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COMBINED ULTRAVIOLET–FLUORESCENCE DETECTION IN HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF PHARMACEUTICALS*

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SUMMARY

The use of combined UV–fluorescence detection for the evaluation of incompletely resolved compounds and trace components in the presence of large quantities of major components is described, analysis for thioridazine and some of its oxidation products by high-pressure liquid chromatography being chosen as a practical example. Mesoridazine and the ring oxide of thioridazine have been determined quantitatively with relative standard deviations ($n = 6$) of 2.0 and 3.6%, respectively, at concentrations below 0.1 μg per injection. Resolution of the two components is difficult and, in this instance, unnecessary. By a similar approach, it was possible to determine the highly fluorescent sulforidazine at a concentration of 0.4% of the thioridazine with 6.2 μg of thioridazine injected. A relative standard deviation of 5% was attainable at this concentration. Fluorescence detection limits for mesoridazine and sulforidazine at a signal-to-noise ratio of 4:1 are between 5 and 10 ng per injection; this corresponds to about 0.1% of the active substance for the above example.

INTRODUCTION

Fluorescence detection as an alternative to UV detection in high-pressure liquid chromatography (HPLC) has been shown to add considerably to the specificity of an analytical method[1–4], particularly if pre-column[1,2] or post-column[3,4] derivatization procedures are used to render non-fluorescent species fluorescent in a selective or semi-selective manner. A combination of the two detection modes can yield additional information and has been utilized successfully (for example, with post-column derivatization techniques, whereby the fluorescence-generating process occurs after the UV detector[3,4]).

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Theoretically, fluorescence detection should result in lower detection limits, but, owing to a lag in technical development (currently available fluorescence detectors are still far from perfect), detection limits for UV and fluorescence methods are about equal if a good fluorophor is present.

Several attempts have been made to adapt commercial fluorescence instruments for HPLC[3–6], and the difficulties in making a compromise between fluorescence yield (high excitation energy; larger cells) and dead volume have been recognized; only Hatano *et al.*[6] have reported an improved detection limit. Nevertheless, the problem (which is primarily optical in nature) is being solved, and, with the availability of improved fluorescence and combined UV–fluorescence detectors[7,8], the adaptation of fluorescence detection to HPLC is clearly increasing.

In this light, we have attempted to investigate the application of this dual detection mode in selected problems in pharmaceutical analysis. The following two general cases have been chosen:

(1) determination of two unresolved components, of which only one is fluorescent, and

(2) determination of traces of a fluorescent degradation product by calibration via a weakly fluorescent major component (active substance) and/or an internal standard.

In both cases one is dealing with problems that cannot easily be solved by using only one of the two detection modes.

As practical examples, compounds from the phenothiazine family (psychotropic drugs) have been studied, in particular, thioridazine and its oxidation products sulforidazine, mesoridazine and the ring oxide of thioridazine. Mesoridazine and sulforidazine are strongly fluorescent, thioridazine is the weakly fluorescent drug substance and the ring oxide does not fluoresce. Caffeine was used in the second case as a non-fluorescent internal standard. The chromatographic separation of these components has been discussed elsewhere[9].

EXPERIMENTAL

The drug substances were available to us (Sandoz). For chromatographic separations, mixtures of isopropanol–di-isopropyl ether–aqueous ammonia in various proportions were used; these solvents were of analytical grade (Merck). In order to obtain reproducible chromatograms, it was important to check the concentration of the ammonia solution in the reagent bottle; old solutions were less concentrated and could cause reversal in separation order between caffeine and sulforidazine (see Fig. 1)[10].

The columns were packed with Merckosorb SI 60 (E. Merck, Darmstadt, G.F.R.) with an average particle size of 10 μm and 60-Å pore opening; the columns were packed by an equal-density method[11].

All the work was carried out with a Hewlett–Packard Model 1010A liquid chromatograph. The detector combination consisted of the LDC (Laboratory Data Control) UV and fluorescence monitors.

2- to 5-μl volumes of solution were injected directly on to the column via a

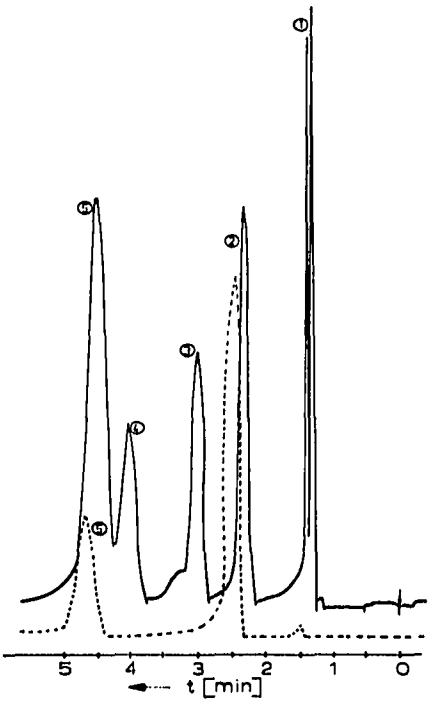

Fig. 1. Separation of thioridazine and some of its oxidation products on a column (25 cm × 3.1 mm I.D.) of Merckosorb SI 60 (10 μm), with isopropanol–di-isopropyl ether–conc. aqueous ammonia [10:90:0.5 (v/v)] as eluent (flow-rate, 1.2 ml/min) and a sample volume of 5 μl. Detection: ———, UV (254 nm); — — —, fluorescence (300–450 nm). Peaks: 1 = thioridazine; 2 = sulforidazine; 3 = caffeine; 4 = ring oxide; 5 = mesoridazine. (Peaks 1 and 3: change of the range by the integrator).

septum injection system (Varian) with a 10-μl Hamilton syringe. Peak areas were measured with a SIP electronic integrator (Perkin–Elmer).

RESULTS AND DISCUSSION

Determination of unresolved peaks

The separation of thioridazine from some of its oxidation products is shown in Fig. 1. As can be seen from the UV detection, the ring oxide and mesoridazine (peaks 4 and 5, respectively) are poorly resolved, but with the fluorescence detector only the mesoridazine peak is detected.

The following procedure is suggested for the quantitative determination of both components. The ratio of peak areas is computed for the UV and fluorescence signals of mesoridazine. The resulting factor, F, is solely dependent on the detectors used, the amplifier setting and the method of peak evaluation. F is therefore a reproducible entity provided that the peak areas fall within a linear concentration range of the corresponding calibration curves and provided that instrumental parameters are kept constant. For the ratios of peak integral A for UV and the product of peak height

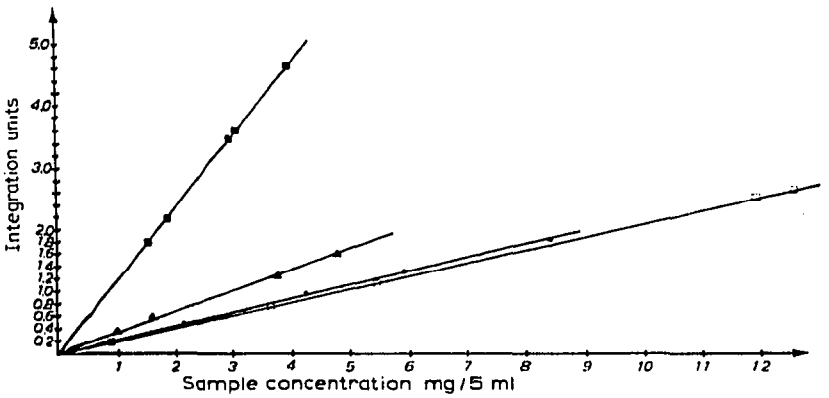

Fig. 2. UV-calibration curves for thioridazine (▲), sulforidazine (●), mesoridazine (□) and the pair mesoridazine plus its ring oxide (■).

and retention time ($h \times t_r$) for fluorescence, the relative standard deviation of the computed values of F ($F = A/ht_r$) did not exceed $\pm$ 1.36%.

A combined integral of the peak pair is then formed. By mean sof the fluorescence signal ($h \times t_r$) for mesoridazine and the factor F, one can compute the area corresponding to the UV signal of mesoridazine. The ring oxide is then computed from the expression $A_{\text{ring oxide}} = A_{\text{total}} - A_{\text{mesoridazine}}$.

This procedure was checked by computing the correlation coefficient for the peak pair (see Fig. 2); the correlation was highly significant (>0.998), and the relative standard deviation for ring-oxide determinations was 3.5% at concentrations <0.1 μg per injection ($n = 8$). The direct determination of mesoridazine via the fluorescence signal had a relative standard deviation of <2%, with a detection limit of 6 μg per injection. Fig. 2 also shows UV calibration curves for mesoridazine, sulforidazine and thioridazine; the correlation coefficients for these compounds are also better than 0.998.

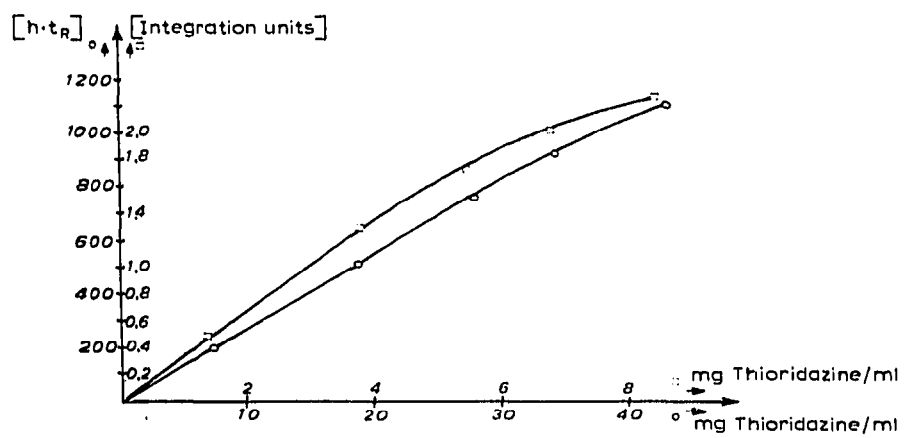

Fig. 3. UV (□) and fluorescence (○) calibration curves for thioridazine over an extended concentration range.

Determination of fluorescent oxidation products

Thioridazine, the principal substance in the investigated samples, shows only weak fluorescence, and this fact can be used to determine traces of strongly fluorescent oxidation products in the presence of large amounts of thioridazine. The weakly fluorescent component can be taken as an internal fluorescence standard, and combined UV–fluorescence detection will then permit [via a suitable internal UV standard (caffeine)] quantitative analysis for traces of degradation products.

Fig. 3 shows calibration curves for the fluorescence and UV detection of thioridazine over the same concentration range. As expected, the linearity of the UV curve is somewhat poorer than that of the fluorescence curve; however, even the fluorescence curve shows serious deviation from linearity due to a concentration quenching effect. This is why, at concentrations of thioridazine above 4 mg/ml, the factor F becomes concentration dependent (see Table I). At concentrations between 4 and 6 mg/ml, the variation in F remains below 3 % (relative), which can be tolerated for trace analysis. At higher concentrations, the factor would have to be checked at narrow concentration intervals. If the values of $(A_T/A_C)/F$ (last column of Table I) are plotted as a function of concentration, a correlation coefficient better than 0.998 is obtained.

TABLE I

ULTRAVIOLET AND FLUORESCENCE SIGNALS AND CONVERSION FACTORS FOR A DILUTION OF THIORIDAZINE

Experiment No.	*Concentration (mg/5 ml)*		*Detector signal**		*Factor F** (A_T/ht_r)	$\frac{A_T}{A_C} \cdot \frac{1}{F}$
	Thioridazine	*Caffeine*	*UV* (A_T/A_C)	*Fluorescence* (ht_r)		
1	7.2	62.7	0.476	200	278	0.00171
2	18.7	62.7	1.190	517	275	0.00433
3	27.8	62.7	1.700	755	265	0.00640
4	34.2	62.7	1.996	922	253	0.00787
5	43.1	62.7	2.053	1095	206	0.00995

* The subscripts T and C denote thioridazine and caffeine, respectively; h is the peak height, A the peak area and t_r the net retention time.

With this fluorescence signal calibrated in terms of the weakly fluorescent component thioridazine, it is possible to carry out quantitative trace analysis for the strongly fluorescent oxidation products. For this purpose, one has to compute fluorescence intensity ratios for the strongly and weakly fluorescent components. Fig. 4 shows the UV and fluorescence detection of thioridazine, two of its oxidation products and the internal standard (caffeine). The sulforidazine determined by this method was 0.44 ± 0.02 % ($n = 6$) of the thioridazine; the amount of thioridazine was 6.2 μg, and 18.6 μg of caffeine was used as internal standard. The detection limit of sulforidazine is about 0.1 % of the thioridazine concentration (signal-to-noise ratio of 4:1). The reproducibility can be expected to be less than 5 % (relative standard deviation), which, for trace analysis at these concentration levels, is acceptable. Absolute fluorescence detection limits for mesoridazine and sulforidazine are between 5 and 10 ng per injection.

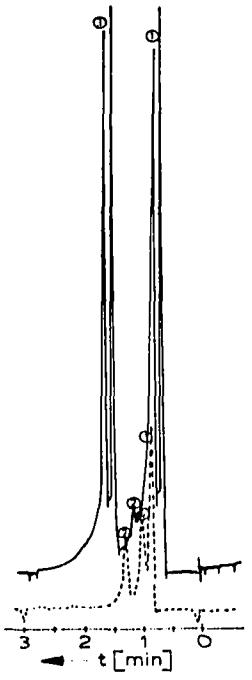

Fig. 4. Chromatogram for the determination of sulforidazine in presence of thioridazine. Conditions as in Fig. 1, except that the solvent ratio was changed to 26:84:1.5 (v/v). Flow-rate, 1.9 ml/min. Peaks: 1 = thioridazine (6.2 μg); 2 = sulforidazine (≈25 ng); 3 = caffeine (18.6 μg); 4 = ≈25 ng of an unidentified oxidation product.

CONCLUSIONS

The above results show that combined UV–fluorescence detection can facilitate quantitative evaluation of mixtures of incompletely resolved compounds and trace components in the presence of large quantities of major components, provided that only one compound is fluorescent. Since UV–fluorescence conversion factors and fluorescence intensity ratio factors remain constant for constant instrumental conditions, occasional checking of the factors is sufficient. Because of the large linear dynamic concentration range for strongly fluorescent compounds (up to 10^3-fold), one can expect fluorescence ratio factors to be independent of concentration as long as measurements are within the linear range of the weakly fluorescent component. As the detection limit for sulforidazine, for example, is about 6 ng per injection, there is no need to use very high concentrations of thioridazine.

It could obviously be argued that external standardization would be a valuable alternative to this approach. For automatic-injection or loop-injection procedures, this is true, but the present technique needs no reference chromatograms and is more reliable with conventional manual-injection procedures.

The fact that, of the pair mesoridazine and its ring oxide, only the first fluoresces could also be used to develop a highly sensitive and selective method for determining mesoridazine.

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