Journal of Chromatography, 307 (1984) 295—304
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2043

ISOTHERMAL GAS CHROMATOGRAPHIC ANALYSIS OF DIPHENHYDRAMINE AFTER DIRECT INJECTION ONTO A FUSED-SILICA CAPILLARY COLUMN

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(First received September 9th, 1983; revised manuscript received December 15th, 1983)

SUMMARY

The packed column injector of a gas chromatograph was modified to accommodate direct injection by syringe onto a wide-bore fused-silica capillary column. No changes were made to the nitrogen—phosphorus detector. The resultant configuration combines fast separations with precise quantitations. The analysis of diphenhydramine in serum is presented as an application. Chromatographic separation of diphenhydramine and orphenadrine (internal standard) from caffeine and other endogenous material takes 2 min. Serum diphenhydramine concentrations are presented for six volunteers following a 50-mg oral dose.

INTRODUCTION

Conventional capillary gas chromatographic analyses rely on split, splitless or on-column sample introduction. Each of these injection techniques usually involves cold trapping of the analytes onto the first few centimeters of the capillary column followed by temperature programming to effect their elution.

Grob and Grob, Jr. [1—3] have shown isothermal analyses to be reliable only under the influence of the solvent effect. To be successful, close attention must be paid to the rate of sample injection and the choice of solvent for a

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given stationary phase and oven temperature. The analytes must of course be soluble in the solvent selected.

Direct injection has been used with wide-bore borosilicate glass columns having inside diameters > 0.5 mm. Kits to convert chromatographs from packed column to wide-bore borosilicate column operation are commercially available from chromatography supply houses. Only two reports [4, 5] have described direct injection with fused-silica capillary columns.

Demedts et al. described the combined use of direct injection, a fused-silica capillary column and a nitrogen—phosphorus detector for the analysis of heroin in contraband [4] and for the analysis of codeine and ethylmorphine in biological materials [5]. In both reports, temperature programming was used.

In this report, direct injection onto a fused-silica capillary column is evaluated at isothermal conditions. It is shown to be a rapid means of sample introduction, resulting in fast separations without the need of the solvent effect.

EXPERIMENTAL

Equipment

A Perkin-Elmer Sigma 3 gas chromatograph equipped with nitrogen—phosphorus and flame ionization detectors, a Model R-100 chart recorder (Perkin-Elmer, Norwalk, CT, U.S.A.) were used. Quantitative results from peak areas were automatically obtained with a Model 3390A reporting integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.). A 007 bonded methyl silicone (0.5-μm film) wide-bore fused-silica capillary column, 15 m × 0.45 mm O.D., 0.32 mm I.D. (Quadrex, New Haven, CT, U.S.A.) was used in the analysis.

The injector of the gas chromatograph was modified as shown in Fig. 1. The packed-column injector rear fitting and glass insert were replaced by a 160-mm long borosilicate glass sleeve, 5.5 mm O.D., 0.65 mm I.D. The sleeve was chamfered at the inlet end to facilitate easy insertion of the 26-gauge (0.47-mm) needle of a 5-μl Hamilton 85 N syringe. Prior to mounting, the following deactivation procedure was used. The sleeve was soaked in 10% Decon 75 detergent (BDH Chemicals, Toronto, Canada) for 2 h, then rinsed with water, ethanol and hexane. It was silanized with 20% dimethyldichlorosilane (Pierce, Rockford, IL, U.S.A.) in toluene for 2 h. The sleeve was rinsed

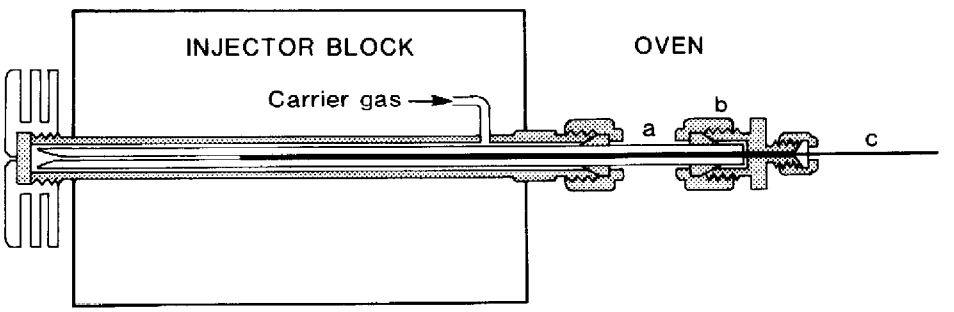

Fig. 1. Modified packed column injector to accommodate wide-bore fused-silica capillary column. (a) Borosilicate glass sleeve, 160 mm × 5.5 mm O.D., 0.65 mm I.D.; (b) 1/4—1/16 in. reducing union; (c) fused-silica capillary column 15 m × 0.45 mm O.D., 0.32 mm I.D.

again with ethanol and hexane, then air dried. A 6.35—1.59 mm (1/4—1/16 in.) stainless-steel Swagelok® zero dead volume reducing union facilitated connection of the fused-silica capillary column to the injector sleeve inside the oven. Vespel-graphite 6.35–1.59 mm (1/4—1/16 in.) reducing ferrules (Supeltex M2A®, Supelco, Bellefonte, PA, U.S.A.) were drilled out to 5.55 mm (7/32 in.). They were used to connect the injector sleeve to the reducing union and to the rear of the injector block.

The column inlet was positioned inside the injector sleeve 3—5 mm from the point of injection (needle tip). Thermogreen septa (Supelco) were used to avoid the addition of contaminant peaks to the chromatogram.

At the detector, the column was held in place by a 6.35—1.59 mm (1/4—1/16 in.) stainless-steel Swagelok reducer mounted onto the 1/4 in. packed-column fitting. The effluent column end was positioned flush with the detector jet tip, 1 mm from the rubidium bromide bead. Surface adsorption within the detector was thus avoided without the need of a make-up gas.

The gas chromatograph was operated isothermally at 180° C, the injector and detector temperatures set at 300° C. Helium carrier gas at 140 kPa (20 psi) provided a linear velocity of 60 cm/sec. Hydrogen and air flow-rates were 3 and 130 ml/min, respectively. The rubidium bromide bead potentiometer was set at 380; the attenuation was 64. Under these conditions, diphenhydramine and orphenadrine eluted at 1.30 and 1.69 min, respectively.

Equipment evaluation

Approximately 100 mg each of diphenhydramine · HCl and orphenadrine · HCl, internal standard (Sigma, St. Louis, MO, U.S.A.) were dissolved in 1 ml of water in a 20-ml extraction tube. The water was made basic with 100 µl of 1 mol/l sodium hydroxide and extracted with 5 ml of HPLC-grade hexane (Fisher Chemical, Winnipeg, Canada). The hexane layers were placed in clean tubes and dried under a stream of dry air. Stock solutions at 100 µg/ml in hexane were prepared from the free bases. Working dilutions of diphenhydramine were prepared at 80, 60, 40, 20, 15, 10, 5 and 1 µg/ml in hexane, each containing orphenadrine at 10 µg/ml. Quantities of 1 µl of these solutions were injected ten times in order to assess the precision, linearity and stability of the instrument.

Serum samples

Stock 100 µg/ml diphenhydramine and orphenadrine standards were prepared by dissolving 22.86 and 22.70 mg of the respective hydrochloride salts in 200 ml of 0.01 mol/l hydrochloric acid. Working orphenadrine internal standard solution, 1000 ng/ml, was a 100-fold aqueous dilution of 1.0 ml of the stock solution. A serum-based diphenhydramine solution (1000 ng/ml) was prepared by diluting 1.0 ml of aqueous stock standard to 100 ml with drug-free serum. Further dilutions were made with drug-free serum to give 50-ml standards of 800, 200, 100, 50 and 10 ng/ml. Aliquots stored at −20° C in 3-ml polypropylene vials were used for precision and recovery studies.

Extraction

New 20-ml Pyrex 16 mm × 150 mm PTFE-capped extraction tubes and 8-ml

Pyrex conical centrifuge tubes were washed, silanized and dried as described above for the custom-made injector sleeve. A 1-ml aliquot of serum was added to an extraction tube with 100 μl of orphenadrine internal standard (1000 ng/ml), 100 μl of 1 mol/l sodium hydroxide and 5 ml of hexane. The tube was gently mixed by inversion (60 rpm) for 15 min, then briefly centrifuged. The hexane layer was transferred to an 8-ml silanized conical centrifuge tube and reduced to about 10 μl under a stream of dry air. The tube was briefly vortexed, then 1 μl was injected onto the gas chromatograph.

Data analysis

Within-day and day-to-day diphenhydramine precision data were determined on 10 and 20 replicates, respectively. Data were obtained for the following serum concentrations: 800, 200, 100, 80 and 10 ng/ml. Recovery experiments at these five concentrations were carried out according to the above extraction protocol except that no internal standard was added to the serum. Rather, 80 ng of orphenadrine in hexane was added to 4.0 ml of the hexane extract just prior to its evaporation. Peak area ratios were compared to peak area ratios of the corresponding free base standards prepared in hexane. The same technique was used to calculate the recovery of orphenadrine at 100 ng/ml by using diphenhydramine as the reference material.

Six healthy volunteers participated in a diphenhydramine pharmacokinetic study. Each person ingested a 50-mg diphenhydramine · HCl capsule (Benadryl®, Warner Lambert/Parke Davis Pharmaceuticals, Ann Arbor, MI, U.S.A.) on an empty stomach. Blood samples were drawn before and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 and 24 h following administration. The blood was centrifuged and the serum collected for analysis of diphenhydramine.

RESULTS AND DISCUSSION

A chromatogram following a 1-μl injection of hexane containing diphenhydramine and orphenadrine at 10 μg/ml is shown in Fig. 2. Symmetrical sharp peaks appear at 1.30 and 1.69 min, respectively, for these two drugs with little baseline disturbance from the solvent. For an OV-1 capillary column and similar chromatographic conditions, Grob and Grob, Jr. [2] have indicated hexane to be far too volatile to elicit a solvent effect. Here, the combined use of a narrow bore injector sleeve and a brisk carrier gas flow-rate (60 cm/sec) accommodate the relatively rapid transfer of analytes onto the fused-silica capillary column upon injection. Unfortunately the transfer rate is far from ideal and is most apparent in the early eluting peaks. Only 3500 and 4600 plates were calculated from the diphenhydramine (k = 6.6) and orphenadrine (k = 8.9) peaks, respectively. The optimal plate count is 38,000 for k = 10.3.

Carrier gas velocities below 40 cm/sec produced longer trailing solvent fronts and wider analyte peaks which sometimes split into doublets. Fortunately, typical van Deemter curves for helium are relatively flat [6] such that the column performance is not unduly compromised at a higher gas velocity.

During the investigative stages of this work, a 0.78-mm I.D. injector sleeve was tried and found to give acceptable chromatograms. However, the smaller

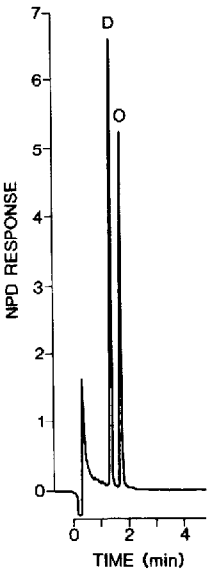

Fig. 2. Chromatogram of diphenhydramine (D) and orphenadrine (O), 10 ng each in hexane, 1 μl injection.

0.65-mm I.D. sleeve is preferable because it gave a two-fold increase in the peak height/peak area ratio. A yet narrower 0.50-mm I.D. sleeve resulted in only marginal further improvement. It appears that the choice of bore diameter is critical but not limiting for optimal performance of the injector.

The position of the capillary column inlet was varied along the length of the injector sleeve. Reproducibility was best with the column inlet positioned 3 to 5 mm from the point of sample deposition. The same inlet location was selected by Demedts et al. [4, 7].

The injector modification resulted in no memory effect. The rate of sample injection did not have to be rigidly controlled to avoid back flashing the sample from the injector sleeve. A convenient 1—2 sec delivery time was adopted for a 1-μl injection. A 10% carry-over of both drugs was inherent to the syringe needle, probably due to hexane distillation in the injector. The problem was eliminated by routinely baking the needle in the second injector for 10 sec after rinsing.

The nitrogen—phosphorus detector response was evaluated with the column effluent end positioned flush and withdrawn 10 and 20 mm from the jet tip. Each position was tested with and without nitrogen make-up gas. Make-up gas did not enhance the nitrogen—phosphorus detector response with the column end held flush with the jet tip. With the column end withdrawn inside the jet, a make-up gas was necessary to prevent peak broadening. Flush mounting is preferred because peak broadening is minimized without the need of a make-up gas, the nitrogen—phosphorus detector response remains optimized and the potential risk of drug adsorption within the detector is avoided.

Within-day retention time precisions (n = 80) were 0.7% for diphenhydramine (1.30 min) and 0.5% for orphenadrine (1.69 min). Over three months, however, the retention times of diphenhydramine and

orphenadrine gradually increased from 1.30 to 1.44 min and from 1.69 to 1.88 min, respectively. Approximately 1200 injections of serum extracts were made during this time. The injector sleeve was subsequently cleaned in detergent, resilanized and replaced. The retention times decreased to 1.33 min for diphenhydramine and to 1.73 min for orphenadrine, nearly identical to those originally observed. It would appear that the bonded methyl silicone stationary phase had not been modified by repetitive injections of hexane. The accumulation of septum particles and charred debris in the injector sleeve was responsible for the shift in retention times.

Repetitive injections of nanogram quantities of diphenhydramine standards in hexane resulted in desirable quantitative data (Table I). Precision ranged from 3.18% to 0.90% at 1 and 80 ng diphenhydramine injected, respectively. The instrument configuration gave a linear response and from the data in Table I, the correlation coefficient was 1.000; the equation of the straight line was $y = 0.099x + 0.053$, where y is the ratio of peak areas for diphenhydramine to orphenadrine and x is the mass of diphenhydramine injected. These results indicate the virtual absence of adsorption sites in the injector and column for these two drugs above 1 ng.

TABLE I

WITHIN-DAY INSTRUMENT PERFORMANCE

Diphenhydramine injected (ng) (n = 10)	Peak area diphenhydramine / Peak area orphenadrine	
	$\bar{X}$	C.V. (%)
1.0	0.119	3.18
5.0	0.533	2.15
10.0	1.041	1.48
15.0	1.558	1.37
20.0	2.039	0.59
40.0	4.002	1.08
60.0	5.984	0.97
80.0	7.910	0.90

Chromatograms obtained from serum extracts appear in Fig. 3. Baseline separations of caffeine, diphenhydramine and orphenadrine occurred at 1.05, 1.33 and 1.73 min, respectively. Extraneous serum peaks were small and did not interfere in the analysis. Caffeine peaks were disproportionately small because it is poorly extracted into hexane. Similar extracts were previously injected onto 1.83 m × 2 mm I.D. glass columns packed with either 3% OV-1 on 80—100 Chromosorb W HP or 3% SP2100 on 100—120 Supelcoport. Carrier gas was nitrogen at a flow-rate of 25 ml/min. With either packed column, caffeine separated poorly from diphenhydramine and tailed profusely. In the presence of > 1 μg/ml caffeine, quantitation of diphenhydramine below 50 ng/ml was unreliable. In addition, the calibration curve became somewhat alinear below 30 ng/ml, probably due to adsorption onto the column support.

Two brands of evacuated serum collection tubes were evaluated for their

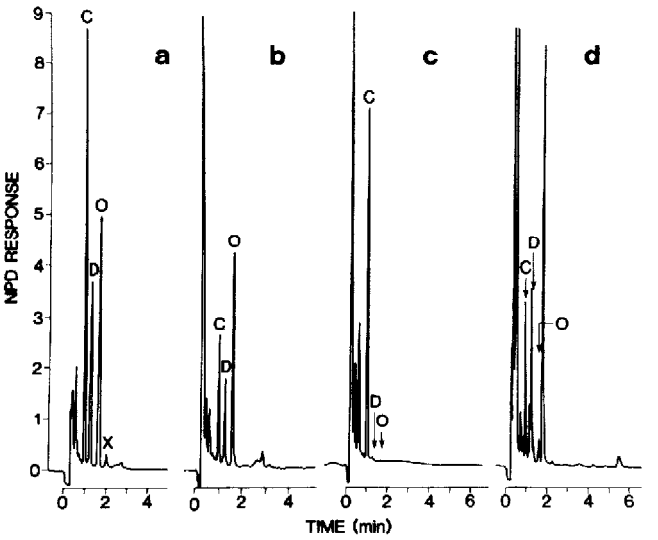

Fig. 3. Chromatograms from serum extracts. (a) Diphenhydramine 50 ng/ml standard; (b) volunteer 12 h post 50-mg oral dose of diphenhydramine · HCl, serum diphenhydramine = 27.4 ng/ml; (c) drug-free serum collected in a Vacutainer (plain) collection tube; and (d) drug-free serum collected in a Venoject (plain) collection tube. Peaks: C = caffeine, D = diphenhydramine, O = orphenadrine, X = unknown.

suitability in clinical studies. Drug-free blood was collected in 10-ml red-stoppered Vacutainer® (Becton-Dickinson, Mississauga, Ontario, Canada) and Venoject® (Kimble Terumo, Elkton, MO, U.S.A.) tubes. The serum was separated and extracted according to the above procedure except that an internal standard was not added. Resulting chromatograms appear in Fig. 3c and d. Numerous interfering peaks appeared in the serum extract from the Venoject tube but not the Vacutainer tube. The limit of detection for diphenhydramine collected in Vacutainer tubes was 0.9 ng/ml. Both collection tubes were further investigated for the presence of the plasticizing agent tris(2-butoxyethyl) phosphate (TBEP). Its presence in Vacutainer tubes has been shown to cause artifactually low serum concentration determinations for a variety of basic drugs, e.g. imipramine and alprenolol [8]. It is not known whether TBEP would affect diphenhydramine in the same way. TBEP if present extracts into hexane at any pH and elutes at 1.6 min at 220°C on this column. Both tubes were found to be TBEP-free.

Table II provides a summary of the precision and recovery data for serum-based standards at 10, 50, 100, 200 and 800 ng/ml diphenhydramine. The coefficients of variation (C.V.) are similar at corresponding serum concentrations for the within-day and day-to-day precision studies. However, from a comparison of corresponding diphenhydramine concentrations in Tables I and II, it is seen that the extraction step roughly doubles the imprecision compared to that observed with pure standards in hexane. To correlate the two tables, extraction of 1 ml of serum at 10 ng/ml results in an injection of about 1 ng of drug, etc. Recovery of diphenhydramine at all concentrations tested ranged from 98.0% to 106.7%. Recovery of orphenadrine at 100 ng/ml was 98.6%. For practical purposes, complete recovery for both drugs can be assumed.

TABLE II

ASSAY PERFORMANCE

Compound (ng/ml)	Precision						Recovery (n = 5)	
	Within-day (n = 10)			Day-to-day (n = 20)				
	$\bar{X}$ (ng/ml)	S.D. (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	S.D. (ng/ml)	C.V. (%)	$\bar{X}$ (%)	C.V. (%)
Diphenhydramine								
10	11.2	0.8	7.6	11.4	0.7	6.6	105.6	5.1
50	50.2	1.4	2.6	50.7	2.3	4.5	106.7	7.2
100	100.0	3.2	3.2	100.9	3.0	3.0	103.1	4.6
200	212.8	7.7	3.6	193.9	7.5	3.9	101.0	2.2
800	841.3	16.0	1.9	776.6	15.0	1.9	98.0	2.2
Orphenadrine								
100	—	—	—	—	—	—	98.6	3.4

Method linearity was evaluated from the within-day precision data presented in Table II by plotting observed diphenhydramine concentrations (y-axis) against the target values of the serum-based standards. Linear regression analysis gave a correlation coefficient of 1.000, a slope of 1.05 and a y-intercept of −1.36 ng/ml.

Single-step serum extractions with hexane provide gas chromatograms with clean backgrounds when a nitrogen–phosphorus detector is used. Hexane is a preferred solvent for drug analysis provided the recovery is sufficiently high. Unfortunately, hexane and biological fluids are notorious for forming emulsions which are not always broken under centrifugation. The addition of 2% isoamyl alcohol or any other low-molecular-weight alcohol to hexane prevents emulsion formation but also causes endogenous biological material to co-extract. Further time-consuming clean up steps such as back extraction into acid followed by re-extraction into an organic solvent from the alkalinized acid fraction are then required. It was discovered that when the silanized extraction tubes were simply rinsed with water after use rather than cleaned in detergent, emulsions rarely formed between hexane and serum. No diphenhydramine carry-over was detected with the following cleaning procedure: (a) rinse with 2 ml of methanol or ethanol; (b) rinse with four 10-ml portions of warm tap water; (c) rinse with two 10-ml portions of distilled water; (d) invert to drain dry.

Serum concentrations of diphenhydramine following a single 50-mg oral dose of the hydrochloride salt were measured in six healthy volunteers. The results are illustrated in Fig. 4. Peak concentrations ranging from 53 to 196 ng/ml occurred between 0.5 and 4 h. Similar serum concentration–time data have previously appeared [9, 10]. A thorough discussion of diphenhydramine pharmacokinetics is in press [11]. The major amine metabolite of diphenhydramine, 2-(benzhydroloxy)-N-methylethylamine, eluted between caffeine and diphenhydramine and hence would not potentially interfere with the

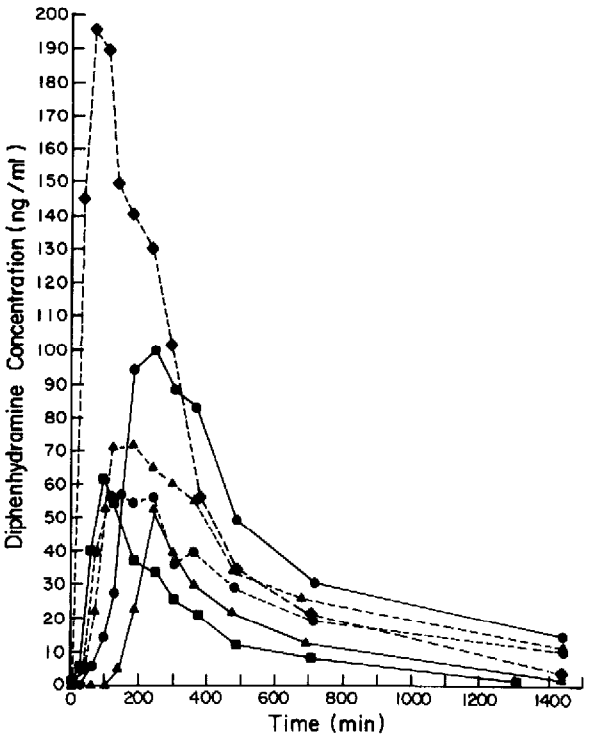

Fig. 4. Serum concentration—time curves for six volunteers following a single 50-mg oral dose of diphenhydramine · HCl. Each point is the average of duplicate determinations. Key: (●—●) subject 1; (● - - ●) subject 2; (▲—▲) subject 3; (▲ - - ▲) subject 4; (■—■) subject 5; (◆ - - ◆) subject 6.

quantitation of diphenhydramine. In fact, the metabolite appeared in only a few of the volunteer serum samples.

ACKNOWLEDGEMENTS

We thank Mr. D. Sansome, Perkin-Elmer Canada, Ltd. and Mr. I. Ward (Glass Shop), University of Manitoba, Canada for their technical assistance. We are grateful to Dr. Tsun Chang, Warner-Lambert Co., Ann Arbor, MI, U.S.A. for providing a pure sample of the monodesmethyl metabolite of diphenhydramine. This research was funded by grants from the Manitoba Health Research Council (MHRC-3215) and the Medical Research Council of Canada (MS-3250).

REFERENCES

1 K. Grob and K. Grob, Jr., J. High Resolut. Chromatogr. Chromatogr. Commun., 1 (1978) 57.
2 K. Grob and K. Grob, Jr., J. Chromatogr., 94 (1974) 53.
3 K. Grob, J. High Resolut. Chromatogr. Chromatogr. Commun., 1 (1978) 263.
4 P. Demedts, M. van den Heede, J. van der Verren and A. Heyndrickx, J. Anal. Toxicol., 6 (1982) 30.

5 P. Demedts, M. De Waele, J. van der Verren and A. Heyndrickx, J. Anal. Toxicol., 7 (1983) 113.
6 W. Jennings, Gas Chromatography With Glass Capillary Columns, Academic Press, New York, 2nd ed., 1980, p. 134.
7 P. Demedts, personal communication.
8 O. Borga, K.M. Piafsky and O.G. Nilsen, Clin. Pharmacol. Ther., 22 (1977) 539.
9 W. Bilzer and U. Gundert-Remy, Eur. J. Clin. Pharmacol., 6 (1973) 268.
10 A.J. Glazko, W.A. Dill, R.M. Young, T.C. Smith and R.I. Ogilvie, Clin. Pharmacol. Ther., 16 (1974) 1066.
11 D.R.P. Guay, R.C. Meatherall, P.A. Macaulay and C. Yeung, Int. J. Clin. Pharmacol. Ther. Toxicol., in press.