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SEPARATION OF ACIDIC DRUGS IN THE μg/ml RANGE IN UNTREATED BLOOD PLASMA BY DIRECT INJECTION ON LIQUID CHROMATOGRAPHIC COLUMNS

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SUMMARY

Conditions are described that enable the injection of untreated, non-diluted blood plasma on liquid chromatographic columns for the determination of two acidic drugs. The mobile phase is chosen so that the plasma proteins are kept in solution. The influence of the number of plasma injections on the column back-pressure and retention time have been studied. Quantitations could be made without sample losses.

INTRODUCTION

The determination of drugs in complex mixtures such as body fluids usually involves a number of procedures, *e.g.*, extraction, evaporation, etc., that can contribute considerably to the total analysis time and work. There is an obvious need to reduce the number of working steps or to automate the total procedure. Liquid chromatography with aqueous mobile phases permits direct application of aqueous samples onto the separation column which means that extraction with organic solvents can be avoided.

The direct injection of blood plasma samples can be complicated by several factors. Aqueous mobile phases containing polar organic solvents will in many cases cause precipitation of the plasma proteins. Therefore plasma samples have often been equilibrated with the mobile phase solvent and the clear supernatant obtained after such a denaturing procedure then injected on the chromatographic column, *e.g.*, as in ref. 1. Alternatively, the proteins are removed by more efficient denaturing agents such as perchloric acid or ammonium sulphate[2] or acetonitrile[3]. However, denaturing can result in serious errors due to losses of the determined compound in the protein precipitate[2], especially for relatively hydrophobic compounds[4], and therefore it may be an advantage to make direct injections of the untreated plasma samples.

Some work has already been done in which untreated plasma samples have been fed to liquid chromatographic columns. Thus, attempts to separate serum proteins were made on size exclusion columns[5] or on ion-exchange columns[6] with buffers as the eluent. For the determination of low-molecular-weight compounds, like many drugs, the conditions are chosen such that the compound to be determined is com-

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pletely retained when the eluent is water. The compound is then rapidly eluted by switching to an aqueous eluent containing a suitable concentration of a polar organic solvent. This preconcentration technique has been performed on separate precolumns[7] or on an automated system where the precolumn was coupled on-line to the separation column[8]. Preconcentration is of particular advantage in trace analysis when a large plasma sample is required.

There are many cases, however, where a drug or a similar compound is present in blood plasma in such high concentrations that a relatively small sample volume (*e.g.*, 10 μl) can be applied to the separation system in order to detect the compound. It should be possible in those cases to apply plasma samples without a previous precipitation of the proteins and without preconcentration steps that involve a change of the eluting solvent. Thus, N-acetyl-*p*-aminophenol has been determined in 1.5 μl serum samples (after dilution 1:1 with buffer) on a 13-μm cation-exchange resin protected by an exchangeable precolumn and with a buffer as eluent, but the separation time was rather long[9]. Recently[10], the same drug was determined on a reversed-phase μBondapak C_{18} column from 2-μl plasma samples (diluted 1:1 with buffer) and with acetonitrile–buffer (7:93) as the eluent, without any precolumn. The same conditions were used for the determination of theophylline and no problems with the column back-pressure occurred[11].

In this paper, a study of the separation conditions for two carboxylic acids from blood plasma by direct injection of the untreated, non-diluted, plasma samples (10 or 20 μl) on reversed-phase liquid chromatographic columns is described. Several of the experimental parameters which influence the performance of such a procedure have been examined. The choice of chromatographic phase system is discussed, as well as the stability of the system with respect to the back-pressure and retention time. Some results on quantitation are also presented.

EXPERIMENTAL

Chemicals

Salicylic acid and naproxen, (+)-2-(6-methoxy-2-naphtyl)propionic acid, were of pharmacopoeial grade. All other chemicals were of analytical grade.

LiChrosorb SI 60 (silica) and RP-2, RP-8 and RP-18 (alkyl-modified silicas) were obtained from E. Merck (Darmstadt, G.F.R.). They had particle diameters between 5 and 10 μm.

Equipment

An LDC Solvent Delivery System 711-46 or 711-26 (Milton-Roy Minipumps with LDC pulse dampener; LDC, Riviera Beach, Fl, U.S.A.), a Rheodyne Syringe Loading Injector 7120 equipped with 10-, 20- or 50-μl loops and a SpectroMonitor III (LDC) variable-wavelength detector or a Schoeffel FS 970 fluorescence detector were used. The separation columns were LiChroma tubes (316 stainless steel, Handy and Harman), 100 × 4.6 I.D. They were equipped with Swagelok end fittings containing Altex 250-21 bed supports with 2-μm steel frits. The precolumns (5 × 3.2 mm I.D.) were constructed from similar materials as the separation column after modification. Altex bed supports were placed in both ends. The precolumns were connected to the separation column by 1/16 in. I.D. tubing via a Swagelok zero dead volume union.

Procedures

All experiments were performed at 25.0°C, with buffers having an ionic strength of 0.1. The liquid–liquid chromatographic system was prepared as described previously[12]. The support was LiChrosorb RP-18, 5 μm.

The separation columns were slurry packed with a high pressure pump. The precolumns were also slurry packed, either with the high pressure pump or by suction with a water pump. In the latter case the slurry was made up in methanol–dichloromethane (5:95).

Plasma samples were carefully centrifuged. Spiking with salicylic acid or naproxen was achieved by mixing 5 μl of concentrated aqueous solutions of these compounds with 500 μl centrifuged plasma. External standards were prepared in the same way except that the eluent solvent was used instead of the plasma.

RESULTS AND DISCUSSION

Solubility of plasma proteins

The success of a procedure to determine a drug in plasma by direct injection on a liquid chromatographic column depends to a great extent on the influence of the normal constituents of the plasma, especially those that are present in high concentrations, *e.g.*, proteins, normally having a total concentration of *ca.* 8% (w/v).

In this study the conditions are such that the plasma proteins are prevented from precipitating when they come in contact with the chromatographic eluent. It is known that water-miscible organic solvents, like those often used as the eluent of reversed-phase liquid chromatographic systems, are amongst the more efficient precipitants for proteins. Some simple experiments showed that concentrations of methanol and acetonitrile exceeding 50% in water gave pronounced precipitation of plasma proteins, whereas at lower concentrations the precipitation effect was much weaker and hardly noticeable below 10%. The results presented were obtained either without any organic solvent in the eluent or with 10% methanol in the eluent, but it is possible that even higher concentrations of organic solvent can be used.

Test compounds

Two carboxylic acids, salicylic acid and naproxen, have been used as test compounds. Salicylic acid is formed in the body after administration of the drug acetylsalicylic acid, whereas naproxen is used as a drug. Both compounds are present in rather high concentrations (well above 1 μg/ml) in blood plasma after administration. They have high molar absorptivities and are highly fluorescent, and can therefore easily be detected by an UV detector or a fluorescence detector. Thus, the conditions are favourable for direct injection of plasma samples of these compounds.

Phase systems

The phase systems tested are summarized in Table I. They represent both liquid–liquid and liquid–solid reversed-phase systems. The liquid–liquid system has been shown to be useful for the separation of carboxylic acids[12]. The liquid–solid systems were prepared with eluents free from organic solvents, which means that the retention can only be regulated by the type of solid stationary phase and the concentration of salts in the eluent.

TABLE I

PHASE SYSTEMS

System	*Mobile phase*	*Stationary phase*
I* (liquid–solid)	Phosphate buffer, pH 7.5	LiChrosorb SI 60, 5 and 10 μm
II* (liquid–solid)	Phosphate buffer, pH 7.5	LiChrosorb RP-18, 5 and 10 μm
III (liquid–liquid)	Methanol + citrate buffer, pH 5.4 (1:9)	Tributyl phosphate
IV (liquid–solid)	Phosphate buffer + sodium octyl sulphate	LiChrosorb RP-18, 5 and 7 μm
V (liquid–solid)	Phosphate buffer + sodium octyl sulphate	LiChrosorb RP-2, 6 μm

* Precolumns only.

Normally the retention of a carboxylic acid should decrease with increasing pH of the mobile phase (above the pK_a value of the acid). Thus, in the liquid–liquid phase system the retention can be regulated in a predictable way and within wide limits[12]. In the liquid–solid phase systems the retention often tends to level off even though the pH is increased. This may be due to the retention of the anions of the carboxylic acids, *e.g.*, as ion pairs with sodium in the buffer[13]. In such cases it can be shown that the addition of a competitor to the mobile phase further decreases the retention. The competitor should compete with the sample anion for the solid stationary phase. For salicylic acid and naproxen it was necessary to add sodium octyl sulphate to the mobile phase as a competitor. The competition effect can be regulated by the concentration of the competitor and its hydrophobicity. Lauryl sulphate will be a more effective competitor than octyl sulphate since the former is more hydrophobic.

Effect of plasma injections on the back-pressure and retention time

In all experiments when plasma was injected the separation column was preceded by a short precolumn in order to protect the separation column from possible deterioration. The volume of the precolumn has not been optimized with respect to the number of plasma injections that can be made, and therefore the precolumns were in most cases routinely changed after about ten plasma injections or when they gave an excessive back-pressure.

TABLE II

EFFECT OF PLASMA INJECTIONS ON BACK-PRESSURE AND RETENTION TIME WITHOUT CHANGE OF THE PRECOLUMN

Phase system	*Flow-rate (ml/min)*	*Plasma injections*		*Average increase of back-pressure (bar per injection)*	*Retention time (min)*		
		Vol. (μl)	*No.*		*Mean*	*Relative S.D. × 100*	*Range*
I, II*	1.4	10	25	0.09–0.6			
III	0.8	20	8	1.5	–	–	3.7–3.9
IV	1.0	10	17	0.6	5.55	0.3	
V	1.0	10	15	0.9	6.42	1.2	
V	1.0	10	24**	2.2	6.47	1.5	

* Precolumns only.

** Precolumn changed twice.

It was essential to obtain a clear plasma solution otherwise the frits of the bed supports soon became blocked.

A possible effect of the injection of plasma is the enrichment of the stationary phase by substances like fats and proteins. This can decrease the porosity of the column which leads to an increase in the resistance to flow. Depending on the type of pump used, this can result in a decreased flow-rate and an increased retention time.

Table II summarizes the effects on back-pressure and retention time by plasma injections in some different systems. There was no clear difference between the ordinary silica (LiChrosorb SI 60) and hydrophobic silica (LiChrosorb RP-18) with respect to the increases in back-pressure over the precolumns, and only slightly better performance was seen with the larger particle sizes (10 μm). In many cases the back-pressure increased stepwise, which means that in one case fourteen injections could be made without any change of the back-pressure. The restrictions to flow were often localized to the frits of the bed supports in the precolumns.

In the liquid–liquid phase system (III) the increases in back-pressure and retention time were somewhat higher than in the other phase systems, which may be a result of the denaturing effect of methanol and tributyl phosphate on the proteins. In the liquid–solid systems (IV and V) the retention time was constant despite a gradual increase of the back-pressure, but the retention time at injection of a plasma sample was always 1 % lower than for an external standard.

The constancy of the retention time obtained with the relatively simple pumping system used in this study indicates that quantitations will be possible if the precolumns are changed at suitable intervals. With a more advanced pumping system the possibilities will be even greater.

On injecting a large number of plasma samples (60 samples, phase system V) the back-pressure occasionally increased drastically. It could always be restored by change of the precolumn and the retention time was always constant. This indicates that the retention properties of the separation system were unaffected by the plasma injections.

A study of the changes of back-pressure during the elution of a plasma sample shows that the viscous plasma components migrate with the solvent front. It is possible that the drastic increases of the back-pressure after many injections of plasma is related to this. The presence of the concentrated plasma plug may have increased the blocking of the pores. Therefore, injection of slightly more dilute plasma samples may result in improved performance.

Separation of salicylic acid

Phase system III. The buffer pH in the eluent was chosen as 5.4 in order to give salicylic acid a capacity ratio of close to 4. A comparison of the peak heights from a few plasma samples with those obtained from external standards indicated a recovery of close to 100 % at a concentration level of 40 μg/ml salicylic acid.

When the plasma sample volume exceeded 20 μl the salicylic acid peak became skewed and for a 40-μl sample was divided into two peaks with the new peak occurring at a shorter retention time than the original. It could be shown that both peaks contained the salicylic acid. The effect seemed to depend on the amount of plasma injected, since if the plasma samples were diluted and a correspondingly larger volume was injected the effect remained. It was suspected that the peak splitting effect

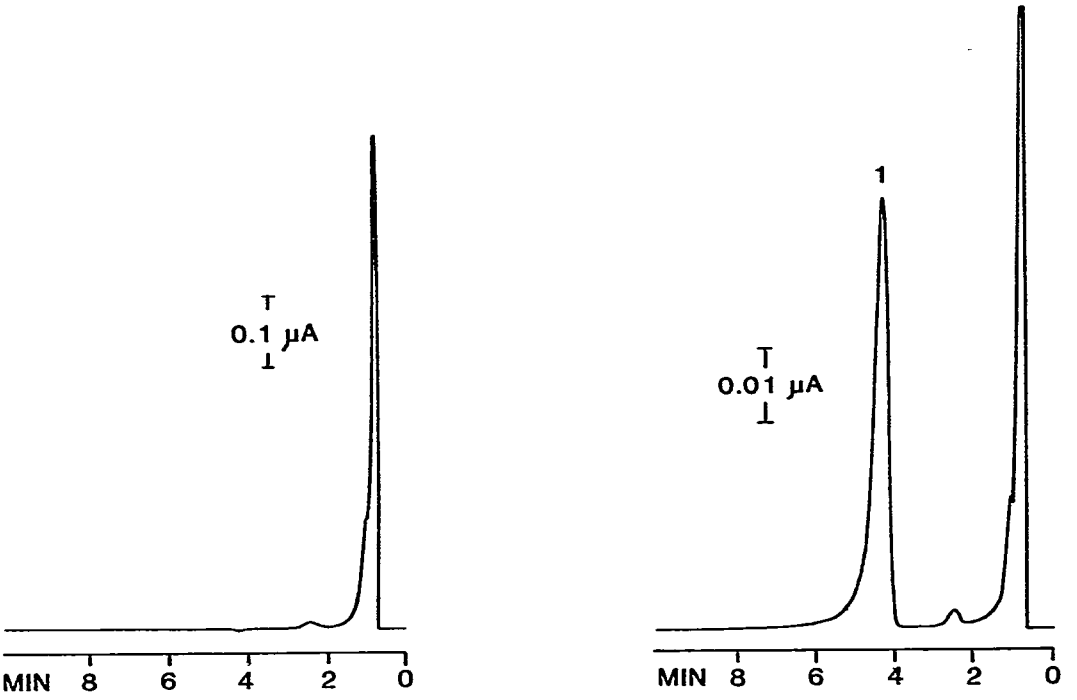

Fig. 1. Chromatograms of plasma. Left, 20 μl plasma; right, 3 μl plasma spiked with 40 μg/ml salicylic acid (1). Separation column: LiChrosorb RP-18, 5 μm, coated with tributyl phosphate. Precolumn: LiChrosorb RP-18, 5 μm. Eluent: methanol–citrate buffer, pH 5.4 (1:9); 0.8 ml/min. Fluorescence detection: excitation at 295 nm. emission at >370 nm.

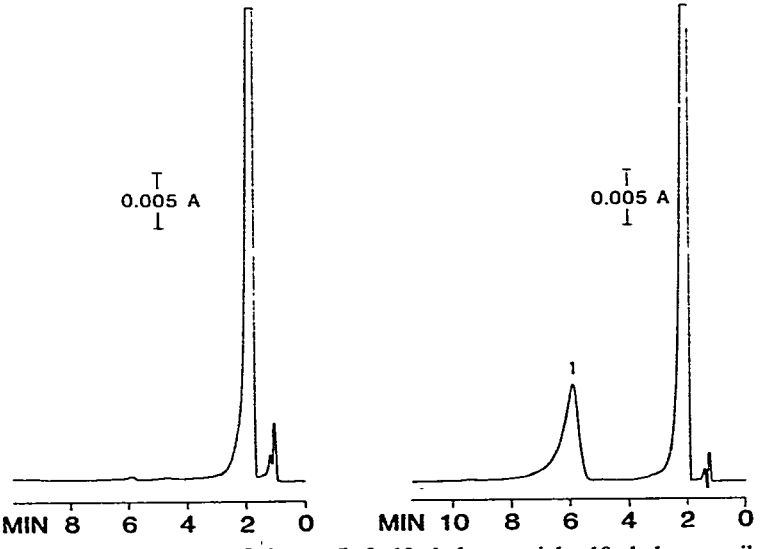

Fig. 2. Chromatogram of plasma. Left, 10 μl plasma; right, 10 μl plasma spiked with 27 μg/ml salicylic acid (1). Separation column: LiChrosorb RP-18, 5 μm. Precolumn: LiChrosorb 18, 10 μm. Eluent: phosphate buffer, pH 5.90–1.02 · 10^{-4} *M* sodium octyl sulphate; 1 ml/min. Detection: UV at 296 nm.

could be due to the strong binding of salicylic acid to plasma albumin (as is also the case for naproxen), but the addition to the plasma sample of a substance (flurbiprofen), which would displace salicylic acid from albumin[14], did not eliminate the effect. It is possible that the injection of plasma changed the properties of the stationary phase. Further studies are necessary to explain this problem.

Fig. 1 gives typical chromatograms from plasma, without and with addition of salicylic acid, obtained with fluorescence detection. Spectrophotometric detection at 296 nm could also be used and gave as good resolution from the leading peak even though this peak was much higher than the salicylic acid peak.

Phase system IV. In this case the separation column contained LiChrosorb RP-18, 5 or 7 μm, and the precolumn the same type of particles but of 10 μm. The eluent was of pH 5.9 and contained sodium octylsulphate, $1.0 \cdot 10^{-4}$ *M* or $3.1 \cdot 10^{-5}$ *M*, which gave salicylic acid a capacity ratio of 4.7 or 3.6.

Quantitation was only briefly studied and a few comparisons with external standards indicated a recovery close to 100% at the 30-μg/ml level. Fig. 2 shows a chromatogram of 10 μl plasma, before and after spiking with 27 μg/ml salicylic acid, with spectrophotometric detection at 296 nm. The peak is well resolved.

Separation of naproxen in plasma with phase system V

The more hydrophobic compound naproxen required a less hydrophobic sta-

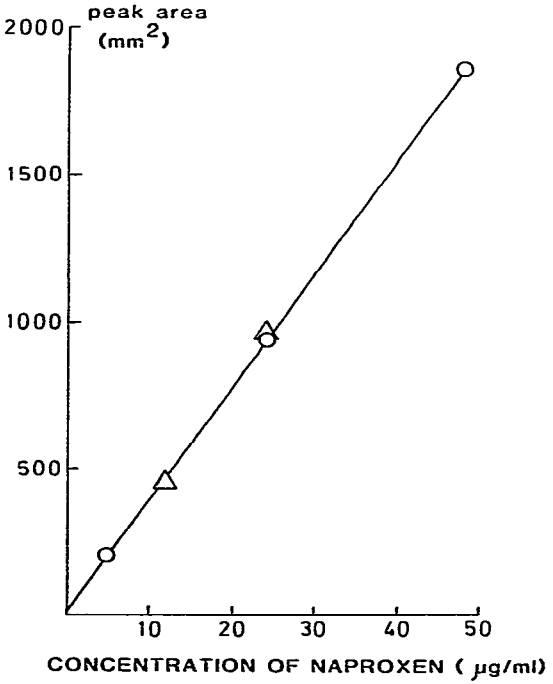

Fig. 3. Quantitation of naproxen in plasma. ○, External standards dissolved in the eluent (the mean of two or three values); △, plasma samples (single values). For conditions see Fig. 4.

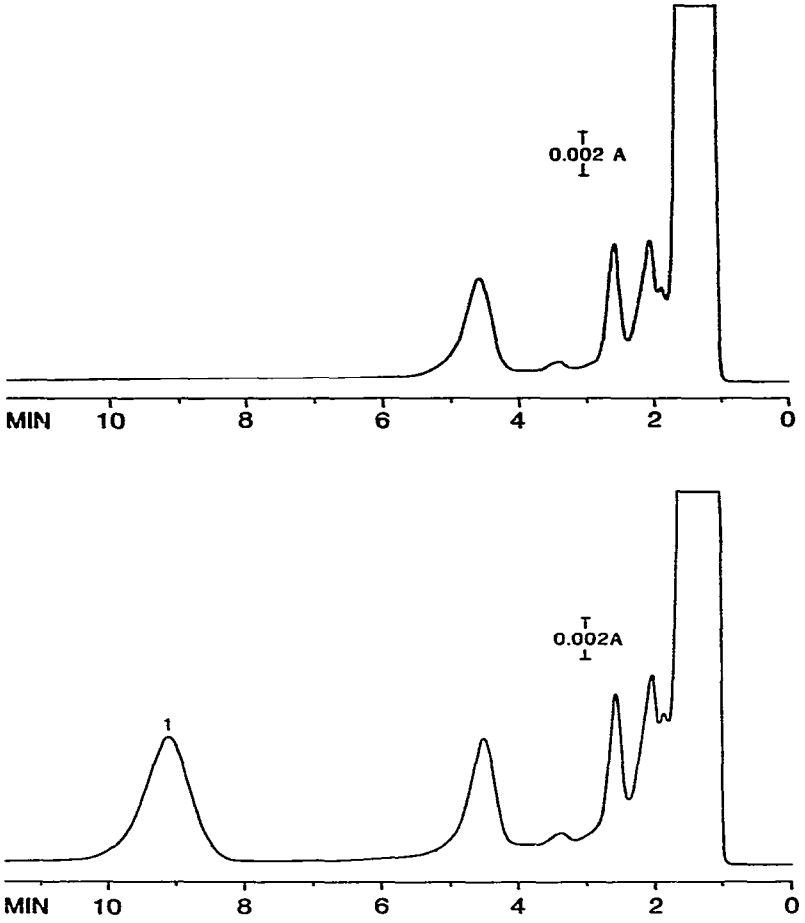

Fig. 4. Chromatograms of plasma. Top, 10 μl plasma; bottom, 10 μl plasma spiked with 24 μg/ml naproxen (1). Separation column: LiChrosorb RP-2, 6 μm. Precolumn: LiChrosorb RP-2, 6 μm. Eluent: phosphate buffer, pH 7.6–0.01 *M* octyl sulphate; 0.8 ml/min. Detection: UV at 261 nm.

tionary phase (LiChrosorb RP-2) than salicylic acid. The capacity ratio was adjusted to 6. The precolumns contained either the same material as the separation column or LiChrosorb RP-8, 10 μm. The latter gave a somewhat better performance with respect to the number of plasma injections that could be made until the back-pressure started to increase drastically.

The possibilities for quantitation were tested by comparing peak heights and peak areas from spiked plasma samples with those from external standards. Some results are given in Fig. 3 and they indicate that quantitation can be achieved with no loss of substance in the studied concentration range. These results were obtained with plasma samples and standards intermittently injected at close time intervals, and showed a quite normal disperision. Over a longer time period the relative standard deviation of peak heights or peak areas was around 5% for both plasma and standards. This dispersion seemed to be random and could not be correlated to, *e.g.*,

changes in back-pressure. The fluctuations in the column efficiency that were observed might, however, have contributed to the dispersion.

Fig. 4 shows illustrative chromatograms of the separation of naproxen.

CONCLUSIONS

The chromatograms of plasma samples are surprisingly free from interfering peaks, which makes possible consecutive injection of plasma samples. There are some indications that most of the plasma proteins are unretained in the liquid–solid phase systems. A chromatogram of albumin gives support to this conclusion.

The column efficiency for salicylic acid and naproxen was rather low in the liquid–solid systems under the conditions used, the reduced plate height being around 20. This was not caused by the plasma injections.

The phase systems described have been optimized for the chromatography of salicylic acid and naproxen. The conditions can of course be changed to suit compounds of a different nature such as bases, anions, cations or uncharged aprotic compounds, but the requirement for a low concentration of organic solvent in the eluent can restrict the applicability of the technique. Since little knowledge of the retention properties of the endogeneous compounds in plasma is available there is always a risk that they will cause interference if a new phase system is used.

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REFERENCES

1 K.-G. Wahlund and U. Lund, *J. Chromatogr.*, 122 (1976) 269.
2 B. Henke and D. Westerlund, *J. Chromatogr.*, 187 (1980) 189.
3 B. E. Cham, D. Johns, F. Bochner, D. M. Imhoff and M. Rowland, *Clin. Chem.*, 25 (1979) 1420.
4 D. Westerlund and K. H. Karset, *Anal. Chim. Acta*, 67 (1973) 99.
5 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, p. 503.
6 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
7 J. X. de Vries, W. Günthert and R. Ding, *J. Chromatogr.*, 221 (1980) 161.
8 W. Roth, K. Beschke, R. Jauch, A. Zimmer and F. W. Koss, *J. Chromatogr.*, 222 (1981) 13.
9 D. Blair and B. H. Rumack, *Clin. Chem.*, 23 (1977) 743.
10 B. R. Manno, J. E. Manno, C. A. Dempsey and M. A. Wood, *J. Anal. Toxicol.*, 5 (1981) 24.
11 B. R. Manno, J. E. Manno and B. Hilman, *J. Anal. Toxicol.*, 3 (1979) 81.
12 K.-G. Wahlund and B. Edlén, *J. Liquid Chromatogr.*, 4 (1981) 309.
13 A. Tilly-Melin, Y. Askemark, K.-G. Wahlund and G. Schill, *Anal. Chem.*, 51 (1979) 976.
14 I. Sjöholm, B. Ekman, A. Kober, I. Ljungstedt-Påhlman, B. Seiving and T. Sjödin, *Mol. Pharmacol.*, 16 (1979) 767.