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# LIQUID CHROMATOGRAPHIC DETERMINATION OF TETRACYCLINE IN PLASMA AND URINE

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## SUMMARY

Tetracycline is extracted from plasma (0.50 ml) as an ion pair with tetrabutylammonium into chloroform–1-heptanol (9:1). After re-extraction into an acidic aqueous phase the separation is performed by reversed-phase liquid chromatography using LiChrosorb RP-2 as the support and acetonitrile–water–phosphoric acid as the mobile phase. The chromatographic system shows a high selectivity for the separation of tetracycline analogues. A high detection selectivity is obtained by the use of photometric detection at 357 nm. Analysis of urine is performed by direct injection of the sample into the liquid chromatograph. The precision in the determination of tetracycline in plasma was about 10% (relative standard deviation) at drug levels of 200 ng/ml and 200 μg/ml. Urine samples containing 20 and 200 μg/ml of tetracycline were determined with a precision of 3%.

## INTRODUCTION

Tetracycline (Fig. 1) is an antimicrobial agent with a broad spectrum of activity. A selective and sensitive analytical method is required for pharmacokinetic studies of the drug in man.

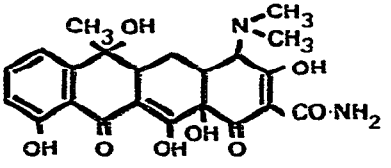


Fig. 1. Structural formula of tetracycline.

Tetracycline has been determined by microbiological[1] and fluorimetric methods[2,3]. Analytical methods including a chromatographic step offer a higher selectivity and have been used for the quantification of tetracycline and its degradation products[4–7] in pharmaceutical preparations. The selectivity and sensitivity of the analytical methods used are inadequate for the determination of tetracycline in biological fluids.

The determination of tetracycline in plasma and urine using high-performance

liquid chromatography has been reported[8–12]. The chromatographic conditions used seem to give a low selectivity and low separating efficiency[13]. In this work, tetracycline was determined in plasma after extraction as an ion pair with tetrabutylammonium and isolation by reversed-phase liquid chromatography. A high detection selectivity is obtained by the use of photometric detection at 357 nm. Analysis of tetracycline in urine is performed by direct injection of the samples (thus omitting the extraction procedure). The chromatographic system shows a high selectivity for the separation of tetracycline derivatives.

## EXPERIMENTAL

### *Apparatus*

A Zeiss PMQ III Spectralphotometer and a Pye Unicam Model 1800 instrument, both equipped with 10.0-mm cell were used. An Orion Research Model 701 digital pH meter equipped with an Ingold Type 401 combined electrode was used for the pH determination.

The chromatographic detector used was the LDC Spectromonitor III with a 10.0-mm path length and a cell volume of 8 $\mu$l.

The pump was of the LDC 711 Solvent Delivery System type, and the columns were made of stainless steel (length 150 mm, I.D. 4 mm, O.D. 1/4 in.). The column end fittings were modified Swagelok connectors. A Rheodyne Model 70-10 injection valve with a sample loop of 300 $\mu$l was used.

The LiChrosorb RP-2, RP-8 and RP-18 supports (E. Merck, Darmstadt, G.F.R.) had a mean diameter of 5 $\mu$m.

The mobile phase were prepared from acetonitrile (Merck, Uvasol) and distilled water. Phosphoric acid (Merck, p.a.) was added to a final concentration of $10^{-2}$ *M*.

The chromatographic system was thermostated at 25.0 $\pm$ 0.1°.

### *Chemicals*

Tetracyclines (tetracycline, ACO Läkemedel AB, Solna, Sweden; chlortetracycline, Glaxo Läkemdel AB, Mölndal, Sweden; demeclomycine, Cyanamid Nordiska AB, Stockholm, Sweden; doxycycline, AB Ferrosan, Malmö, Sweden; metacycline, Roerig AB, Täby, Sweden; oxytetracycline, Pfizer AB, Täby, Sweden) were used as obtained from the suppliers.

Tetrabutylammonium hydrogen phosphate was kindly supplied by AB Hässle (Mölndal, Sweden). All other chemicals were of analytical-reagent grade and used without further purification.

### *Chromatographic technique*

The chromatographic columns were packed by the slurry packing technique using glycerol–methanol (1:3 by volume) as suspending medium[14]. The slurry was forced into the chromatographic column with a flow-rate of 9 ml/min or a pressure of 5000 p.s.i., whichever was the limiting factor. The mobile phases were passed through the chromatographic system until constant retention was obtained. Usually less than 50 ml were required.

*Determination of partition ratio*

The partition experiments were performed in centrifuge tubes with mechanical shaking in a thermostated bath at 25.0 ± 0.5° using an equilibrium time of 30 min. Tetracycline was dissolved in the aqueous phase. The concentration of tetracycline in the aqueous phase at equilibrium stage was determined by photometric measurements at 270 nm. The concentration of tetracycline in the organic phase was calculated as the difference between the initial concentration in the aqueous phase and the concentration in the aqueous phase found at the equilibrium stage.

*Plasma and urine samples*

Blood samples (5–7 ml) were collected in 10-ml glass test-tubes (Vacutainer) containing 250 I.U. of heparin (freeze dried) immediately before and at appropriate times after the start of drug administration. The samples were immediately centrifuged at 4000 *g* for 10 min. The plasma fraction was carefully separated and frozen at −20° until taken for assay. Urine samples were stored at −20° until taken for assay.

*Spiking of plasma and urine samples*

The accuracy and precision of the analytical methods were studied by spiking drug-free plasma and urine with tetracycline dissolved in 0.1 *M* phosphoric acid (50 μl/ml of plasma or urine). After neutralization with 0.1 *M* sodium hydroxide solution the spiked samples were analysed according to the analytical methods below.

## ANALYTICAL METHODS

*Plasma samples*

*Extraction procedure.* The plasma sample (0.500 ml) was mixed with 0.50 ml of 0.4 *M* tetrabutylammonium and 0.10 ml of carbonate buffer of pH 9.4 (ionic strength 1.0). The mixture was extracted twice with 2.50 ml of chloroform–1-heptanol (9:1), the extraction time being 10 min. After centrifugation the combined organic phases were transferred into a new centrifuge tube containing 1.00 ml of 0.1 *M* perchloric acid and equilibrated for 10 min. The aqueous (upper) phase from the extraction procedure was transferred into a centrifuge tube with a tapered base (0.2 ml) containing 2 ml of *n*-hexane and centrifuged (this step has been included to facilitate the transfer of the aqueous phase into the chromatographic column without contamination with organic phase).

*Liquid chromatographic isolation and quantification.* Part of the aqueous (lower) phase (0.050–0.300 ml) was injected into the chromatographic column (support, LiChrosorb RP-2, 5 μm; mobile phase, 6.7 *M* (35%) acetonitrile in 0.01 *M* phosphoric acid; mobile phase flow-rate 0.8–1.0 ml/min. The absorbance of the eluate was measured at 357 nm. Quantification was based on peak area measurements and the molar absorptivity of the migrating compound.

*Urine samples*

The urine (0.050 ml) was diluted with 0.050 ml of 0.1 *M* phosphoric acid and injected into the liquid chromatograph. To avoid interfering peaks a mobile phase containing only 3.4 *M* (18%) of acetonitrile was used.

## RESULTS AND DISCUSSION

*Extraction properties*

Tetracycline has previously been extracted into an organic phase as a complex with calcium and barbiturates[2], as the degree of extraction in an uncomplexed form is very low[15,16]. In this study tetracycline was extracted as an ion pair with tetrabutylammonium (TBA). A theoretical elucidation of the extraction mechanism was not possible probably as a result of the highly complicated protolytic properties of tetracycline[17,18] in combination with a probable high tendency for formation of dimeric and tetrameric forms[19]. The following empirical conclusions could, however, be drawn from the extraction studies:

(1) The extraction of tetracycline as an ion pair with TBA is most favourable at pH $> 9.0$.

(2) The partition ratio is increased with increasing concentration of tetracycline when the tetracycline concentration ($C_T$) is less than $2 \cdot 10^{-5}$ *M* (Fig. 2). The distribution seems to be almost independent of $C_T$ at higher concentrations.

(3) The degree of extraction of the tetracycline–TBA ion pair is increased with increasing concentration of TBA (Fig. 3). The non-linear relationships observed might be due to the formation of ion pairs in the aqueous phase[20].

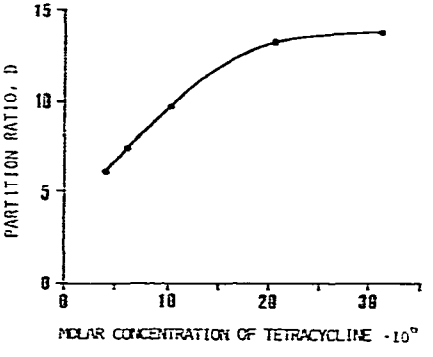


Fig. 2. Partition ratio of tetracycline as TBA ion pair as a function of drug concentration. Organic phase: chloroform–1-heptanol (9:1). Aqueous phase: $8.7 \cdot 10^{-2}$ *M* tetrabutylammonium, in carbonate buffer of pH 9.4.

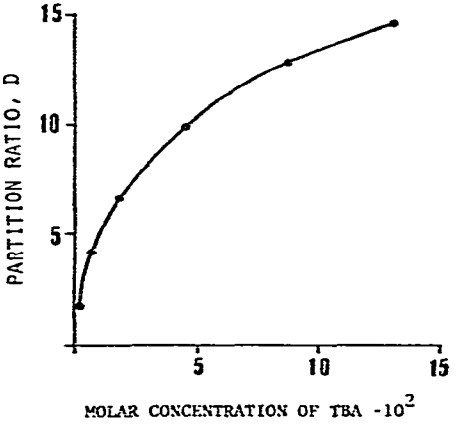


Fig. 3. Partition ratio of tetracycline as TBA ion pair as a function of TBA concentration. Organic phase: chloroform–1-heptanol (9:1). Aqueous phase: tetrabutylammonium in carbonate buffer of pH 9.4. Initial concentration of tetracycline: $4 \cdot 10^{-5}$ *M*.

A partition ratio of 14 was obtained with equal phase volumes, *i.e.*, about 93% of tetracycline was transferred from the aqueous into the organic phase using $C_{TBA} = 0.1$ *M*, pH = 9.4 (initial concentration of tetracycline greater than $2 \cdot 10^{-5}$ *M*). By repeating the extraction with a new organic phase, 99.6% of tetracycline was extracted from the aqueous phase.

Human plasma contains large and variable amounts of anionic components extractable with TBA[21], which decreases the concentration of TBA available for the

tetracycline extraction. An excess of extraction reagent is therefore used for the extraction of tetracycline from plasma samples.

After initial extraction from the biological samples tetracycline is re-extracted into an acidic aqueous phase, which is injected into the liquid chromatograph. The biological extract is partly purified by this procedure and a concentration of tetracycline is obtained.

Excess of TBA, coextracted with endogenous anions, must be removed from the aqueous phase to be injected into the liquid chromatograph, as it will be adsorbed on the chromatographic support resulting in a gradually decreased retention of tetracycline. By using perchloric acid in the re-extraction step TBA is partitioned into the organic phase as a perchloric ion pair. A large excess of perchloric acid should be avoided as tetracycline is also extractable as a perchlorate ion pair.

In the analytical method 0.1 *M* perchloric acid is used in the re-extraction step, which proved to inhibit the influence of coextracted TBA on the chromatographic retention of tetracycline. More than 80% of tetracycline was found to be re-extracted into 1.0 ml of 0.1 *M* perchloric acid from 5.0 ml of the organic phase [chloroform–1-heptanol (9:1)].

*Stability of tetracycline*

The stability of tetracycline at 25° was found to be strongly dependent on the composition of the aqueous phase. In 0.1 *M* perchloric acid more than 90% of the tetracycline was unchanged after storage for 24 h. The degradation was considerable faster in alkaline solution. Less than 40% of the drug was recovered after storage in carbonate buffer of pH 9.84 for 5 h. Thus, the initial extraction of tetracycline from the plasma samples should be performed immediately after addition of the buffer solution (pH $\approx$ 9.4).

*Liquid chromatography*

*Retention and selectivity.* Reversed-phase liquid chromatography using an acidified mobile phase with acetonitrile as organic modifier showed a high selectivity in the separation of tetracycline analogues (Table I)[22]. The retention of tetracycline was strongly dependent on the concentration of organic modifier in mobile phase. Fig. 4 gives the capacity factor, $k'$, as a function of the molar concentration of acetonitrile in the mobile phase, [$CH_3CN$]. A minimal value of the capacity factor was observed on the LiChrosorb RP-8 and RP-18 supports within the range 0.9 <

TABLE I

RETENTION OF SOME TETRACYCLINES

Support: LiChrosorb RP-2. Mobile phase: 2.9 *M* (15%) acetonitrile in 0.01 *M* phosphoric acid.

| *Compound* | *Capacity factor, k'* |
|---|---|
| Tetracycline | 2.85 |
| Chlortetracycline | 6.62 |
| Demeclomycine | 4.08 |
| Doxycycline | 9.71 |
| Metacycline | 8.95 |
| Oxytetracycline | 1.97 |

log [$CH_3CN$] < 1.1. It is likely that the retention behaviour using the LiChrosorb RP-2 support is similar, but the capacity factors within the range 0.8 < log [$CH_3CN$] < 1.1 were too low to be determined with sufficient precision. It should be noted that the capacity factors at log [$CH_3CN$] = 0.76 and log [$CH_3CN$] = 1.23 are almost identical.

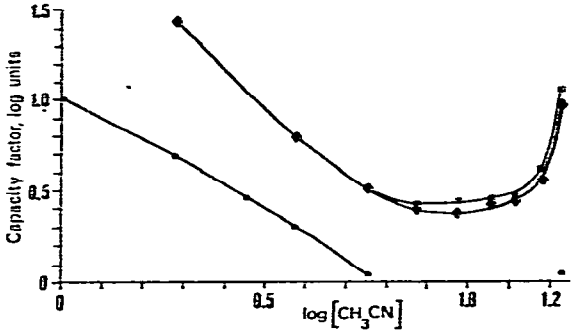


Fig. 4. Concentration of acetonitrile in mobile phase and retention of tetracycline. Support: ●, LiChrosorb RP-2; ◆, LiChrosorb RP-8; ■, LiChrosorb RP-18. Mobile phase: acetonitrile in 0.01 *M* phosphoric acid. Solute: 1 μg of tetracycline in 100 μl of 0.1 *M* phosphoric acid.

The unusual retention behaviour of tetracycline, as well as those of some other drugs with a tetracycline structure[22], may be explained by the formation of a drug adduct containing water and organic modifier in combination with adsorption on the support and/or distribution into a stationary phase. It was possible to obtain a sufficiently low capacity factor for an optimal separation speed[23] only by the use of the LiChrosorb RP-2 support.

Analysis of tetracycline in urine was performed by direct injection of the untreated samples. No detoriation of the chromatographic column was observed using this technique. Injection of untreated plasma samples gave rise to a continuously decreasing retention of tetracycline.

*Detection selectivity and sensitivity.* The adsorption spectrum of tetracycline

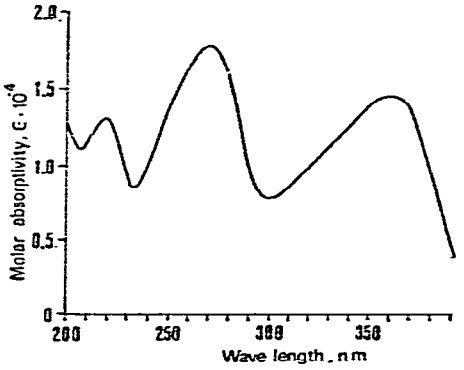


Fig. 5. Absorption spectrum of tetracycline. Tetracycline, $4 \cdot 10^{-5}$ *M*, dissolved in 3.8 *M* (20%) acetonitrile in 0.01 *M* phosphoric acid.

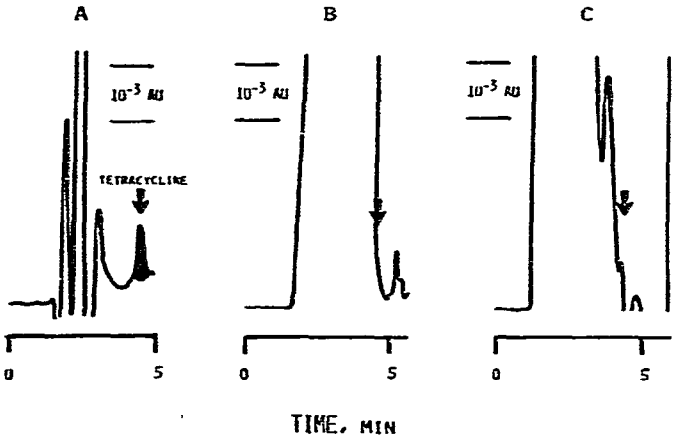


Fig. 6. Detection selectivity. Blank plasma samples analysed according to the proposed analytical method, with chromatographic detection at (A) 357 nm, (B) 270 nm and (C) 220 nm.

shows several maxima (Fig. 5). A comparison of blank plasma chromatogram runs at 220, 270 and 357 nm shows that the highest detection selectivity is obtained at 357 nm (Fig. 6) with a highly separated chromatographic peak of tetracycline. The high detection selectivity at 357 nm allows frequent sample injections as no chromatographic peaks with a capacity factor higher than that for tetracycline appeared. The detection selectivity at 357 nm is sufficient for the analysis of urine samples with the extraction procedure being omitted. The high molar absorptivity of tetracycline at 357 nm permits the detection of less than 1 ng of the drug per injected sample.

*Quantification.* The quantitative determination of tetracycline is based on peak area measurement:

$$M = Yub\varepsilon^{-1} \quad (1)$$

where $M$ = amount of sample (mmole), $Y$ = peak area ($mm^2$), $u$ = chart paper speed (ml/min), $b$ = absorbance per mm chart paper and $\varepsilon$ = molar absorptivity of the migrating compound[20]. The molar absorptivity of tetracycline was $1.47 \cdot 10^4$ l $mol^{-1}cm^{-1}$ at 357 nm.

TABLE II

RECOVERY AND PRECISION

| *Biological sample* | *Drug level (μg/ml)* | *Recovery (%)** |
|---|---|---|
| Plasma | 0.20 | 60.2 ± 12.5 |
| Plasma | 200.0 | 64.9 ± 10.0 |
| Urine | 4.00 | 83.1 ± 8.7 |
| Urine | 20.0 | 92.1 ± 3.2 |
| Urine | 200.0 | 95.7 ± 2.8 |

* Recovery ± relative standard deviation ($n$ = 10).

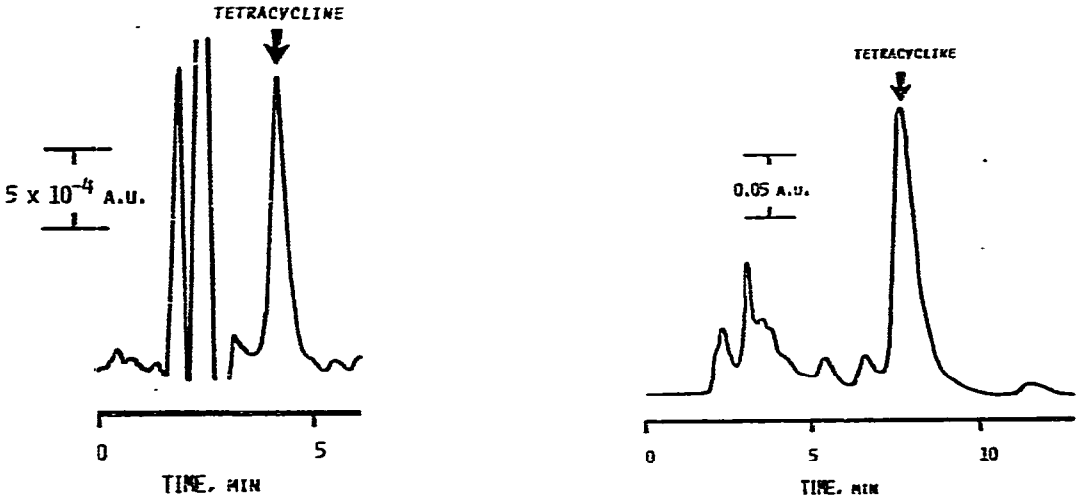


Fig. 7. Chromatogram of a plasma sample from a patient treated with tetracycline. Found concentration: 520 ng/ml plasma. Plasma sample taken 1 h after administration of 250 mg of tetracycline.

Fig. 8. Chromatogram of a urine sample from a patient treated with tetracycline. Found concentration: 105 μg/ml urine. Urine sample taken 14 h after administration of 250 mg of tetracycline.

*Recovery and precision*

The recovery and precision of the proposed analytical methods are presented in Table II.

*Plasma and urine samples from patients*

Chromatograms of plasma and urine from a patient treated with tetracycline are shown in Figs. 7 and 8. The plasma levels after oral administration of tetracycline (3.6 mg/kg body weight) are presented in Fig. 9. The urinary excretion of tetracycline from the same patient is illustrated in Fig. 10.

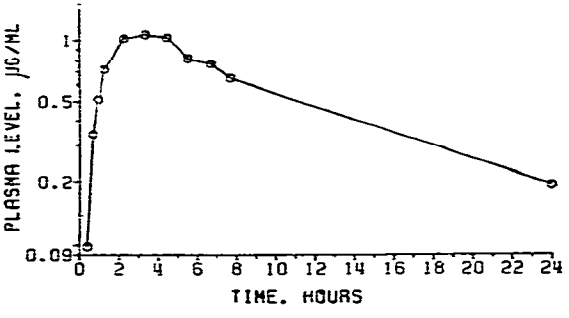


Fig. 9. Plasma levels of tetracycline after oral administration. Administered dose: 3.6 mg/kg body weight.

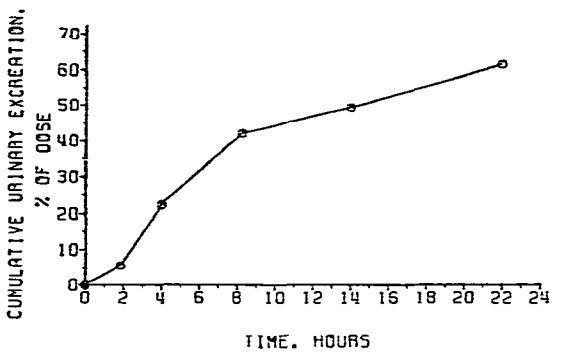


**Fig. 10. Urinary excretion of tetracycline after oral administration. Administered dose: 3.6 mg/kg body weight.**

## ACKNOWLEDGEMENTS

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## REFERENCES

1 D. C. Grove and W. A. Randall, *Assay Methods for Antibiotics*, Medical Encyclopedia Inc., New York, 1955.
2 K. W. Kohn, *Anal. Chem.*, 33 (1961) 862.
3 H. Poiger and C. Schlatter, *Analyst (London)*, 101 (1976) 808.
4 K. Tsuji and R. H. Robertson, *J. Pharm. Sci.*, 65 (1976) 400.
5 G. D. Mack and R. B. Ashworth, *J. Chromatogr. Sci.*, 16 (1978) 93.
6 R. F. Lindauer, D. M. Cohen and K. P. Munnelly, *Anal. Chem.*, 48 (1976) 1731.
7 S. L. Ali and T. Strittmatter, *Int. J. Pharm.*, 1 (1978) 185.
8 J. P. Sharma, E. G. Perkins and R. F. Bevill, *J. Chromatogr.*, 134 (1977) 441.
9 J. P. Sharma, G. D. Koritz, E. G. Perkins and R. F. Bevill, *J. Pharm. Sci.*, 66 (1977) 1319.
10 E. R. White, M. A. Carroll, J. E. Zarembo and A. D. Bender, *J. Antibiot.*, 28 (1975) 205.
11 I. Nilsson-Ehle, T. T. Yoshikawa, M. C. Schotz and L. B. Guze, *Antimicrob. Ag. Chemother.*, 9 (1976) 754.
12 E. R. White, M. A. Carroll and J. E. Zarembo, *J. Antibiot.*, 30 (1977) 811.
13 J. H. Knox and J. Jurand, *J. Chromatogr.*, 110 (1975) 103.
14 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 142 (1977) 213.
15 J. L. Colaizzi and P. R. Klink, *J. Pharm. Sci.*, 58 (1969) 1184.
16 H. Terada and T. Inagi, *Chem. Pharm. Bull.*, 23 (1975) 1960.
17 C. R. Stephens, K. Murai, K. J. Brunings and R. B. Woodward, *J. Amer. Chem. Soc.*, 78 (1956) 4155.
18 N. E. Rigler, S. P. Bag, D. E. Leyden, J. L. Sudmeier and C. N. Reilley, *Anal. Chem.*, 37 (1965) 872.
19 J. B. Bogardus and R. K. Blackwood, Jr., *J. Pharm. Sci.*, 68 (1978) 188.
20 S. Eksborg and G. Schill, *Anal. Chem.*, 45 (1973) 2092.
21 A. White, P. Handler and E. L. Smith, *Principles of Biochemistry*, McGraw-Hill, New York, 1964.
22 S. Eksborg, *J. Chromatogr.*, 149 (1978) 225.
23 B. L. Karger, in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, p. 3.