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DETERMINATION BY COUPLED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–GAS CHROMATOGRAPHY OF THE β-BLOCKER LEVOMOPROLOL IN PLASMA FOLLOWING OPHTHALMIC ADMINISTRATION

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SUMMARY

Levomoprolol is a β-blocking agent used in the topical treatment of glaucoma. The necessity for comparing the plasma levels of a drug administered by the ophthalmic route with those obtained following systemic treatment requires increasingly sensitive methods in order to determine the low plasma concentrations produced by the administration of eye drops. On-line high-performance liquid chromatography–gas chromatography and concurrent solvent evaporation proved to be advantageous in the determination of levomoprolol in human plasma. Levomoprolol was determined by capillary gas chromatography (GC) with electron-capture detection (ECD) after solid-phase extraction from plasma and derivatization. Quantitation was based on the internal standard method. The detection limit of 0.2 ng/ml is 50 times lower than that obtained with previous GC methods involving on-column injection and ECD.

INTRODUCTION

In trace analysis, detection limits are commonly restricted by the level of simultaneously eluted interfering material and/or non-volatile materials in the sample matrix. Hence, detection limits depend primarily on the efficiency of the sample clean-up. Optimum sample preparation is based on few, but highly efficient steps. High-performance liquid chromatography (HPLC) is probably the most efficient method for isolating trace components of interest from a complex material. Direct coupling of HPLC to gas chromatography (GC) simplifies the procedure and renders it rapidly. Levomoprolol or 1-(2-methoxyphenoxy)-3-isopropylamino-2-propanol is a β-blocking agent[1] in use for a long time by the oral route in antihypertensive therapy[2]. Recently, it proved to be successful in the topical treatment of glaucoma[3–5]. A previous method[6] used for the determination of levomoprolol in plasma and urine of healthy volunteers after oral treatment provided a detection limit of 10 ng/ml.

Determination of the drug in human plasma after topical administration, in particular by the ocular route, requires analytical methods with very low detection limits. Its purpose is to assess the drug absorption and to carry out bioavailability

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studies in comparison with a systemic reference dosage form to evaluate toxicological aspects.

This paper reports the results obtained by an on-line HPLC–GC method and a concurrent solvent evaporation technique[7–13]. This method permits the detection limit to be lowered to 0.2 ng/ml, allowing the determination of levomoprolol in the plasma of subjects treated by the ocular route. This approach also enables the extent of absorption to be compared with that of the systemic reference dosage form (oral route).

EXPERIMENTAL

Materials and reagents

Levomoprolol hydrochloride and 1-(3-chloroisoxazol-5-yl)-2-(*tert.*-butylamino)ethanol (used as an internal standard) were supplied by Zambon Group (Milan, Italy). All solvents and reagents were purchased from Merck (Darmstadt, F.R.G.) and were of analytical-reagent grade, except the derivatizing agent, trifluoroacetic anhydride, obtained from Supelco (Bellefonte, PA, U.S.A.).

Bond-Elut CN columns (1-ml capacity) were purchased from Analytichem International (Harbor City, CA, U.S.A.) and a solid-phase extraction (SPE) vacuum manifold from Supelco. The centrifuge was a Sorvall RT 6000 B supplied by DuPont Instrument Systems (Wilmington, DE, U.S.A.). *n*-Pentane and diethyl ether were distilled before use.

The HPLC system consisted of a Jasco (Tokyo, Japan) Model BIP-1 pump, a Jasco Uvidec 100-V UV detector operating at 223 nm on-line with the pump and the HPLC–GC interface and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 561 recorder.

The gas chromatograph was a 5300 Mega series from Carlo Erba (Milan, Italy), equipped with a Model ECD 400 ^{63}Ni electron-capture detector. The output of the detector was recorded on a Spectra-Physics (San Jose, CA, U.S.A.) Model 4270 integrator.

The interface between the HPLC and GC instruments, not commercially available, was assembled in our laboratories, using a Porter Instrument (Hatfield, PA, U.S.A.) Model VCD 1000 A-10 flow regulator and a Valco (Houston, TX, U.S.A.) Model C-6-W six-port switching valve, equipped with a Supelco 500-μl sample loop. When the switching valve was in the load position, the HPLC eluent from the UV detector passed through the sample loop. The switching valve was then turned to transfer position when the HPLC fraction was fully eluted in the sample loop, *i.e.*, at the end of the fraction as observed on the liquid chromatogram recorder. At this time the carrier gas was introduced into the sample loop behind the plug of liquid and pushed it into the capillary precolumn. The vapour pressure in front of the liquid plug counterbalanced the pressure of the carrier gas behind the plug, allowing the liquid plug to evaporate within the section of the precolumn inlet. Evaporated liquid was automatically replaced, as the plug was moved forward, by the carrier gas, up to completion of solvent evaporation.

Sample preparation

Sample preparation was performed with a Bond-Elut CN column containing silica modified with bound cyanopropyl groups. It was attached to the Luer fittings

over the vacuum manifold cover, having the capacity for twelve columns. The column was conditioned by rinsing with one volume of dichloromethane, and successively with one volume of methanol and one volume of carbonate buffer (pH 10), taking care to avoid drying of the column. To 1 ml of plasma were added 1 ng of internal standard (10 μl of a 0.1 μg/ml aqueous solution) and 0.5 ml of the carbonate buffer. The sample was applied to the activated Bond-Elut CN column, which was then washed with two volumes of carbonate buffer. The Bond-Elut CN column was centrifuged at 4000 rpm for about 15 s, then transferred to a test-tube and eluted with two volumes of dichloromethane by centrifugation at 1000 rpm for 3 min. The organic phase was evaporated to dryness under a gentle stream of nitrogen at room temperature, then 1 ml of diethyl ether and 0.25 ml of trifluoroacetic anhydride were added and allowed to react at room temperature for 45 min. Final evaporation under a stream of nitrogen afforded the residue for the chromatographic analysis. The residue was dissolved in 0.5 ml of HPLC eluent [*n*-pentane–diethyl ether (55:45)] and 100 μl were injected into the HPLC system.

Chromatography

The HPLC column was a Hibar LiChrosorb CN (5-μm) column (250 mm × 4 mm I.D.) (Merck). The HPLC pre-separation was carried out with *n*-pentane–diethyl ether (55:45) as eluent at a flow-rate of 1 ml/min. The column was kept at room temperature.

The column performance and eluent flow-rate were carefully checked by periodic injections of an amount of sample and internal standard that could be revealed by the UV detector (see also Fig. 1, right). Further, the HPLC eluent was kept under a slight pressure of nitrogen to avoid the formation of bubbles, which could change the HPLC retention time. Under the conditions described, levomoprolol and the internal standard were eluted at approximately the same retention time. The HPLC fraction corresponding to the levomoprolol was then transferred to the gas chromatograph by means of the HPLC–GC interface.

For GC analysis a Permabond SE-54 fused-silica column (25 m × 0.32 mm I.D.) supplied by Macherey-Nagel & Co. (Düren, F.R.G.) was used. A fused-silica capillary precolumn (2.5 m × 0.32 mm I.D.) silylated with diphenyltetramethyldisilazane was coupled to the separation column by means of a press-fit connection. Hydrogen was used as the carrier gas at a flow-rate of 3 ml/min and an inlet pressure of 300 kPa behind the flow regulator. Nitrogen was used as make-up gas for electron-capture detection (ECD) at a flow-rate of 30 ml/min. Eluent introduction occurred at 79°C. The column temperature was programmed linearly at 10°C/min to 200°C and subsequently at 40°C/min to 250°C. It was then kept constant at 250°C for 3 min. Under the conditions described, 300 analyses can be done before loss of efficiency.

Calibration

A calibration graph covering the range from 0.2 to 5 ng/ml was obtained as follows. Into each of the seven human plasma samples (1 ml) a constant amount of internal standard (1 ng) was added by means of a microsyringe, together with increasing amounts of levomoprolol (0.2, 0.5, 0.8, 1, 2, 3 and 5 ng).

Extraction was performed as described under *Sample preparation*. Blank plasma was also extracted to ensure that no interfering peaks were present. The cali-

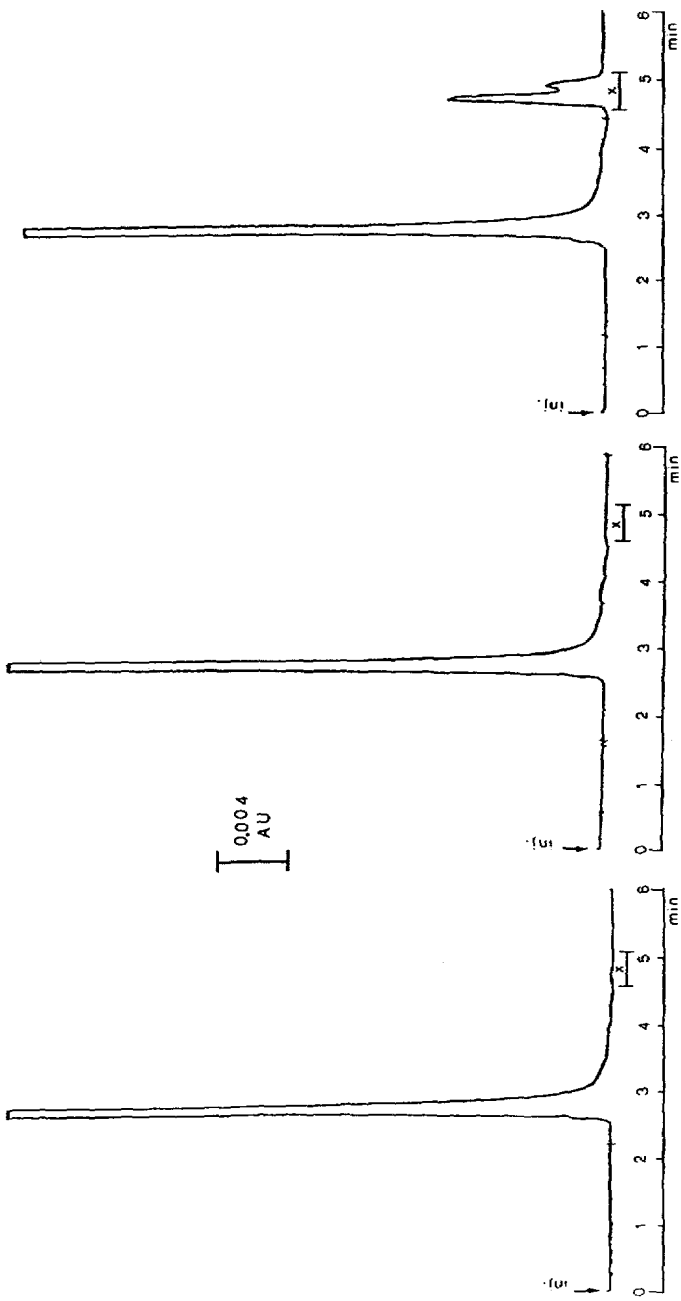

Fig. 1. Typical liquid chromatograms of (left) blank human plasma, (middle) blank human plasma spiked with 1 ng/ml of levomoprolol and 1 ng/ml of internal standard and (right) blank human plasma, containing 0.1 μg/ml of levomoprolol and 0.1 μg/ml of internal standard, to provide evidence for the transfer of the cut to the GC system (marked ×).

bration graph was plotted with levomoprolol concentration on the abscissa and the ratio of the levomoprolol and internal standard peak heights on the ordinate.

RESULTS

In Fig. 1, liquid chromatograms are presented for blank human plasma and for blank human plasma to which 1 ng/ml of levomoprolol and 1 ng/ml of internal standard had been added. Under these conditions, 1 ng/ml of levomoprolol cannot be detected on the chromatogram. Fig. 1 also shows the liquid chromatogram of a blank human plasma to which 0.1 μg/ml of levomoprolol and 0.1 μg/ml of internal standard had been added. This chromatogram shows that only a very large amount of sample (in this instance 100 times higher than 0.1 ng/ml) can be detected by UV spectrophotometry, indicating the liquid fraction that must be transferred to the GC system.

The gas chromatograms of three samples transferred from the HPLC system to the gas chromatograph are shown in Figs. 2, 3 and 4. They show that levomoprolol and the internal standard are eluted from the GC column without significant in-

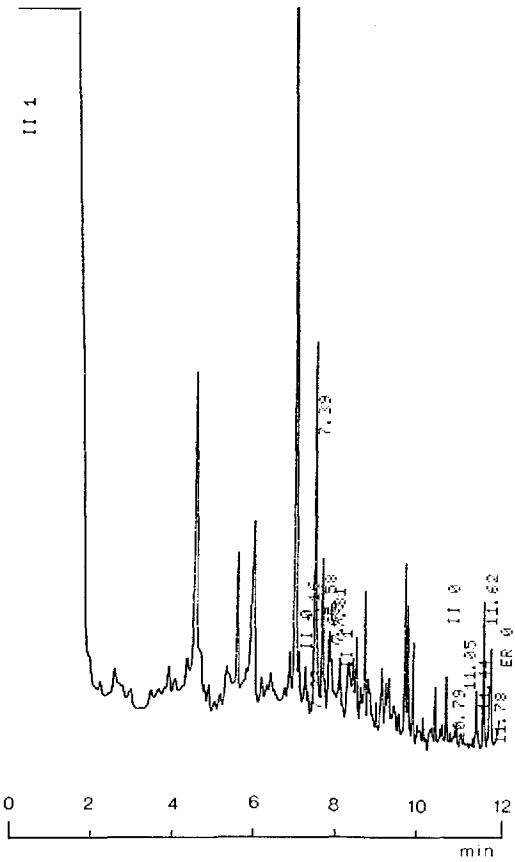

Fig. 2. Typical gas chromatogram of blank human plasma.

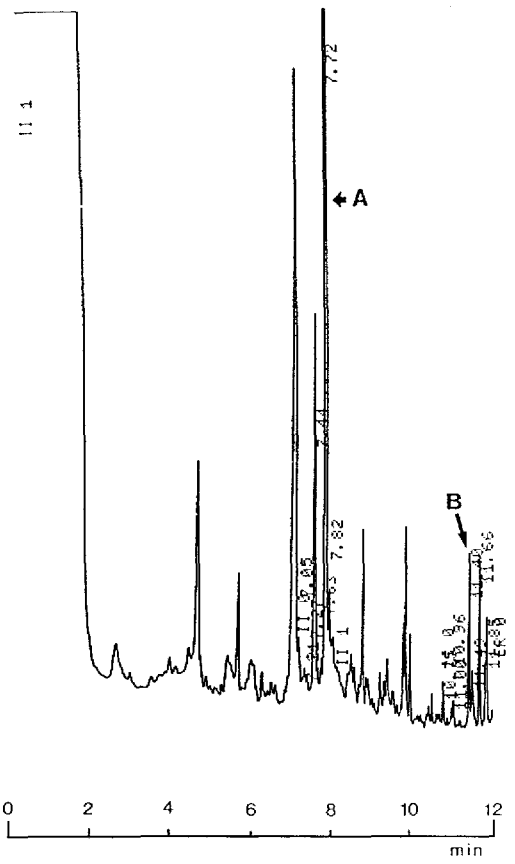

Fig. 3. Typical gas chromatogram of blank human plasma spiked with (B) 1 ng/ml of levomoprolol and (A) 1 ng/ml of internal standard.

terference (Fig. 2); the gas chromatogram of a blank plasma to which 1 ng/ml of levomoprolol and 1 ng/ml of internal standard had been added (Fig. 3) and that of a blank plasma spiked with 0.2 ng/ml of levomoprolol and 1 ng/ml of internal standard (Fig. 4) indicate a detection limit below 0.2 ng/ml.

The calibration graph was obtained as described under Experimental. Each concentration was determined at least in triplicate. The calibration graph was linear in the range 0.2–5.0 ng/ml. Linear regression gave the equation $y = 0.145x - 0.003$ and a correlation factor of $r^2 = 0.995$.

The results for precision and accuracy evaluation are given in Table I. They were obtained by determining seven different concentrations of levomoprolol in 1 ml of human plasma, ranging from 0.2 to 5 ng/ml. This analysis was repeated three times on 0.8, 1 and 2 ng/ml samples and six times on 0.2, 0.5, 3 and 5 ng/ml samples. The results indicate a recovery ranging from 92.7 to 113.8% and a relative standard deviation of 15.7% for a 0.2 ng/ml concentration. These values were obtained by comparing the amount of levomoprolol recovered with the known amount added to blank human plasma (see Table I).

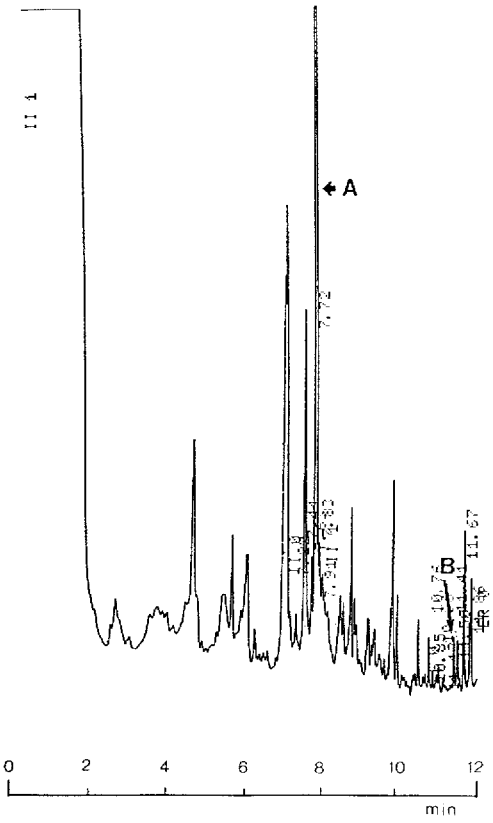

Fig. 4. Typical gas chromatogram of blank human plasma containing (B) 0.2 ng/ml of levomoprolol and (A) 1 ng/ml of internal standard.

TABLE I

ACCURACY AND PRECISION OF LEVOMOPROLOL DETERMINATION

Levomoprolol added to plasma (ng/ml)	*Recovery*		*C.V. (%)*
	ng/ml	*%*	
0.2	0.20 (n = 6)	100.0	15.7
0.5	0.53 (n = 6)	106.0	17.5
0.8	0.91 (n = 3)	113.8	16.1
1	0.98 (n = 3)	98.3	20.8
2	1.95 (n = 3)	97.5	18.5
3	2.78 (n = 6)	92.7	19.4
5	5.12 (n = 6)	102.4	13.8

TABLE II

INDIVIDUAL AND MEAN (± S.E.M.) LEVOMOPROLOL PLASMA CONCENTRATIONS FOLLOWING OCULAR AND ORAL TREATMENT

Administered dose: ocular, 540 μg; oral, 75 mg.

Subject	*Levomoprolol (ng/ml)*													
	Oral administration							*Ophthalmic administration*						
	Time (min)							*Time (min)*						
	30	*60*	*120*	*180*	*240*	*360*	*480*	*30*	*60*	*120*	*180*	*240*	*360*	*480*
1	362.3	350.0	252.7	125.4	107.8	72.1	36.8	0.50	0.70	0.80	0.57	0.52	0.31	0.21
2	314.4	382.7	441.8	281.5	263.0	141.8	92.3	2.3	3.1	1.5	1.4	0.97	0.53	0.32
3	115.8	130.8	102.1	57.9	49.6	30.3	18.5	1.2	1.7	1.1	0.85	0.50	0.36	0.20
4	299.2	360.9	400.3	265.5	201.3	105.1	39.9	2.9	3.5	1.6	1.1	0.93	0.58	0.37
5	260.5	355.5	341.5	211.9	210.1	97.8	41.2	2.0	2.9	1.8	1.5	1.1	0.63	0.35
6	160.0	192.4	180.2	100.0	75.6	46.5	23.5	1.5	1.9	1.6	0.93	0.62	0.87	n.d.
Mean ± S.E.M.	252.0 ± 38.9	295.4 ± 43.3	286.4 ± 53.7	173.7 ± 37.7	151.2 ± 34.8	82.3 ± 16.7	42.0 ± 10.7	1.7 ± 0.35	2.3 ± 0.43	1.4 ± 0.15	1.1 ± 0.14	0.78 ± 0.1	0.45 ± 0.06	0.24 ± 0.05

Pharmacokinetics of levomoprolol

The method was used for the evaluation of the plasma concentrations of levomoprolol after ocular administration. It allowed a comparison of the results with those obtained for the same subjects after oral administration. The study was carried out on six healthy volunteers (four males and two females) aged 28–51 (37 ± 8) years and weighing 48–90 (72 ± 15.6) kg. Each subject was apparently healthy, in particular as far as the renal, hepatic and gastrointestinal functions are concerned. A "cross-over" experimental design was adopted. Each subject received the two preparations (0.9% levomoprolol eye drops, 1 drop into each eye = 540 μg of levomoprolol, and Levotensin tablet = 75 mg of levomoprolol, 7.5 μg/kg body weight and 1.04 mg/kg body weight, respectively), in two treatment sessions, carried out 2 weeks apart, randomizing the order of administration. During each of the two sessions, basal samples of venous blood were drawn from each fasting subject prior to administration of the eye drops or the tablet. Further blood samples were also collected 30, 60, 120, 180, 240, 360 and 480 min after treatment. The plasma samples obtained after centrifugation were analysed as described under Experimental. The individual and mean (± S.E.M.) values of levomoprolol plasma concentrations obtained at the different sampling times for the two treatments are given in Table II.

The following pharmacokinetic parameters were evaluated: the plasma concentration peak (C_{max}), the time at which the plasma concentration peak was reached (T_{max}), the half-life of the elimination phase ($T_{1/2}$) and the extrapolated area under the concentration–time curve from time zero to infinity (AUC_{tot}). C_{max} reached 2.3 ng/ml at 70 min after ocular administration and 313.9 ng/ml at 75 min following oral administration. $T_{1/2}$ was 150.8 min after ocular administration and 145.1 min following oral treatment. The AUC_{tot} values were 545.8 and 83 750.8 ng · min/ml after ocular and oral administration, respectively. The extent of absorption after ocular treatment was thus 90.5% of the absorption after oral administration, calculated by comparison of the total AUC values and taking into account the ratio of the administered doses.

DISCUSSION

The results confirm that coupled HPLC–GC is a versatile method, overcoming problems due to inadequate detection limits of methods involving GC alone. The method proved to be very useful for preliminary clean-up of samples and for trace enrichment, allowing large volumes to be injected into the capillary GC column. When analysing tissues and biological fluids, detection limits are primarily constrained by interfering sample components. The possibility of reducing the detection limits renders coupled HPLC–GC very suitable for pharmacokinetic and metabolic studies. The on-line HPLC GC technique proved to be essential for the pharmacokinetic study of levomoprolol after ocular administration. In fact, it allowed the determination of plasma concentrations in humans following ocular administration and comparison with those obtained after oral administration of the tablets used as a reference dosage form.

The results obtained from the pharmacokinetic study indicate that levomoprolol is very well absorbed through the cornea after ocular administration, as demonstrated by the ratio of the total AUC values compared with the administered dose.

However, the plasma concentration peak after ocular administration is more than 100 times lower than that obtained following oral treatment. In spite of the large extent of absorption, the plasma levels are not so high as to compel us to perform systemic toxicity studies following ocular administration. Generally, accurate and ultrasensitive methods such as on-line HPLC–GC for the determination of low drug concentrations after ocular treatment permit the establishment of pharmacokinetic *vs.* pharmacodynamic relationships and pharmacokinetic studies comparing the ocular (or generally topical) administration and a reference dosage form for bioavailability, activity and tolerance evaluation purposes. Further, it allows the detection of the drug in the eye and monitoring of pharmacokinetic studies designed to define long-acting formulations or formulations in which drug absorption is not desired.

REFERENCES

1 G. Ferrari, R. Ferrini and C. Casagrande, *Boll. Chim. Farm.*, 107 (1968) 234.
2 P. Ghirardi and F. Grosso, *J. Card. Pharm.*, 2 (1980) 471.
3 M. Virno, J. Pecori-Giraldi and G. Garofalo, *Glaucoma*, 8 (1986) 14.
4 J. Pecori-Giraldi, M. Virno, R. Ralli and A. Planner-Terzaghi, *Fortschr. Ophthalmol.*, 83 (1986) 564.
5 M. Virno, A. Ciarnella, R. Cozza and G. Garofalo, in L. Cappelli (Editor) *Proceedings of the 44th Congress of the Italian Ophthalmological Society, Rome, 1984*, Nuova Casa, Bologna, 1984, p. 647.
6 J. P. Desager, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 129.
7 F. Munari, A. Trisciani, G. Mapelli, S. Trestianu, K. Grob, Jr. and J. M. Colin, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 601.
8 K. Grob, Jr. and B. Schilling, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 726.
9 K. Grob, Jr., H. P. Neukom and R. Etter, *J. Chromatogr.*, 357 (1986) 416.
10 K. Grob, Jr., Ch. Walder and B. Schilling, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 95.
11 H. J. Cortes, B. E. Richter, C. D. Pfeiffer and D. E. Jensen, *J. Chromatogr.*, 349 (1985) 55.
12 H. J. Cortes, C. D. Pfeiffer and B. E. Richter, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 469.
13 K. Grob, Jr. and J. M. Stole, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 518.