Journal of Chromatography, 149 (1978) 587–598

CHROM. 10,799

DETERMINATION OF METHOTREXATE IN PLASMA BY ON-COLUMN CONCENTRATION AND ION-EXCHANGE CHROMATOGRAPHY*

J. LANKELMA

Department of Clinical Pharmacy, St. Radboud Hospital, Geert Grooteplein Zuid 10, Nijmegen (The Netherlands)

and

H. POPPE

Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, Amsterdam (The Netherlands)

SUMMARY

A method for the concentration of dilute samples on a column and its application to the analysis of biomedical mixtures is described. Two columns are used, one for the concentration step and the other for the separation. The concentration column is used in the position of the loop in an injection valve. Concentration is effected by eluent switching.

The influence of the chromatographic parameters such as capacity ratio and void volume on separation and detectability are described and the optimal choice of conditions is discussed.

The method has been applied to the analysis of the cytostatic drug methotrexate. An octyl-modified silica was used as the stationary phase in the concentration column and a chemically bonded anion exchanger on silica in the separation column. The procedure shows good selectivity and precision. The detection limit corresponds to $2 \cdot 10^{-8}$ *M* (9 ppb**) in plasma, which is sufficiently low for therapeutic concentrations to be measured.

An example of the use of the same stationary phase in both columns is given for the analysis of phenothiazines in blood, using alkyl-modified silica and electrochemical detection.

INTRODUCTION

Detection problems in column liquid chromatography (LC) can occur with either too small absolute amounts or too low concentrations[1,2]. In the former instance not much can be done without changes in the chromatographic detection or separa-

* Title in symposium programme: "HPLC systems involving sample concentration on columns and their application to the analysis of drugs in serum".

** Throughout this article, the American billion (10^9) is meant.

tion equipment, but in the latter, concentration of the analyte before or during chromatography can effect significant improvements.

Often clinical analyses are quoted as examples in which the detection limit in amount, rather than that in concentration, is important, as in most instances only limited amounts of sample are available. However, as shown in the two examples in this paper, concentration steps are also frequently necessary in this type of analytical work. The reason for this is obvious: the small amounts of sample or extract available (of the order of a few millilitres) are still large compared with the volumes in which chromatographic detection occurs (10–500 μl).

On-column concentration procedures for use in high-performance liquid chromatography (HPLC) have been described by several workers[3–6]. In these methods, a combination of stationary and mobile phases is chosen in such a way that the retention of analytes is excessively large in the sample medium and moderate with the mobile phase. During injection of the large amount of sample medium, the analytes are concentrated on the top of the column. The concentration effect is of the order of $\kappa_i^{ci}/\kappa_i^{ce}$ (for symbols, see under Theoretical).

In this paper, a two-column system, in which concentration and separation are effected in the different columns, is described. The system is shown schematically in Fig. 1. The concentration column is the loop of valve I and the amount present in the loop volume injected by valve II is adsorbed in this concentration column. After switching valve I to the injection position, the flow of sample medium (the one with the high capacity ratio) in the concentration column is reversed and eluent (with the low capacity ratio) can transport the concentrated compounds immediately to the analytical column. This "back-flush" mode was chosen in order to avoid increasing contamination of the concentration column.

An essential condition for this concentration procedure is that the capacity ratio in the sample medium is large and that in the eluent is moderate.

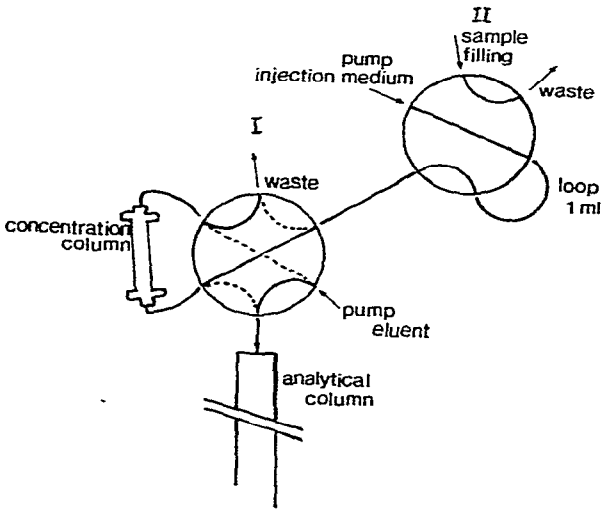

Fig. 1. Concentration system with two valves (I and II). Solid line, concentration; broken line, injection.

This method of sample concentration has the following advantages compared with extraction or evaporation procedures:

(a) Losses due to glass adsorption[7] (*e.g.*, during evaporation) are avoided.

(b) The system is suitable for automation.

(c) Handling of small volumes, with inherent loss of material, is avoided.

In comparison with the on-column concentration procedures in which the separation column is used for this purpose, the following advantages apply:

(d) The separation column is flushed with only minor (V_{0c}) amounts of foreign (sample) medium, where in the one-column system the total amount of this medium passes through at every injection. This can disturb the column and the detection system.

(e) The two-column method can bring about a pre-separation; if the same stationary phase is used in both columns, compounds of low retention will leave the concentration column and will not be injected on the main column. When different stationary phases are used, even more effective pre-separation effects can be obtained. This will result in cleaner, more easily interpretable chromatograms, and less overloading of the column and the detector.

The points mentioned under (d) and (e) will enhance the stability and lifetimes of expensive efficient separation columns. The concentration columns do not need to be very efficient and can be easily replaced. These use of disposable columns would be the most practical procedure.

Methotrexate (Mtx) has been used for more than 20 years as a cancer therapeutic agent[8]. Its determination in body fluids is of great importance in obtaining more insight into its pharmacokinetics[9–12]. Mtx is now currently determined by radioimmunoassay[13,14], competitive protein binding[15] or enzymatic inhibition[16]. Although the detection limits of these methods are very low, they have some disadvantages. The first two methods have not yet been tested for metabolites of Mtx. It has recently been shown[17] that 4 h after intraveneous injection of ^{3}H-labelled Mtx in cynomolgus and rhesus monkeys, more than 80% of the radioactivity no longer represented intact Mtx. This fraction is described as representing the metabolites of the compound. A disadvantage of the kinetic method is that calibration has to be carried out at least twice a day because of a decrease in either the enzyme or the dihydrofolic acid concentration, both of which are used for the enzymatic reaction. Another disadvantage of the method is that the method shows a linear range of only a factor of 10.

The method described here is highly specific for Mtx and has a linear range of more than 500.

The determination of phenothiazines in serum at the nanograms per millilitre level by reversed-phase liquid chromatography in combination with coulometric detection has been described before[18]. In this method, sample pre-treatment involves an extraction based on acid–base properties. In the final step of the procedure the sample is concentrated by evaporation of the organic layer and the extract is dissolved in a small amount of eluent. In this paper, a method is described in which the aqueous extract is concentrated on a column.

THEORETICAL

The most suitable dimensions of the concentration column are determined by

the chromatographic parameters. When the internal volume is too large, the injection of concentration eluent into the analytical column can cause a large distortion of the detector signal and a considerable change in capacity ratio. On the other hand, when the volume is too small, the analytes can leave the column during the sampling step.

An expression can be derived for the concentration factor in the entire system, assuming that no dispersion occurs in the concentration column. This assumption results in a block shape for the peak of concentrated compound. This model allows an estimation of the contribution of the width of the injection plug compared with the dispersion in the whole system.

The following symbols are used.

A = area of the cross-section of the column, with subscript c or s;
c_{i0} = concentration of the analyte in the sample solution;
H = theoretical plate height, with subscript c or s;
κ_i^{ci} = capacity ratio with injection medium in concentration column;
κ_i^{ce} = capacity ratio with eluent in concentration column;
κ_i^{si} = capacity ratio with injection medium in separation column;
κ_i^{se} = capacity ratio with eluent in separation column;
L = length of the column, with subscript c or s;
N = number of theoretical plates, with subscript c or s;
Q_i = absolute amount of i;
V_{0s} = void volume of separation column;
V_{0c} = void volume of concentration column;
V_0 = sample volume;
σ_{vs} = volume standard deviation produced by the column;
$\sigma_{v\ inj}$ = volume standard deviation of injection peak.

When the sample has been fed completely into the concentration column, the volume of mobile phase containing the compound i, V_i has been contracted to

$$V_i = V_0 \cdot \frac{1}{1 + \kappa_i^{ci}} \tag{1}$$

The concentration in this part of the injection medium remains equal to the initial concentration, c_{i0}.

The elution function of the concentration after back-flushing with immediate switching to the eluent is shown in Fig. 2. With large values of κ_i^{ci} and low values for V_{0c} the front can be neglected, as it contains only a minor amount of the analyte. Moreover, this front will be contracted to some extent on the separation column also. We can therefore treat the injection on the separation column as one of a block function with a volume

$$V_0 \cdot \frac{\kappa_i^{ce}}{\kappa_i^{ci}} \tag{2}$$

and a concentration

$$c_{i0} \cdot \frac{\kappa_i^{ci}}{\kappa_i^{ce}} \tag{3}$$

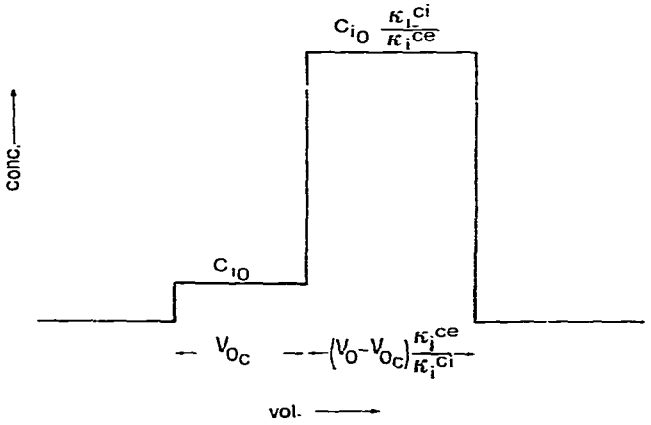

Fig. 2. Concentration profile for the analyte leaving the concentration column.

The limit of the injection volume, set by a tolerated loss in resolution, as discussed by Huber *et al.*[1], is of course valid in this instance also, and should be applied to the concentrated injection plug. This means that the maximum volume of the sample in the concentration column is set by

$$V_0 \cdot \frac{\kappa_i^{ce}}{\kappa_i^{ci}} \leqslant V_{\max.} \tag{4}$$

in which $V_{\max.}$ is the maximum volume that can be injected into the analytical column under normal conditions without an intolerable loss of resolution.

Assuming plug injection and a 20% loss in the resolution, we can derive that the maximum injection volume is

$$\begin{aligned} V_{\max.} &= \tfrac{3}{4}\sqrt{12} \cdot V_{0s}(1 + \kappa_i^{se})\, N_s^{-\frac{1}{2}} \\ &\approx 2.60\, V_{0s}(1 + \kappa_i^{se})\, N_s^{-\frac{1}{2}} \end{aligned} \tag{5}$$

It follows from eqns. 4 and 5 that the maximum value of V_0, the volume injected on the concentration column, is

$$V_0(\max.) = 2.60 \cdot \frac{\kappa_i^{ci}}{\kappa_i^{ce}} \cdot V_{0s}(1 + \kappa_i^{se})\, N_s^{-\frac{1}{2}} \tag{6}$$

This maximum volume is a factor $\kappa_i^{ci}/\kappa_i^{ce}$ larger than that with normal operation of the column. This forms the rationale of the concentration procedure.

Suitable dimensions of the concentration column should be chosen. In order to avoid loss of analyte during the sampling, the concentration column should have a void volume of

$$V_{0c} > \frac{V_0}{1 + \kappa_i^{ci}} \tag{7}$$

On the other hand, an excessively large concentration column would lead to the injection of large amounts of foreign phase into the main column, and would also decrease the concentration effect. Therefore

$$1.2 \cdot \frac{V_0}{1 + \kappa_i^{ci}} < V_{0c} < 2.0 \cdot \frac{V_0}{1 + \kappa_i^{ci}} \tag{8}$$

seems to be a reasonable compromise for low values of κ_i^{ci}. For larger values of κ_{ci} the latter inequality can be disregarded because it would result in excessively low values of V_{0c}.

It should be noted that a concentration on the main column, as used by other workers mentioned above, also leads to a concentration factor of $\kappa_i^{ci}/\kappa_i^{ce}$. A drawback of the two-column system proposed here is that it operates properly only for large absolute values of κ_i^{ci} (of the order of 100), while concentration on the main column depends only on the ratio $\kappa_i^{ci}/\kappa_i^{ce}$. However, for low values of κ_i^{ci}, the separation in the main column of the same stationary phase would be very poor because of too low capacity ratios.

The influence of dispersion on the concentration process can be discussed in general terms, but this leads to rather complicated expressions, which are not easily interpreted. We shall therefore discuss this effect for the particular situations in our experiments.

For the analysis of fluphenazine we used a value of 1000 μl for V_0 and 150 for κ_i^{ci}. The concentration column was 3 mm in diameter. Assuming a total porosity of 0.8, the depth of penetration of the analyte into the column can be calculated to be 1.2 mm. Assuming a maximum plate height of 100 μm and considering that a total migration of twice this distance occurs for part of the analyte, this results in an increase in volume variance of 376 μl^2 for a capacity ratio of 6 in the eluent. This has to be compared with the volume variance produced by the separation column, which was about 14000 μl^2. In this instance, therefore, no contribution to peak broadening is to be expected from dispersion within the concentration column. It should be noted, however, that with even larger values of V_0 this can become a significant effect.

In the analysis of Mtx, the extra separation effect brought about by the concentration column was enhanced by elution with water in order to remove less retained compounds. This elution was continued up to the point where the analyte is almost at the end of the concentration column. Taking into account observed values for the different parameters of $\kappa_i^{ci} = 80$, $\kappa_i^{ce} = 4$, $L_c = 4.6$ cm, $V_{0c} = 260\,\mu$l, and taking into account the double migration through the same column length, the volume standard deviation at the entrance of the separation column can be estimated as

$$\sigma_v^2 = 2 \cdot 260^2 \cdot \left(\frac{46}{0.1}\right)^{-1} (1 + 4)^2 = 7.3 \cdot 10^3\,\mu l^2$$

The volume standard deviation of the separation column was observed to be about $2 \cdot 10^5\,\mu l^2$. It can be concluded that even under these more difficult conditions, a negligible loss in resolution (1.8%) occurs.

EXPERIMENTAL

Apparatus

The liquid chromatograph and the coulometric electrochemical detector used for the detection of fluphenazine have been described previously [18,19]. The detection potential was +0.8 V *versus* Ag–AgCl–0.05 *M* Cl^-. For the detection of Mtx, a UV detector (Model 770; Schoeffel Instruments, Westwood, N.J., U.S.A.) was used at a wavelength of 306 nm. The switching in the LC system was effected by means of sampling valves (CV-6-UHPa-N60; Valco, Houston, Texas, U.S.A.). Gas chromatographic syringes (Glenco Scientific, Houston, Texas, U.S.A.) were used for the introduction of the 1-ml samples. All experiments were carried out at ambient temperature (18–25°).

Chemicals

All chemicals used were of analytical grade. Fluphenazine (FPZ) was obtained from Squibb (Rijswijk, The Netherlands), perphenazine (PPZ) from Schering Pharmaceuticals (Kenilworth, N.J., U.S.A.) and methotrexate (Mtx) from Lederle (American Cyanamid, Pearl River, N.Y., U.S.A.).

In the determination of FPZ it was found that some factory-prepared solutions were not suitable as the eluent buffer because of contamination of the working electrode of the coulometric detector. For all purposes doubly distilled water was used, except for concentration of Mtx, where water from the town water supply could be used. When RP 8 is used in the concentration column and a compound is concentrated using water as the eluent, it is important not to use water that is contaminated with organic material that can serve as a moderator on the reversed-phase surface, which might decrease the capacity ratio considerably. Before passing through the valve to the concentration column this water was eluted over a pre-column filled with active charcoal (40–60 mesh, material for gas chromatographic purposes; Chrompack, Middelburg, The Netherlands). The eluents in the other columns were purified by means of elution over methyl-modified silica (0.063–0.200 mm; Merck, Darmstadt, G.F.R.).

Columns

Concentration columns were of dimensions 4.6 cm × 3 mm I.D. filled with an octyl-modified silica, particle size 10 μm (RP 8; Merck) for Mtx and 3 cm × 3 mm I.D. filled with methyl-modified silica, particle size 8–9 μm, according to Tjaden[20] and Tjaden and Huber[21] for FPZ. Separation columns of dimensions 25 cm × 4.6 mm I.D. filled with a silica-bound strong anion exchanger, particle size 10 μm (Partisil SAX; Whatman, Maidstone, Great Britain) for Mtx and 10 cm × 3 mm I.D. for FPZ with the same filling as in the concentration column.

RESULTS AND DISCUSSION

The phase system and other analytical conditions for the chromatography of trace amounts of FPZ have been studied previously[18,20,21]. The modified procedure for use in the column concentration methods was as follows. A 3-ml volume of serum was extracted and back-extracted twice into *n*-hexane in order to remove lipids

present in the serum[18,22]. The resulting 1.0 ml of aqueous sulphuric acid solution (0.1 *N*) was injected after neutralization with 30–40 mg of sodium hydrogen carbonate. This layer was eluted in the concentration column with a phosphate buffer (0.025 *M*, pH 6.9). When the aqueous layer was not neutralized before injection, PPZ and FPZ were less retained, which resulted in a loss during concentration.

At pH 6.9, the capacity ratios were higher than 100 in the injection medium. After injection, the concentration column was eluted with a further 100 μl of this medium in order to remove unretained material present in the sample. Then the valves were switched to the injection mode (Fig. 1) and the concentration column was eluted with an eluent consisting of methanol and phosphate buffer (0.025 *M*, pH 6.9, volume ratio 55:45). A chromatogram obtained in the analysis of FPZ is presented in Fig. 3.

For the electrochemical detection used here, it was found that the advantage mentioned under (d) in the Introduction was especially important. Injection of large volumes of a solution with a composition different from that of the normal effluent results in serious and prolonged distortion of the detector baseline. Use of the two-column concentration procedure gave a significant improvement in comparison with concentration on the main column.

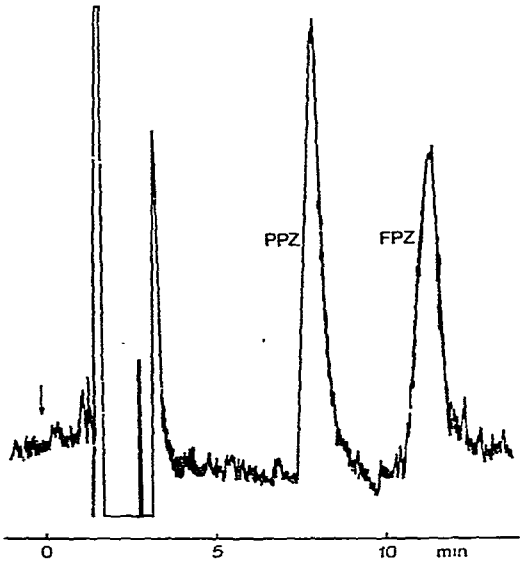

Fig. 3. Analysis of serum, spiked with PPZ and FPZ, concentration 3 ng/ml. Serum volume, 3 ml; volume of the aqueous extraction layer, 1 ml; coulometric detection at + 0.8 V *versus* Ag–AgCl–0.05 *M* Cl^-. Concentration column: methyl-modified silica, particle size 8–9 μm; length 3 cm, I.D. 3 mm; eluent, water. Analytical column: methyl-modified silica, particle size 8–9 μm; length 10 cm, I.D. 3 mm; eluent, 0.025 *M* phosphate buffer, pH 6.9, 0.05 *M* sodium chloride–methanol (45:55); pressure, 200 bar; flow-rate, 12 μl/sec.

Changes in capacity ratios were also decreased considerably. Nevertheless, increases from 7.0 to 11.5 and from 12.0 to 18.2 for PPZ (internal standard) and FPZ, respectively, were observed in the final procedure compared with injection in an eluent on the separation column.

Mtx can be extracted from acidified de-proteinized plasma with butanol[23].

However, butanol is a polar and not very selective extraction fluid. Moreover, we found that, even after removing proteins, that had been denatured with trichloroacetic acid, part of the proteins remained in solution. After shaking with butanol a new layer of denatured proteins was formed in the butanol layer. It was found that in this layer a large fraction of the Mtx was adsorbed.

In the present method, using an alkyl-modified silica concentration column, it was found to be sufficient to de-proteinize the plasma samples by means of a 10% solution of trichloroacetic acid in 0.1 *N* hydrochloric acid. This solution was added to the plasma samples in a volume ratio of 1:1. The mixture was allowed to stand for 5 min. After centrifuging at 2000 *g* for 5 min, the clear upper layer could be directly injected into the concentration column. When using trichloroacetic acid alone, a turbid supernatant was obtained in some instances.

For the chromatographic analysis of Mtx, a phase system described by Stout *et al.*[24] was taken as a starting poinnt. For the mobile phase, a mixture of sodium phosphate buffer (0.05 *M*, pH 4.9) containing sodium chloride (0.001 *M*)–methanol (4:1) was finally chosen.

The addition of methanol was found to improve the peak symmetry. During this study, no significant deterioration of the column, as was observed by Stout *et al.*[24], was noticed after the analysis of 350 plasma samples. Whether this is a result of the absence of gradient elution or our injection method is not known.

Fig. 4 shows the chromatogram obtained when 1 ml of de-proteinization supernatant was injected directly into the separation column (one-column concentration procedure) and Fig. 5 shows the result after concentration on the reversed-phase column. The concentration effect on this column, *i.e.*, the decrease in capacity ratio

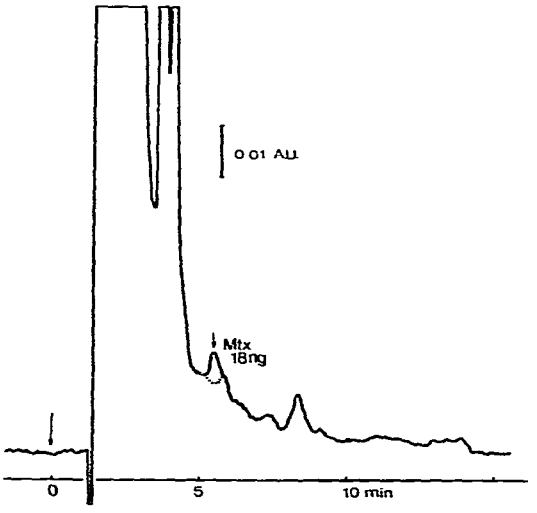

Fig. 4. Injection of de-proteinized plasma, spiked with 18 ng of Mtx, directly on the analytical column (Partisil SAX). Injection volume, 1 ml. Analytical column: Partisil SAX, particle size 10 μm; length 25 cm, I.D. 4.6 mm; eluent, 0.05 *M* phosphate buffer (pH 4.9), containing 0.001 *M* sodium chloride + methanol (4:1); pressure, 150 bar; flow-rate, 42 μl/sec.

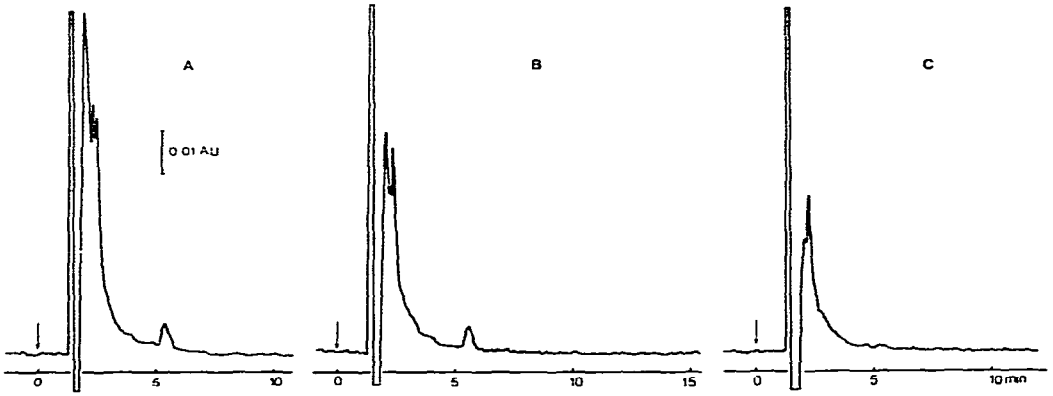

Fig. 5. Analysis by the concentration method for different concentration eluent volumes: (A) 11 ml, (B) 20 ml and (C) 30 ml. Plasma spiked with methotrexate, concentration $8 \cdot 10^{-8}$ *M*. (Concentration column: RP 8, particle size, 10 μm; length 4.6 cm, I.D. 3 mm; pressure, 90 bar; flow-rate, 40 μl/sec). Chromatographic conditions as in Fig. 4.

when switching from the injection medium to the eluent, is of course related to the increased methanol content of the latter solution.

As mentioned above, the analyte was eluted in the same direction for some time, with a medium comparable to the injection medium (water in this instance), in order to remove as much less retained material as possible. Fig. 5 gives the result for three different cases, A, B and C, differing in the volume of water that was pumped into the concentration column after injection, and before switching to the position for injection on the main column.

It can be seen that more prolonged elution leads to a "cleaner" chromatogram, but elution with 30 ml or more causes the Mtx peak to disappear. Elution with 20 ml seems to be optimal under the present conditions. Fig. 5b should be compared with Fig. 4 in order to demonstrate the usefulness of the method. The peak broadening is hardly affected by the concentration when compared with a direct injection of 20 μl of Mtx solution in the analytical column. This result is in agreement with the small influence as calculated in the example under Theoretical.

The precision of the quantitative determination of Mtx, excluding the de-proteinization step, by means of this method was measured by injection of 1 ml of the supernatant after de-proteinization of plasma that had been spiked with different amounts of Mtx. Fig. 6 shows the dependence of peak height on concentration for these conditions. The broken lines show the confidence limits for $\pm$ twice the standard deviation. The relative standard deviation was 2% for a concentration of $8 \cdot 10^{-8}$ *M* and higher and 5% for a concentration of $4 \cdot 10^{-8}$ *M* ($n = 3$).

The variation in peak height at the higher concentrations can be attributed to uncertainties in the injected volumes and to variable recoveries. At lower concentrations, the baseline noise of the detector gives the main contribution to the variation in peak height. The detection limit was observed to correspond to $2 \cdot 10^{-8}$ *M* of analyte in plasma. This low value is also partly due to the strong absorption of the compound at 306 nm ($\varepsilon = 25{,}000$). The detection limit can be decreased further by injection of more than 1 ml of plasma, if larger amounts of blood are available.

Although Mtx is relatively stable, all folic acid analogues can be easily oxi-

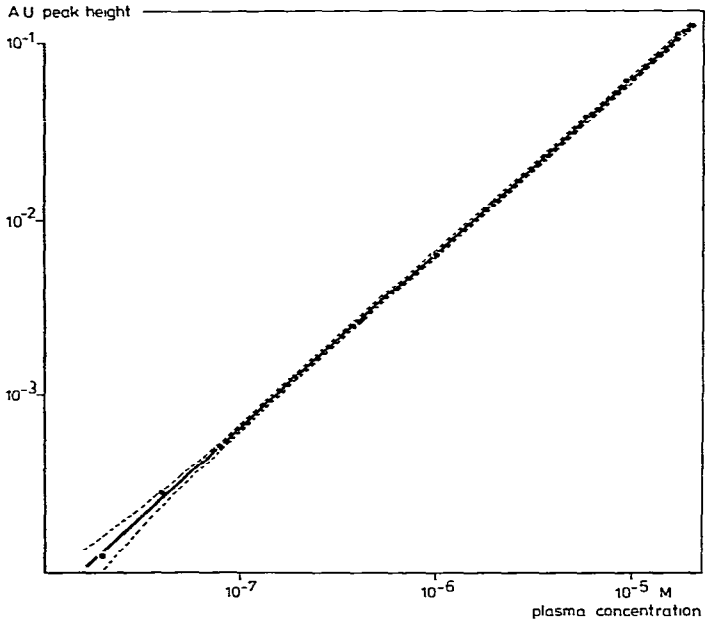

Fig. 6. Peak height *versus* concentration for injection of 1 ml of de-proteinized mixture, corresponding to 0.5 ml of plasma. The broken lines show ±twice the standard deviation. Conditions as in Fig. 4.

dized, especially in very dilute solutions[25]. The concentration of a $4 \cdot 10^{-8}$ *M* solution in the medium obtained after de-proteinization decreased by 19% and that of a $2 \cdot 10^{-6}$ *M* solution by 8% in 3 days. A very large time delay in the analysis must therefore be avoided.

The recovery of the whole procedure, including de-proteinization, was 70.2%, with a relative standard deviation of 3.5%. The missing 30% was lost by adsorption on the removed proteins, as no loss was observed in a solution that did not contain proteins.

Kinkade *et al.*[23] used a comparable deproteinization procedure with trichloroacetic acid, but applied it to fivefold diluted plasma samples. They found recoveries of 85–97%, although for higher concentrations of Mtx. As the dilution of the sample was not tolerable in our work in view of the lower concentrations occurring and the migration of Mtx in the concentration column, an alternative method for deproteinization was tried, consisting of membrane filtering (Amicon, exclusion limit 20.000 MW). Even larger percentages of Mtx were lost with this method. As it was meanwhile found that the recovery with the original procedure (70%) was well reproducible for human plasma samples, it was decided to maintain this procedure.

Other described methods for Mtx are enzymatic or by radioimmunoassay. Comparison with these methods with respect to recovery and accuracy is difficult, because an overall calibration is used in these methods.

The column system used for the Mtx determination was used for several months, during which time hundreds of plasma samples were analysed, and no serious deterioration of the columns was observed.

CONCLUSION

The on-column concentration procedure described here is a practical alternative to trace enrichment and concentration procedures based on extraction and evaporation. Development work, such as the choice of phase system, dimensions of the concentration column, etc., is of about the same complexity and difficulty as in the case of extraction/evaporation. Simple guidelines can be given for the proper choice of conditions.

In view of the results obtained, it is to be expected that the method will be of great value for future chromatographic work on trace analysis. Plasma concentrations and pharmacokinetic results will be published elsewhere. The method could also be used for the determination of Mtx metabolites.

ACKNOWLEDGEMENTS

Dr. E. van der Kleijn, Head of the Department of Clinical Pharmacy, is kindly thanked for providing the opportunity for developing the procedure for methotrexate determination. Dr. R. G. Muusze initiated and stimulated the application of the method to fluphenazine. The study of the determination of methotrexate was financed by a grant from the Koningin Wilhelmina Fonds, the Dutch cancer foundation.

REFERENCES

1 J. F. K. Huber, J. A. R. J. Hulsman and C. A. M. Meijers, *J. Chromatogr.*, 62 (1971) 79.
2 B. L. Karger, M. Martin and G. Guiochon, *Anal. Chem.*, 46 (1974) 1640.
3 J. N. Little and G. J. Fallick, *J. Chromatogr.*, 112 (1975) 389.
4 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27, 429.
5 F. Erni, R. W. Frei and W. Lindner, *J. Chromatogr.*, 125 (1976) 265.
6 P. Schanwecker, R. W. Frei and F. Erni, *J. Chromatogr.*, 136 (1977) 63.
7 T. Walle and H. Ersson, *Acta Pharm. Suecica*, 8 (1971) 34.
8 D. G. Johns and J. R. Bertino, in J. F. Holland and E. Frei (Editors), *Cancer Medicine*, Lea and Febiger, Philadelphia, 1973, pp. 739–754.
9 B. A. Chabner, C. E. Myers, C. N. Coleman and D. G. Johns, *N. Engl. J. Med.*, 292 (1976) 1107 and 1159.
10 K. B. Bischoff, *J. Pharmacokinet. Biopharm.*, 1 (1973) 465.
11 R. J. Lutz, R. L. Dedrick, J. A. Straw, M. M. Hart, P. Klubes and D. S. Zaharko, *J. Pharmacokinet. Biopharm.*, 3 (1975) 77.
12 D. H. Huffman, S. H. Wan, D. L. Azarnoff and B. Hoogstraten, *Clin. Pharm. Ther.*, 14 (1973) 572.
13 L. Levine and E. Powers, *Res. Commun. Chem. Pathol. Pharm.*, 9 (1974) 543.
14 V. Raso and R. Schreiber, *Cancer Res.*, 35 (1975) 1407.
15 C. E. Myers, M. E. Lippman, H. M. Eliot and B. A. Chabner, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 3683.
16 L. C. Falk, D. R. Clark, S. M. Kahlman and T. F. Long, *Clin. Chem.*, 22 (1976) 785.
17 H. K. Kimelberg, S. M. Biddlecome and R. S. Bourke, *Cancer Res.*, 37 (1977) 157.
18 U. R. Tjaden, J. Lankelma, H. Poppe and R. G. Muusze, *J. Chromatogr.*, 125 (1976) 275.
19 J. Lankelma and H. Poppe, *J. Chromatogr.*, 125 (1976) 375.
20 U. R. Tjaden, *Thesis*, Amsterdam, 1976.
21 J. F. K. Huber and U. R. Tjaden, to be published.
22 U. Breyer and R. Villumson, *Eur. J. Clin. Pharmacol.*, 9 (1976) 457.
23 J. M. Kinkade, W. R. Vogler and P. G. Dayton. *Biochem. Med.*, 10 (1974) 337.
24 R. W. Stout, A. R. Cashmore, J. K. Coward, C. G. Horvath and J. R. Bertino, *Anal. Biochem.*, 71 (1976) 119.
25 R. L. Blackley, *The Biochemistry of Folic Acid and Related Pteridines*, North-Holland, Amsterdam. 1969.