fig. 7) shows, derivatization does not afford any great improve-

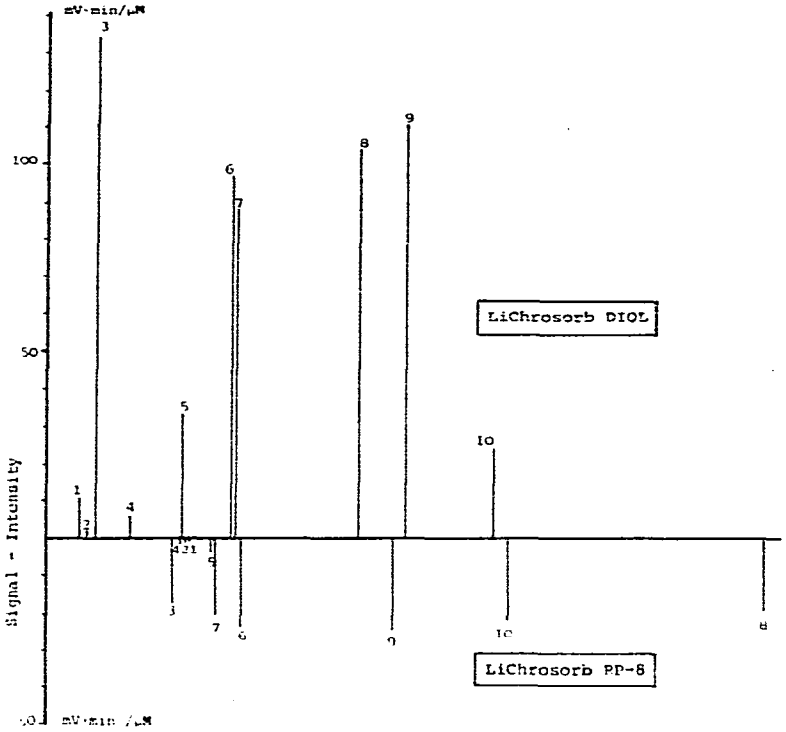

Fig. 6. Effect of the mobile phase on the signal intensities of drugs in post-column derivatization. Mobile phase of the LiChrosorb DIOL system: phosphate buffer, pH 3.0 (0.1 *M*). Mobile phase of the LiChrosorb RP-8 system: phosphate buffer, pH 3.0 (0.02 *M*)–methanol (2:3). Peaks: 1 = hyoscyamine; 2 = ephedrine; 3 = emetine; 4 = pindolol; 5 = guanfacin; 6 = dihydroergotamine; 7 = ketotifen; 8 = pizotifen; 9 = clemastine; 10 = bromocriptine.

ment in sensitivity in comparison with UV detection. However, a remarkable improvement in selectivity can be observed, as the interfering peaks due to other compounds in the urine are no longer detected. To improve the sensitivity beyond that of UV detection, it would be necessary to employ either a mobile phase with a lower concentration of methanol or the LiChrosorb DIOL system with an aqueous buffer solution as mobile phase only.

DISCUSSION

The results show that post-column derivatization by the fluorimetric ion-pair technique is advantageous with respect to sensitivity and selectivity for the assay of pharmaceuticals. For hyoscyamine, for example, the sensitivity of this method was 200 times greater than that of UV detection. However, the improvement in sensitivity would be less with amines that have a stronger chromophore.

The mobile phase can be a limiting factor because, depending on its composition, the blank value may be excessively high or the ion pair may not be fully extracted into the organic phase. This problem was solved by using a LiChrosorb DIOL phase with exclusively aqueous eluents. Taking the more selective reversed phase, *i.e.*, LiChrosorb RP-8, the non-aqueous mobile phase reduces the sensitivity.

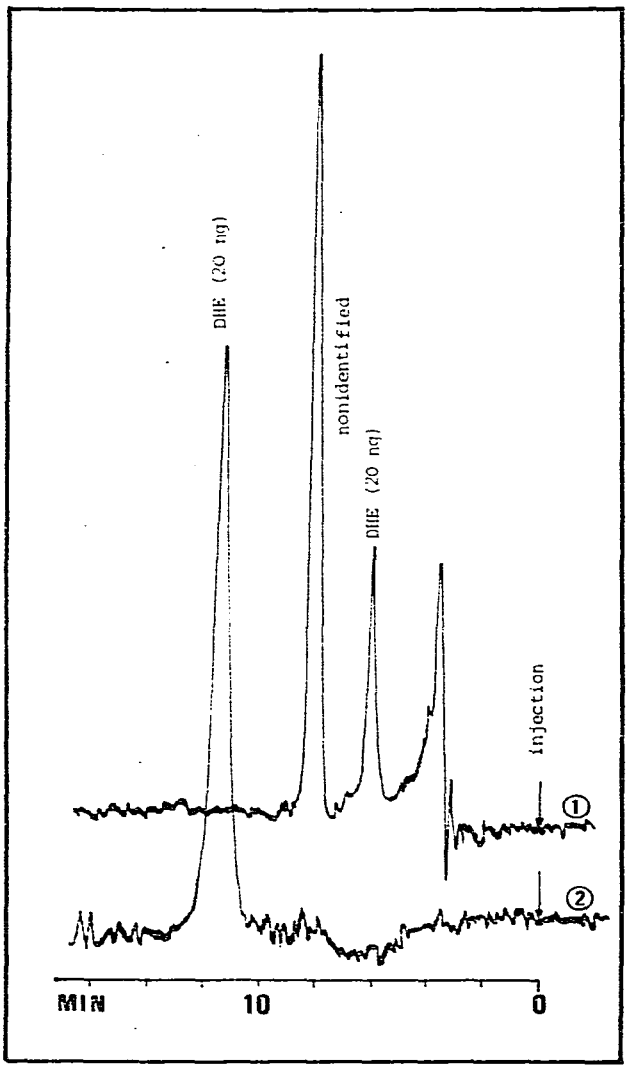

Fig. 7. Chromatogram of DHE in a complex matrix (urine): comparison of (1) UV detection (208 nm) and (2) fluorimetric detection after post-column derivatization. For chromatographic conditions see LiChrosorb RP-8 system described in Fig. 6.

Another alternative is ion-exchange chromatography, which couples chromatographic selectivity and sensitivity, because of the use of aqueous mobile phase[10].

The peak width is increased by derivatization (by about 40%), mainly owing to the dead volume of the phase separator. Miniaturization of the fluorescence reactor by using a smaller separator and polypropylene mixing spirals of 1 mm I.D. did not provide the hoped-for improvement. The ion pair may possibly be adsorbed on the surface of the polyethylene, so that it would be advisable to employ other materials, *e.g.*, glass spirals of the type incorporated in the newer AutoAnalyzer generation. The DIOL system has been employed for about 1 year in these laboratories for the routine assay of pharmaceutical amines in the parts per billion range.

REFERENCES

1 J. A. Marinsky and Y. Marcus, *Ion Exchange and Solvent Extraction*, Marcel Dekker, New York, 1974.
2 D. Westerlund and K. O. Borg, *Anal. Chim. Acta*, 67 (1973) 89.
3 J. C. Gfeller and G. Frey, *Z. Anal. Chem.*, 291 (1978) 332.
4 J. C. Gfeller, G. Frey and R. W. Frei, *J. Chromatogr.*, 142 (1977) 271.
5 J. C. Gfeller, G. Grey, J. M. Huen, J. P. Thevenin, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1978) 213.
6 *Wissenschaftliche Tabellen*, Ciba-Geigy Ltd., Basle, 1968, p. 274.
7 K. H. Meyer, *Justus Liebigs Ann. Chem.*, 379 (1911) 37.
8 R. P. W. Scott, *Liquid Chromatography Detectors*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 11.
9 A. P. Graffeo and B. L. Karger, *Clin. Chem.*, 22 (1976) 665.
10 J. M. Huen and J. P. Thevenin, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, submitted for publication.