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THE USE OF A PRE-COLUMN FOR THE DIRECT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE ANTI-DEPRESSANTS CLOVOXAMINE AND FLUVOXAMINE IN PLASMA

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SUMMARY

A high-performance liquid chromatographic system for the direct determination of the antidepressants clovoxamine and fluvoxamine in plasma is described. The primary amines are derivatized with the fluorogenic reagent fluorescamine in order to increase the sensitivity and selectivity, but also to decrease the polarity of the amines. The band broadening of some combinations of pre-column and analytical column is compared for the fluorescamine derivative of fluvoxamine. The combination of a pre-column containing RP-2 and an analytical column containing RP-8 has been successfully used for the determination of the two antidepressants in plasma. At relatively low concentrations of the drugs, a simple step-gradient elution is required for the removal of a large proportion of the more polar components of the samples. Concentrations in the range of 10—1000 ng clovoxamine per ml plasma were determined by means of external standardization and show a good correlation with the data of a more laborious gas chromatographic method. The detection limits for clovoxamine and fluvoxamine are approximately 3 ng/ml in plasma.

INTRODUCTION

During the last decade, high-performance liquid chromatography (HPLC) has become an important method of analysis, especially in the field of pharmaceutical chemistry. Here, a simple gas chromatographic separation is often not possible because of the nonvolatility, thermolability, and polarity of the substances. At this moment, many HPLC papers are also devoted to the determination of drugs and their metabolites in biological samples. Generally, the combined problems of a complex matrix and the trace level make this type of analysis rather difficult. For pre-concentration and/or clean-up, a more or less extensive and time-consuming pretreatment of the samples is often necessary. Moreover, during this step, considerable losses

may occur, so that the choice of a good method of internal standardization is very critical. Therefore, one of the present research topics in HPLC is the reduction of the pretreatment of biological and other samples by use of selective and sensitive detection modes. Another interesting development is the application of pre-columns for clean-up [1—3] and enrichment [4—8]. Further, the pre-columns also act as so-called guard columns and thus increase considerably the life of the main column.

The preparation of such a pre-column must be as simple and economic as possible in order to limit the time and cost of renewal. In addition it is essential that the pre-column has sufficient capacity and that it should make only a small contribution to the band broadening. For minimization of the band broadening in the enrichment mode, back-flushing of the pre-column has been described [5,7]. However, the filter effect of the pre-column is then less efficient. The contributions of the pre-column (p) and analytical column (a) to the total variance are additive, i.e. from the equation for the total variance,

$$\sigma^2_{\text{total}} = \left(\frac{t_R^2}{N}\right)_p + \left(\frac{t_R^2}{N}\right)_a, \quad (1)$$

it can be seen that for a pre-column dry-packed with relatively large, and thus inexpensive, particles, the low plate number (N) must be compensated by a relatively small retention time (t_R). This can be effected by a small length of the pre-column [8], but this may lead to an insufficient capacity. Alternatively, one can use a pre-column packing material different from that of the analytical column. This principle has been reported by Eisenbeiss et al. [6] for the determination of polycyclic aromatic hydrocarbons in water. The pre-column and analytical column were packed with a non-specified Merck packing and RP-18, respectively.

In the present study, some combinations of stationary phases for a relatively long pre-column (5 cm) and the analytical column have been compared for the analysis of the fluorescamine derivative of the antidepressant fluvoxamine. Subsequently, a system with a relatively small band broadening was investigated for the determination of fluvoxamine and clovoxamine in plasma after a pre-chromatographic derivatization with fluorescamine.

EXPERIMENTAL

Materials

Fig.1 shows the structures of the antidepressants clovoxamine and fluvoxamine* (Philips-Duphar, Weesp, The Netherlands), which are now under clinical investigation. These were dissolved in distilled water at a concentration of 250 mg/l. Dilutions of this stock solution were made as required. The fluorogenic reagent fluorescamine (Fluram, Hoffmann-La Roche, Nutley, NJ, U.S.A.) was dissolved in acetone (analytical-reagent grade) at a

*Actually, the drugs are the fumarate and maleate salt, respectively, of the amines in Fig.1.

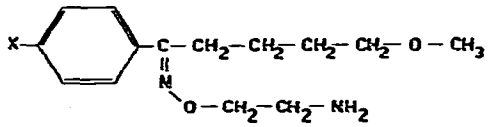

Fig.1. Structures of clovoxamine (X = Cl) and fluvoxamine (X = CF_3).

concentration of 1 mg/ml. The pre-column was a stainless-steel tube (5 cm × 4.6 mm I.D.) dry packed with different LiChrosorb and LiChroprep RP-materials (Merck, Darmstadt, G.F.R.). The analytical column was a stainless-steel tube (15 cm × 4.6 mm I.D.) packed by a slurry technique with LiChrosorb RP-8 (Merck) of average particle size 7 μm. All other chemicals were of analytical-reagent grade.

Apparatus

The HPLC pump was an Orlita DMP-AE-10.4 pump (Orlita, Giessen, G.F.R.); the injection port was a Rheodyne six-port valve (Rheodyne, Berkeley, CA, U.S.A.) with a 200-μl loop. For on-line/off-line switching, a second Rheodyne six-port valve was inserted between the pre-column and the main column. Detection was carried out with a Perkin-Elmer Model 204A fluorescence spectrophotometer ($\lambda_{exc.}$ = 380 nm, $\lambda_{em.}$ = 470 nm). For comparison purposes, a variable-wavelength UV detector (Schoeffel Spectroflow Monitor SF 770, Schoeffel Instruments, Westwood, NJ, U.S.A.) was used.

Procedure

The human and canine plasmas were stored frozen. After thawing and centrifugation, 1 ml of plasma was spiked with a known amount of clovoxamine or fluvoxamine, and subsequently 1 ml of a 0.01 *M* phosphate buffer pH 7 and 1 ml of the fluorescamine solution were added. After centrifugation, 200 μl of the supernatant were injected into the HPLC system. Methanol—0.01 *M* phosphate buffer pH 7 (62:38, v/v) was used as the mobile phase at a flow-rate of approximately 1 ml/min. After approximately five injections, the system was purged for about 5 min with methanol.

However, after injection of sample solutions with concentrations of the amines below approximately 100 ng/ml, the pre-column containing 32-μm RP-2 was first flushed for about 5 min with methanol—phosphate buffer pH 7 (50:50, v/v) with the switching valve in the waste position. Next the valve between the two columns was switched on-line and the derivatives of the amines were eluted with methanol—phosphate buffer pH 7 (62:38, v/v), i.e. a mobile phase containing a higher proportion of methanol. At the end of each analysis, the pre-column and analytical column were flushed for about 5 min with methanol. Finally, the columns were equilibrated for about 10 min with methanol—buffer (50:50, v/v) and methanol—buffer (62:38, v/v), respectively. With real plasma samples, the same procedure was followed.

RESULTS AND DISCUSSION

Fluorescamine reaction

The fluorescence signal of the fluorescamine derivatives of primary amines is influenced by the kinetics of the derivatization reaction and also by dielectric effects and some other effects of the medium [9—11]. For the direct injection of a polar sample such as plasma, a reversed-phase chromatographic system is necessary. Fortunately, the results of Frei et al. [10] with a similar system, i.e. oxytocin—fluorescamine, point to the fact that the net fluorescence decreases with a decrease in solvent polarity, so that it is advantageous to use a polar mobile phase. Data published for different primary amines indicate that the pH optimum is between about 6 and 10.5 [10,12,13]. For the present system, the maximum fluorescence intensity was obtained at pH 7—8. Because the life of RP-8 columns appeared to be longer at pH 7 than at pH 8, the mobile phase was buffered to pH 7. A phosphate buffer was preferred to a borate buffer, since borate ions may complex with the fluorophore and, in this way, suppress the fluorescence [14].

For the excess of fluorescamine, very different values have been reported, the required molar excess varying from 3.6 [15] to approximately 1000 [13]. For concentrations of 0.5—1 μg/ml clovoxamine and fluvoxamine, a molar fluorescamine-to-amine ratio of approximately 300—400 was found to be sufficient. Although fluorescamine itself was known to be non-fluorescent [9,16], the fluorescamine solution was observed to be fluorescent. Probably, this is due to one or more hydrolysis products. For a derivatization reaction prior to separation, this does not interfere, because the excess of reagent and its hydrolysis products are separated from the derivative in the chromatographic system. It is true that the reaction rate is dependent on the concentrations of the amine and fluorescamine; with the conditions used in this study the maximum signal was always obtained within a few minutes.

Packing material of the pre-column

As already described in the introduction, the choice of the pre-column is rather critical. Briefly, the following demands have to be met: (1) rapid and economic preparation; (2) sufficient capacity; (3) small contribution to band broadening, and (4) suitable for clean-up and pre-concentration.

A relatively large pre-column (5 cm × 4.6 mm I.D.) can easily be filled by a simple dry-packing technique and the capacity of this column was found to be sufficient for at least 250 μl plasma. For the present analytical system, i.e. RP-8 with methanol—0.01 *M* phosphate buffer pH 7 (62:38, v/v) as the mobile phase, the use of RP-8 as the packing material for this pre-column causes an intolerable band broadening because of too long a retention time. With the use of RP-2, the retention time of the derivatives of clovoxamine and fluvoxamine and, consequently, the band broadening caused by the pre-column are much less. Retention times and capacity factors of the analytical column and some pre-columns are compared in Table I.

Plate numbers for the analytical column and the pre-column were typically about 4000 and 200, respectively. Using eqn. 1, one can now calculate that for the RP-8 pre-column $\sigma_{total} = 1.9\sigma_{anal.}$ and for the 32-μm RP-2 pre-

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF THE FLUORESCAMINE DERIVATIVE OF FLUVOXAMINE FOR SOME COLUMNS

Mobile phase: methanol—0.01 *M* phosphate buffer pH 7 (60:40, v/v).

Column	Retention time (min)	Capacity factor
Analytical column* RP-8 7 μm	16.8	15.8
Pre-column** RP-8 25—40 μm	6.2	15.9
Pre-column RP-2 32 μm	2.2	8.5
Pre-column RP-2 10 μm	1.7	5.6

*15 cm × 4.6 mm I.D.
**5 cm × 4.6 mm I.D.

column $\sigma_{total} = 1.1\sigma_{anal.}$. The behaviour of the much more expensive 10-μm RP-2 is similar to that of 32-μm RP-2. It should be noted that the observed total standard deviation is often higher than the theoretical one, because connections and the valve between the columns can contribute to band broadening [17—19]. A possible disadvantage of RP-2 with respect to the more apolar RP-8 is the smaller recovery of a pre-concentration step. However, for 1-ml sample solutions with concentrations in the nanogram range, the recovery with water as the mobile phase during the pre-concentration was found to be 100%. Therefore RP-2 is also useful for the pre-concentration of the derivatives of clovoxamine and fluvoxamine. It should be noted that the derivatization of the amines with fluorescamine makes them much less polar and therefore suitable for pre-concentration in reversed-phase systems. Next to the use of chemical reactions in liquid chromatography as an aid to improving the detection properties and selectivities for certain groups of compounds, the modification of the polarity seems also a very useful aspect of derivatization.

Determination of clovoxamine and fluvoxamine in plasma

The system of pre-column 32-μm RP-2 and analytical column 7-μm RP-8 with as the mobile phase methanol—buffer pH 7 (62:38, v/v) was used for the determination of clovoxamine and fluvoxamine in plasma. This system is suitable for the direct analysis of plasma; a chromatogram of a plasma sample is presented in Fig. 2a. For the concentration range of 100—2500 ng of fluvoxamine per ml, a linear calibration curve was obtained ($r = 0.9999$).

The detection limit (signal-to-noise ratio 2:1) for this procedure is approximately 25 ng/ml plasma. By means of repeated analyses of a plasma sample containing approximately 600 ng of fluvoxamine per ml, the reproducibility was shown to be 3% (rel. S.D.; $n = 6$). The efficiency of the analytical column decreases slowly, but the column can be used for several weeks. As for the pre-column, because of deterioration it is necessary to replace this

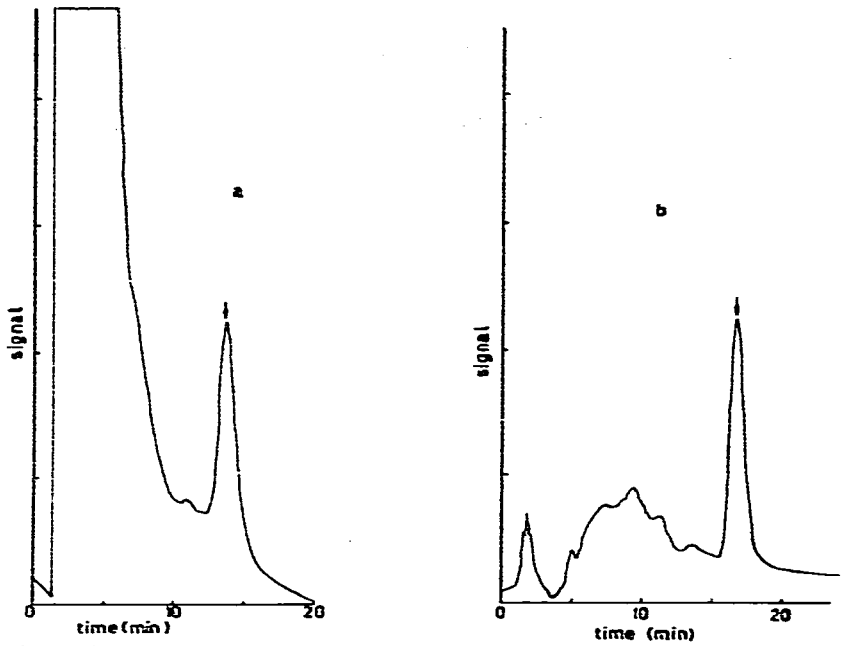

Fig. 2. HPLC chromatograms of a canine plasma sample containing 652 ng fluvoxamine per ml. Chromatographic conditions: pre-column, 32-μm RP-2, 5 cm × 4.6 mm I.D.; main column, 7-μm RP-8, 15 cm × 4.6 mm I.D.; mobile phase, (a) methanol—0.01 *M* phosphate buffer pH 7 (62:38, v/v), (b) methanol—0.01 *M* phosphate buffer pH 7 (62:38, v/v) after previous elution for 5 min with methanol—0.01 *M* phosphate buffer pH 7 (50:50, v/v); flow-rate, 1 ml/min. Detection: pre-separation reaction with fluorescamine; fluorescence detector, $\lambda_{exc.}$ = 380 nm and $\lambda_{em.}$ = 470 nm. The retention time of the fluvoxamine derivative in chromatogram (b) is higher, because the first part of the elution takes place in a gradient.

column after 5—10 samples. Moreover, it is useful to flush the whole system regularly with, for example, methanol in order to ensure the elution of relatively lipophilic substances.

The fluorescence signal of a spiked plasma sample is lower than that of the standard solution in water with the same concentration. Confirmation was obtained that the partial deproteination of plasma by the addition of the acetonic solution of fluorescamine did not cause any loss of the amine of interest. The reduction of the signal is due to the decrease of the excess of fluorescamine brought about by its reaction with biogenic primary amines, especially amino acids. This was proved by an experiment whereby the plasma was added after the reaction between fluvoxamine and fluorescamine; the peak heights of the standard and the plasma sample were then equal. It is true that the excess of fluorescamine can be adjusted, but this also widens the band in the front of the chromatogram. Fortunately, the excess concentration of fluorescamine had no noticeable influence on the linearity of the calibration curve.

The above-mentioned detection limit is determined by the tailing band in the chromatogram, which is caused by polar endogenous compounds and to

a large extent, as demonstrated, by compounds formed during the derivatization reaction. Therefore, for concentrations below approximately 100 ng/ml, a simple step gradient is required in order to remove a large proportion of the more polar compounds from the pre-column prior to the elution of the derivatives of clovoxamine or fluvoxamine. It was found to be possible to flush the pre-column for about 5 min with methanol—buffer pH 7 (50:50, v/v) without the derivatives being eluted. Fig. 2b shows the chromatogram of the same sample as in Fig. 2a obtained after the use of this step gradient. The latter procedure enables the use of the higher sensitivity ranges of the fluorescence detector; consequently, it is then necessary to flush the columns with methanol each time after the analytical step, i.e. elution with methanol—buffer (62:38, v/v). It is evident that this procedure is more time-consuming than the isocratic method, especially in consequence of the equilibration times of the columns. The detection limit then becomes approximately 3 ng/ml plasma for both clovoxamine and fluvoxamine and a further decrease was shown to be possible by means of larger injection volumes. Plasma samples have almost always a higher concentration, but then such a preconcentration step can be useful for increasing the precision of the concentrations just above the detection limit. The detection limit of clovoxamine and fluvoxamine with UV detection at 254 nm was improved about twenty times by the use of fluorescence detection after derivatization with fluorescamine; also the selectivity of the latter method is considerably superior.

TABLE II

COMPARISON OF CONCENTRATIONS IN ng/ml OF CLOVOXAMINE IN PLASMA SAMPLES AS FOUND BY GAS AND LIQUID CHROMATOGRAPHY

The HPLC data of samples No. 1—3 have been obtained with the isocratic method and the data of samples No. 4—7 with the step-gradient method. For experimental details, see text.

Sample No.	Method	
	GC	HPLC
1	803	803
2	686	703
3	561	604
4	72	79
5	23	26
6	23	21
7	11	15

Table II compares some typical data of the method described in this paper with the results obtained by the gas chromatographic analysis used in our laboratory [20]. Similar results have been obtained for fluvoxamine samples. If it is remembered that the relative standard deviation of the gas chromatographic analysis generally is about 3—15% (depending on the range), the correlation between the two methods can be considered good. The gas chromatographic method is rather time-consuming as compared with the HPLC method; it includes extraction with isooctane, two re-extractions with

phosphoric acid, hydrolysis at 90°C, and again extraction with isooctane. The recovery is about 60% and, therefore, internal standardization is necessary. In conclusion it can be stated that the HPLC method is simpler and more suitable for automation. As for the analysis time, for the gas chromatographic analysis many samples can be pretreated at the same time. Therefore, the saving of time is only considerable if one or a few samples are analysed.

CONCLUSION

For the analysis of plasma by means of HPLC, direct injection of the (diluted) sample is possible if a suitable pre-column, and a selective derivatization and/or detection mode are used. Probably, this is also true for the analysis of other complex samples. The actual choice of the packing materials of pre-column and analytical column depends on the polarity of the compound (or derivative) to be assayed, on the ratio of the capacity factors of the two columns and on the nature of the sample. The principle of using different materials in pre-column and analytical column appears to be promising for certain applications of pre-columns for clean-up and pre-concentration of biological and other samples. The application of step gradients can enhance the potential of such systems (see also ref. 21).

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