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DETERMINATION OF CLONIDINE IN HUMAN PLASMA BY GLASS CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas chromatographic assay for clonidine in human plasma has been developed. The buffered serum is extracted on silica columns, alkylated with pentafluorobenzyl bromide, cleaned up-by extractions and analysed by glass capillary gas chromatography with falling-glass-needle injection and electron-capture detection. A gas chromatographic–mass spectrometric analysis of the derivatives formed and an investigation of the structure dependence of the electron-capture response are presented.

INTRODUCTION

Clonidine, 2-(2,6-dichlorophenylamino)-2-imidazole, is a potent antihypertensive agent, and its concentration in the plasma of humans after oral or intravenous administration of a therapeutic dose (75–150 μg) is in the range of ng/ml to pg/ml.

Clonidine has been determined in serum by radioactive labelling[1], gas chromatography–mass spectrometry (GC–MS)[2], GC[3] and radio-immunoassay[4]. The aim of this work was to modify our original GC method (for small animal samples) to analyse large samples and so to improve the chromatographic system that the practical detection limit could be decreased to the lower picogram range.

EXPERIMENTAL

The internal standard 2-(2,4-dichlorophenylamino)-2-imidazoline was synthesized as previously described[3] and dissolved in distilled water to give a final concentration of 150 or 50 ng/ml.

Clonidine and related compounds were generously supplied by Boehringer (Ingelheim/Rhein, G.F.R.).

Pentafluorobenzyl bromide (PFB bromide) and Dri-film were purchased from Pierce (Rockford, Ill., U.S.A.). All chemicals used were of analytical-grade quality and were purified as follows. Potassium carbonate was dried in an oven at 120°

for 2 h. Acetone was refluxed with phosphorus pentoxide and distilled. Cyclohexane and butanol were distilled.

Extraction of plasma samples was performed with columns of silica (Extrelut®; Merck, Darmstadt, G.F.R.). All extractions were performed in silanized centrifuge tubes of capacity 50 or 10 ml. Micro test-tubes (3 ml) were used in the final step of the procedure. The micro test-tubes were agitated three times with methanol in an ultrasonic bath before silanization; silanization was done with 2% Dri-film solution in cyclohexane, followed by three washes with methanol and drying at ambient temperature.

Instruments

A Hewlett-Packard gas chromatograph was used; it was equipped with a ^{63}Ni electron-capture detector (ECD) operating in the constant current–varying pulse-frequency mode. A glass sleeve injection port was used with packed columns. The injection port was enlarged to take a Packard glass solid injector with a falling needle. Argon, with 5% methane as make-up gas for the detector, was connected with a tee-piece and glass-lined tubing. A platinum–iridium capillary (15 × 0.3 mm) was fitted into the glass capillary on the injection side and sealed by melting. A Packard gas chromatograph, with associated equipment similar to that described above, was also used, but with nitrogen as make-up gas.

Columns

The packed column (2 m × 3 mm I.D.) was of silanized glass and was packed with 3% of OV-17 on 80–100-mesh Gas-Chrom Q, a stock packing from Applied Science Labs. (State College, Pa., U.S.A.). All capillary columns were 25 m long. Glass capillary columns from different producers were tested, and one was coated in our laboratory. The following columns were used: SE-30, OV-17 and SP-1000) (0.35 mm I.D.) from LKB (Bromma, Sweden); SE-30 and OV-17 (0.25 mm I.D. from Packard instruments; OV-17 and SP-2340 (0.3 mm I.D.) Ultrasep® columns (Separation Research, Helsinki, Finland); SP-1000 (0.25 mm I.D.) prepared from borosilicate glass deactivated by the barium carbonate method according to Grob[5].

Procedure

Direct extraction. Plasma (5 ml), 0.2 ml of internal standard solution and 0.1 ml of 13 *M* ammonia are mixed, then extracted twice with 10 ml cyclohexane–butanol (9:1); the organic phase is further treated as described in "*General procedure*".

Column extraction on silica. A mixture of 5 ml of plasma, 0.2 ml of internal standard solution and 15 ml of 0.02 *M* ammonia is poured into an Extrelut column; after 10 min, elution is carried out with cyclohexane–butanol (9:1) until 30 ml have been collected, and the organic phase is further treated as described in *General procedure*, except that the 3-ml washing step can be omitted.

General procedure. The organic phase is collected in a 50-ml centrifuge tube and extracted with 1 ml 0.1 *M* sulphuric acid; the organic phase is separated by centrifugation and discarded. The aqueous phase is washed one with 3 ml of cyclohexane–butanol, then made alkaline with 0.1 ml of 13 *M* ammonia and extracted with 10 ml of cyclohexane–butanol. The organic phase is evaporated in a stream of

nitrogen at 60° in a heating bath, and 1 ml of 1% (v/v) PFB bromide solution in acetone and 5–25 mg of potassium carbonate are added to the residue. The mixture is then refluxed for 45 min in a heating block under an air condenser.

The acetone is evaporated with a gentle stream of nitrogen, and 1 ml of heptane and 1 ml of 1 *M* sulphuric acid are added; after mixing and centrifugation, the organic layer is discarded. The heptane wash is repeated, and the aqueous layer is transferred to another tube, and 0.25 ml 13 *M* ammonia and 3 ml of cyclohexane–butanol are added. The test-tube is shaken and centrifuged, the organic layer is finally evaporated to dryness in a micro test-tube under a stream of nitrogen, 0.1 ml of ethyl acetate is added, and the contents of the tube are thoroughly mixed on a Whirlimixer; 1–3 μl of this mixture is injected into the gas chromatograph. Clonidine concentrations are evaluated by using a standard curve established with use of six standard samples in the concentration range 0.1–1 ng/ml.

RESULTS AND DISCUSSION

Structure of derivatives

Derivatives prepared in mg amounts according to the procedure described above were examined by GC, with a flame ionisation detector (FID). The chromatograms showed a single peak, indicative of the purity of the derivative. As a first attempt to confirm the structure of the derivative, a mass spectrum was recorded on an LKB mass spectrometer with a direct inlet probe; a molecular-ion peak at *m/e* 409 was detected. The mass spectrum was in good agreement with that to be expected from a monoalkylated derivative of clonidine, except that the PFB^+ ion at *m/e* 181 was missing (Fig. 1).

The derivative chromatographed with less tailing than would be expected for a compound containing an iminogroup. Further, the PFB derivative of 2-(2,6-diethylphenylamino)-2-imidazoline gave a very low ECD response Table I).

The mass spectra of the PFB derivatives of clonidine and the internal standard were then recorded with a mass spectrometer coupled to the gas chromatograph. The molecular-ion peak was detected at *m/e* 389, but no peak appeared at *m/e* 409. The most likely explanation of these results is that the PFB derivatives cyclicize to form resonance-stabilized compounds of high stability and ECD response.

A structure for the clonidine derivative is proposed in Fig. 1; the clonidine-related compounds studied reacted in the same manner. Attempts to isolate the non-cyclicized clonidine derivative by extraction or liquid chromatography were not successful. The benzyl derivative of clonidine was prepared in order to study the chromatographic properties of a non-cyclicized compound. However, the derivative peak showed severe tailing and gave low ECD response.

Electron-capture response

The derivatives were prepared in mg amounts according to the procedure described, and were analysed GC–FID analysis. The samples were then diluted and injected into a gas chromatograph with an ECD. The ECD/FID response ratio was calculated, and the ratio of the clonidine derivative was taken as 100% for reference; the values obtained are presented in Table I.

The relationship between structure and ECD response has been studied by

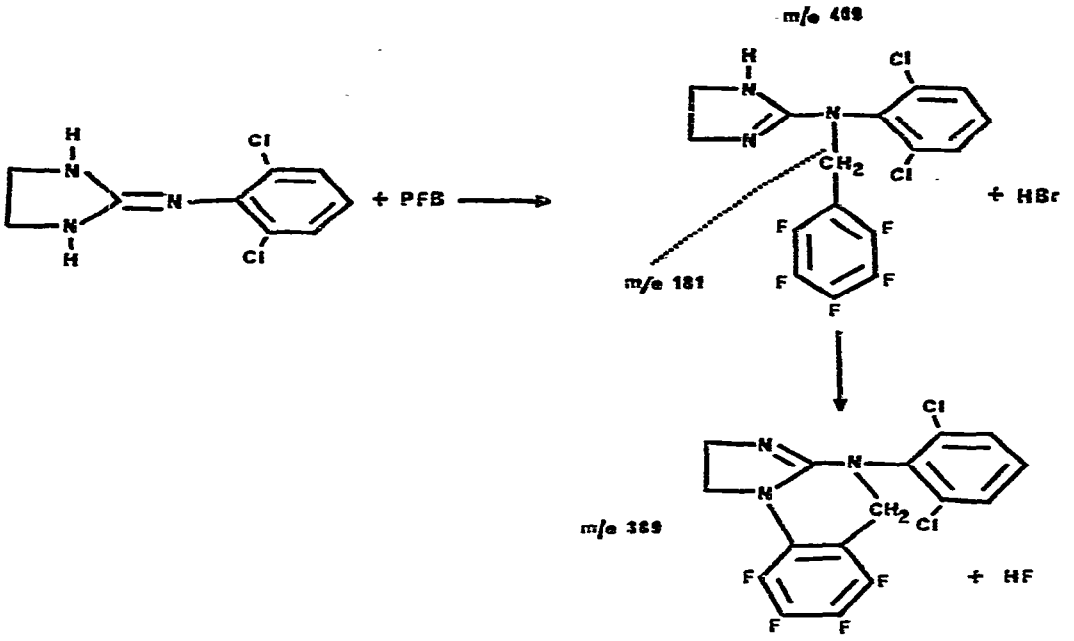

Fig. 1. Alkylation of clonidine with PFB-bromide. Identification of products was by GC–MS.

Zielinski *et al.*[5] for chloro-nitrobenzenes and -anilines. They found that the sensitivity increased, in general, with the number of chlorine atoms; *m*-chloro attachment contributed more than *o*-chloro attachment, and two vicinal chlorine atoms contributed more than when in non-vicinal positions. As can be seen from Table I, the derivatives studied in this work exhibit similar relationships. Replacement of a hydrogen by a chlorine atom increased the ECD response by a factor of seven.

The temperature dependence of the ECD response was determined by making repeated injections of a stock solution of the derivatives at different detector temperatures. The graphs of measured peak area *versus* temperature are presented in Fig. 2. Several fundamental papers have discussed the general response of the ECD, including the theory of mechanisms and temperature dependence[6–13]. An increased response with temperature indicates a dissociative mechanism; the derivatives

TABLE I

ECD/FID AREA-RESPONSE RATIOS FOR CYCLICIZED PFB DERIVATIVES OF SOME PHENYLIMINOIMIDAZOLINES RELATED TO CLONIDINE

Phenyl group substituents	*Retention relative to clonidine on OV-17*	*ECD/FID area (%)*
—	0.66	2
2,6-Ethyl	0.66	2
4-Cl	1.32	13
3,5-Cl	1.97	82
2,4-Cl	1.17	87
2,6-Cl (clonidine)	1.00	100
4-OH, 2,6-di-Cl	6.62	232

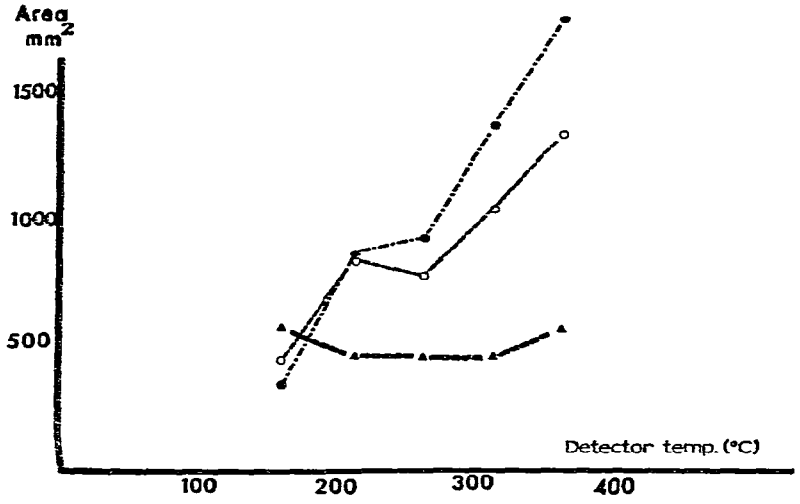

Fig. 2. Graphs of ECD *versus* detector temperature. ●, internal-standard derivative; ○, clonidine derivative. ▲, PFB derivative of theophylline.

of both clonidine and the internal standard exhibit such an increased response (see Fig. 2). The PFB derivative of theophylline was available from previous work[14], and this was also examined as a reference compound.

Thus, for the clonidine derivative and related compounds, it seems probable that the electron is initially captured by the heterocyclic resonance system, which has a high cross-sectional area for collisions and offers the possibility of interaction with free electrons. It is then delocalized to a chlorine atom in the phenyl moiety, with dissociation of the chlorine–carbon bond, following a dissociative electron-capture mechanism. It is of interest to note that the ion for loss of chlorine was the most abundant ion in the mass spectrum. As a consequence, the detector temperature can be used to control the selectivity of the detector for optimal performance.

Extraction

When a large volume of serum was taken for analysis and the direct extraction procedure was used, a precipitate sometimes appeared at the acid re-extraction step. Further, when serum samples stored frozen for 2 years were analyzed, interfering peaks appeared in the chromatograms. Column extraction solved these problems, and clean extracts were obtained, with high recovery (Table II).

TABLE II

ABSOLUTE RECOVERY AND PRECISION

Method	*Clonidine concentration*	*Recovery (%)*	*Precision (% S.D.)*
Batch extraction	1 ng/ml	40–60	3
Packed column	100 pg/ml	40–60	15
Column extraction			
Packed column	100 pg/ml	60–80	13
OV-17 capillary column	100 pg/ml	60–80	5

Protein precipitation with perchloric acid was used for some sets of standards. Precipitation gave clean extracts, but the recovery decreased, and it was not further investigated. Because of the many steps involved, it is a complex problem to establish the absolute optimum conditions for each, as the recovery for a particular step may be affected by how the others are performed; for example, if a very polar solvent is used in the first step, the co-extracted compounds might affect the derivatization and would certainly affect the background in the chromatographic step.

Chromatographic system

The ECD is very sensitive to the clonidine derivative used. The detection limit with pure derivative, a wall-coated open-tubular WCOT) column, coated with OV-17 and a detector in good condition was 0.2 pg. The falling-glass-needle injector proved to be useful for injection of large fractions of the total sample, in combination with capillary columns. However, when large fractions of processed biological samples are injected, small randomly appearing peaks impair the sensitivity. This problem can be partially solved by extensive clean-up of the samples as described above and by the use of a column with high resolution and suitable selectivity.

The packed column used gave about 2500 theoretical plates. To increase the resolution, commercially available capillary columns were tested; the results are shown in Table III. Capillary columns from different producers differed in activity and stability. In general, columns coated with such polar phases as SP-1000 and SP-2340 showed less tailing than non-polar columns. The OV-17 and SP-2340 showed less tailing than non-polar columns. The OV-17 and SP-2340 columns possessed the selectivity to resolve the peaks for clonidine and the internal standard from the background. The Ultrasep® columns were superior to the others tested both with respect to deactivation and stability. The Ultrasep® OV-17 and SP-2340 columns gave 30,000 theoretical plates at the flow-rates used in practical analysis. Chromatograms obtained from plasma samples are presented in Figs. 3–5.

TABLE III

SELECTIVITY AND STABILITY OF COLUMNS TESTED

+ = Good performance; + − = intermediate; − = unsuitable.

Stationary phase	*Selectivity*	*Stability*
SE-30	−+	−
OV-17	+	+−
SP-1000	−	+
SP-2340	+	+

Absolute recovery and precision

The clonidine and internal-standard derivatives were prepared in the mg scale as described under *Procedure*, the final solution being evaporated to dryness and further dried in a vacuum oven. The purity of the derivatives obtained was checked by reversed-phase liquid chromatography and GC. Stock solutions of the derivatives showed only one peak with these methods, and dilutions of these solutions were used as reference in determining absolute recoveries. The recovery and precision values are shown in Table II.

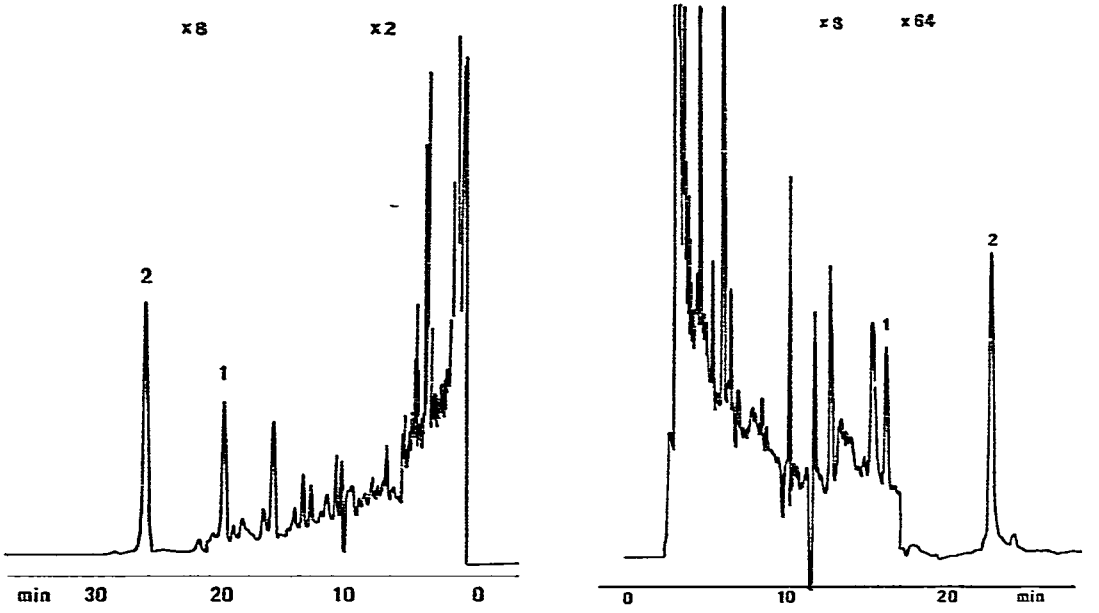

Fig. 3. Chromatogram for a 5-ml serum standard with 200 pg/ml of clonidine (1) and 10 ng of internal standard (2). Injection: 2 μl from 100 μl by falling needle on a 25 m × 0.25 mm WCOT column (Packard). Stationary phase, OV-17; carrier gas, nitrogen (35 cm/sec); injection temperature, 250°; column temperature, 230°; detector temperature, 310°; detector, Packard ECD (^{63}Ni source); make-up gas, nitrogen (30 ml/min).

Fig. 4. Chromatogram for a 5-ml serum standard with 100 pg/ml of clonidine (1) and 30 ng of internal standard (2). Injection: 2 μl from 100 μl by falling needle on a 25 m × 0.30 mm Ultrasep® column (WCOT SP-2340). Carrier gas, helium (50 cm/sec). Other conditions as for Fig. 3, except detector temperature (350°) and make-up gas (5% of methane in argon).

It was possible quantitatively to determine down to 100 pg/ml of clonidine in plasma (the background corresponded to 10 pg/ml with a standard deviation of less than 35%). Scrupulous cleaning of the glassware used is essential if cross-contamination is to be avoided; all glassware is therefore re-silanized after each use to deactivate and clean the surfaces. Ultrasonic agitation with methanol is efficient in removing derivative traces from the glass. Reaction times longer than 45 min with PFB bromide for standard samples (1 ng/ml of clonidine) were investigated to ascertain if such longer times were needed for low concentrations in biological samples; no significant increase in recovery was established.

2-(2,6-Diethylphenylamino)-2-imidazoline was added in μg amounts to see if any "carrier" effect could be observed; there was no difference in recovery, probably because of the carefully silanized glass used. Addition of the diethyl compound gave rise to peaks with long retention times, thereby increasing the interval between injections, and therefore it was not further used. The use of a rather large amount (30 ng) of internal standard was preferred.

The method described has been in use in our laboratory during 2 years with excellent results. A technician can analyse 10 samples a day. A serum profile from a patient after intravenous administration of 200 μg of clonidine is shown in Fig. 6.

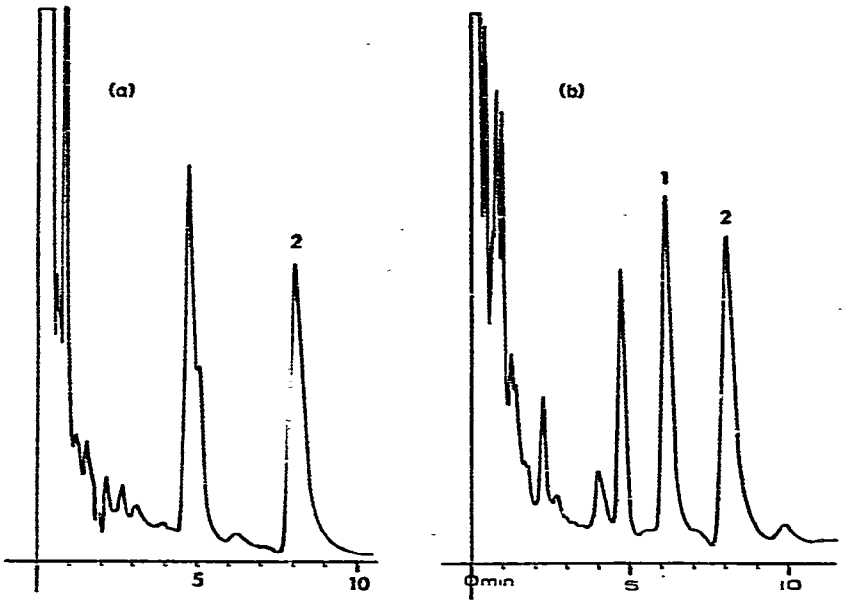

Fig. 5. Chromatogram for a 5-ml serum standard (a) without clonidine but with 10 ng of internal standard (2), and (b) with 1 ng of clonidine (1). Direct injection on a packed column (2 m × 2.2 mm; 3% of OV-17). Carrier gas, 5% of methane in argon (30 ml/min); injection temperature, 240°; column temperature 240°: detector temperature 350°.

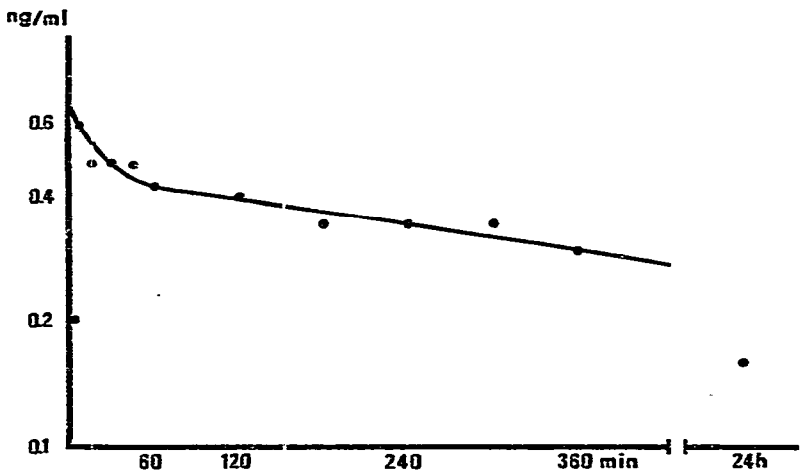

Fig. 6. Serum profile from a patient after a 200-μg intravenous dose of clonidine.

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REFERENCES

1 A. K. Cho and S. H. Curry, *Biochem. Pharmacol.*, 18 (1969) 511–520.
2 C. T. Dollery, D. S. Davies, G. H. Draffan, H. J. Dargie, C. R. Dean, J. L. Reid, R. A. Clare and S. Murray, *Clin. Pharmacol. Ther.*, 19 (1976) 11–17.
3 P. O. Edlund and L. K. Paalzow, *Acta Pharmacol. Toxicol.*, 40 (1977) 145–152.
4 D. Arndts, B. Stähle and C. J. Struck, *Arzneim.-Forsch.*, 3 (1979) 532–538.
5 W. L. Zielinski, Jr., L. Fishbein and R. O. Thomas, *J. Chromatogr.*, 30 (1967) 77–85.
6 E. D. Pellizzari, *J. Chromatogr.*, 98 (1974) 323–361.
7 S. B. Martin and M. Rowland, *J. Pharm. Sci.*, 61 (1972) 1235–1240.
8 W. L. Zielinski, Jr. and L. Fishbein, *J. Chromatogr.*, 28 (1967) 293–299.
9 P. Devaux and G. Guiochon, *J. Chromatogr. Sci.*, 8 (1970) 502–508.
10 B. C. Pettit, P. G. Simmonds and A. Zlatkis, *J. Chromatogr. Sci.*, 7 (1969) 645–650.
11 A. Zlatkis and B. C. Pettit, *Chromatographia*, 2 (1969) 484–492.
12 W. E. Wentworth and E. Chen, *J. Gas Chromatogr.*, 5 (1967) 170–179.
13 W. L. Zielinski, L. Fishbein and L. Martin, *J. Gas Chromatogr.*, 5 (1967) 552–655.
14 A. Arbin and P. O. Edlund, *Acta Pharm. Suecica*, 12 (1975) 119–126.