Journal of Chromatography, 454 (1988) 293–301
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 819

DETERMINATION OF COTININE IN URINE USING GLASS CAPILLARY GAS CHROMATOGRAPHY AND SELECTIVE DETECTION, WITH SPECIAL REFERENCE TO THE BIOLOGICAL MONITORING OF PASSIVE SMOKING

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(First received March 7th, 1988; revised manuscript received July 20th, 1988)

SUMMARY

A capillary gas chromatographic (GC) method using selected-ion monitoring (SIM) was developed for the analysis of cotinine (C.A.S. No. 486-56-6) in human urine. The method is based on basic extraction of cotinine from 2 ml of urine into dichloromethane. After evaporation of the dichloromethane solution to dryness, 100 μl of toluene were added, prior to GC–mass spectrometric (MS) analysis. Trideuterated cotinine (C.A.S. No. 97664-65-8) was used as the internal standard. More than 1000 automatic chromatographic analyses were made without column degradation. Molecular ions (M) of cotinine and trideuterated cotinine, (m/e = 176 and 179), were monitored in the electron impact (EI) mode and m/e = 177 (M + 1) and m/e = 180 (M + 1) in the chemical ionization (CI) mode with isobutane. The correlation coefficient with SIM and EI was 0.998 (5–20 ng/ml) and with CI was (0.2–2 ng/ml). For thermionic specific detection the correlation coefficient was 0.998 (10–510 ng/ml). Only capillary columns with an apolar bonded stationary phase film thickness of 1 μm showed sufficient inertness for cotinine analysis at the sub ng/ml level. The relative standard deviations for 5 and 20 ng/ml were 5.2 and 3.5% respectively (n = 12) using EI. Spiked urine samples from six non-smokers (5 ng/ml) showed a relative standard deviation of 5%. The overall recovery (25 ng/ml) was 100 ± 4%. The minimum detectable concentration, using SIM, was *ca.* 2 ng/ml in the EI mode and *ca.* 0.2 ng/ml in the CI mode. The half-time for cotinine was *ca.* 18 h for both active smokers and non-smokers.

INTRODUCTION

Passive smoking, *i.e.*, the exposure of non-smokers to tobacco smoke, has been shown to be an health hazard. Monitoring the exposure to environmental tobacco

0021-9673/88/$03.50

smoke[1–3] is important because passive smoking has created health problems in homes and work places[4–10]. Due to its relatively long half-life, cotinine, a major nicotine metabolite, has been shown to be the intake marker of choice[11–14]. The purpose of a marker of passive smoking is to evaluate exposure, quantitate the intake, validate non-smokers status[15–17] and provide feedback to encourage smokers to stop smoking.

Several analytical techniques have been used for the determination of cotinine in biological fluids. The methods described in the literature have been compared by Daenens[18]. The most frequently used methods of the low nanograms per ml level are radioimmunoassay (RIA), liquid chromatography (LC)[19] and gas chromatography (GC) with flame ionization, electron-capture, and nitrogen selective detection[20–23] or selected-ion monitoring (SIM)[18,24–26]. Interlaboratory studies have recently been performed for the determination of cotinine in urine[23,27].

Glass capillary GC and nitrogen selective detection has successfully been used[21,23] with detection limits, for cotinine in plasma, of 0.4 ng/ml; N-ethyl-norcotinine was used as the internal standard. SIM of cotinine in plasma or urine, with methylprylone or trideuterated cotinine as the internal standard, has been demonstrated. The detection limit was found to be below 1 ng/ml with electron impact ionization.

In this work we present a method for determination of cotinine at the low ng/ml level in human urine samples using fused-silica capillary columns and SIM. The detection is performed both in the electron impact (EI) mode and in the chemical ionization mode with isobutane as the reagent gas. The main purpose is to evaluate the involuntary exposure to tobacco smoke.

EXPERIMENTAL

Apparatus

A Varian 3500 gas chromatograph equipped with Varian thermionic specific detection (TSD) and a Varian 8035 automatic on-column injector was employed. The injector was cooled with liquid nitrogen. The temperature of the injector was initially 105°C for 5 s and thereafter programmed at 150°C/min to 280°C. After 2 min isothermal, the column oven was programmed at 20°C/min to 290°C and then held for 5 min. Typical settings for the detector were: bead heating current 3.10 A; bias voltage −3.9 V; detector temperature 270°C, gas flow-rates 3 ml/min of hydrogen, 175 ml/min of air and 20 ml/min of nitrogen as the make-up gas. Chromatograms were recorded on Servogor Model 310 linear recorders and Shimadzu CR3A integrators were used for peak evaluation.

A Shimadzu GC–MS QP1000 EI/CI quadrupole mass spectrometer was connected to a Shimadzu GC 9A gas chromatograph equipped with a split/splitless injection system spl-69 and a Shimadzu autosampler AOC-9. The starting temperature of the column oven was set near to the solvent boiling point, and in the case of toluene it was 95°C isothermal for 3 min. The split exit valve was kept closed for 1 min after injection. After elution of the solvent, the column was programmed at 5°C/min to 110°C, then at 30°C/min to a final temperature of 280°C which was maintained for 5 min. The capillary column outlet was mounted directly in the ion source. The gas chromatograph–mass spectrometer interface and the ion source were held at 250°C. The pressure in the ion source was *ca.* $1.5 \cdot 10^{-5}$ Torr. Instrument characteristics for

the QP 1000 were set as follows: *m/e* 176 and 179, molecular ions (M) of cotinine and trideuterated cotinine, were monitored in the EI mode and *m/e* = 177 (M + 1) and *m/e* = 180 (M + 1) in the chemical ionization (CI) mode. The filament in the ion source was turned on after 6 min and off after 14 min. Five measurements were made per second (rate 3). The tuning of the instrument (autotuning) was performed using a standard sample inlet system and the standard sample used was perfluorotributylamine (PFTBA), according to standard procedures (70 eV). The resolution was set to maximum. Three reagents gases, isobutane, ammonia and methane, were tested for CI of cotinine. The pressure in the ion source was kept at *ca.* $3 \cdot 10^{-5}$ Torr. The gas chromatography–mass spectrometry (GC–MS) conditions were set as follows: Samples were introduced into the chromatographic system with an autosampler using a splitless injection technique at 250°C with an inlet pressure of helium of 0.2 kg/cm^2. The amount injected was typically 1–2 μl using an Hamilton 701RN syringe with a point style 5 needle with a conical point and a sidehole to minimize septum coring. A Sigma 3E-1 centrifuge (Sigma Harz, F.R.G.) was employed for phase separation.

Columns

Four different types of fused-silica capillary columns with chemically bonded stationary phases were used: NORDION® (Helsinki, Finland) flexibond capillary columns NB-54 (25 m × 0.32 mm I.D.) with film thickness of 0.1, 0.25, 0.5 and 1.0 μm.

Chemicals

Chemicals used were toluene and dichloromethane from Janssen (Beerse, Belgium), cotinine (C.A.S. no. 486-56-6) and trideuterated cotinine (C.A.S. no. 97664-65-8) picrate from the Swedish Tobacco Company (Stockholm, Sweden) and PFTBA from E. Merck (Darmstadt, F.R.G.).

Preparation of standard solutions

Standard solutions of cotinine were prepared by dissolving accurately weighed amounts of cotinine (*ca.* 50 mg) in 100 ml aqueous solution of 0.1 *M* hydrochloric acid, and further dilution of this solution in hydrochloric acid to the desired concentrations. Typically, solutions containing 5 or 20 ng/ml were used in the work-up procedure where the addition of 1 ml of water was exchanged for the described standard solution. Standard solutions of trideuterated cotinine were prepared by dissolving *ca.* 10 mg of the compound in the manner described above.

Storage of standards

Standard solutions were stored in acidic aqueous solutions at room temperature and diluted solutions of concentrations less than 100 ng/ml were kept in a refrigerator. Diluted standard solutions could be used only for *ca.* 2 weeks.

Sampling and storage of samples

In the application the total urine volume from the test persons was sampled and each batch was analysed for creatinine, pH, density and cotinine concentration according to the method presented. All urine samples were kept frozen until analyzed, and no losses of cotinine were observed during this storage. Urine samples were stored in 10 ml test-tubes kept in a deep-freezer at −20°C.

Procedure

A 2-ml urine sample was added to a mixture of 1 ml water, 0.5 ml of a standard solution containing 202.5 ng/ml of trideuterated cotinine as the internal standard, 2 ml 5 *M* sodium hydroxide and 3 ml dichloromethane. The mixture was shaken for 10 min and centrifuged at 4000 rpm for 20 min. The main part of the aqueous layer was removed. The sample tube was then taken to a cooling solution consisting of solid carbon dioxide and acetone until the remaining water was frozen. The dichloromethane solution was then transferred to the test-tube. The solvent was evaporated to dryness at 30°C in a vacuum desiccator connected to an aspirating pump where several test-tubes containing sample solutions could simultaneously be evaporated. The vacuum desiccator was equipped with an electrically heated oven for the simultaneous treatment of 25 sample tubes. The residue was dissolved in 100 μl toluene and transferred to an autosampler vial, with an 100-μl conical insert, ready for the GC–MS analysis. For each sample, two determinations with duplicate injections were performed and the average values of the peak area or peak height ratios were used.

Standard solutions of cotinine in water were added to blank urine and injected in series with real samples. Every third sample was a standard (5 and 20 ng/ml).

For GC-TSD, the dry residue was dissolved in 100 μl of toluene containing the isobutyl chloroformate derivative of di-*n*-butylamine (200 ng/ml).

RESULTS AND DISCUSSION

Standards

Cotinine and trideuterated cotinine were identified by GC–MS and the purity was checked by GC-TSD and GC-FID. The purity was found to be better than 99%.

Chromatography

The NB-54 apolar columns with a film thickness of 1 μm gave symmetrical peaks and virtually no adsorption in the investigated range of 10–1000 ng/ml of cotinine in toluene. The elution temperature for cotinine (*ca.* 280°C) resulted in no column bleeding, after conditioning the column for 48 h, that interfered with SIM.

Several capillary columns were tested. Due to the adsorption, only capillary columns with an apolar bonded stationary phase film thickness of 1 μm showed sufficient inertness for cotinine analysis in the sub ng/ml range. The bonded Carbowax columns showed considerable column bleeding at the elution temperature of cotinine, giving unstable conditions and higher detection limits for cotinine compared with those obtained on apolar stationary phases. The removal of water (freezing out), described in the Procedure, increased the column lifetime considerably, making possible 1000 chromatographic experiments without any noticeable column degradation and absorption losses. Frequent testing is recommended to evaluate the adsorption behaviour of cotinine and N-ethylnorcotinine in the chromatographic system.

Detection

Nitrogen selective detection. TSD is as sensitive as SIM in the CI mode and ten times more sensitive than in the EI mode. The use of high resolution capillary columns has made it possible to use TSD for detection of cotinine. However, when analysing

cotinine in urine several nitrogen-containing compounds appear in the chromatogram and the matrix[28] is not constant. Considerable care must be taken to avoid contaminants in the concentration range of passive smoking (a few nanograms per ml). The detection limit is mainly set by the matrix. Inter-individual variations in urine composition can influence the analysis.

In order to obtain detection limits with TSD in the same order of magnitude as with SIM, further work-up is necessary.

Mass selective detection. Trace determination of cotinine with the procedure described in the Experimental demands introduction of 1 μl or more of the resulting toluene solution into the chromatographic system. However, the ion source and the high vacuum system can be severely damaged and contaminated when large volumes of solvents are introduced. Usually the solvent is eliminated using a solvent cut and a jet separator. This may result in sample losses and poor reproducibility. The low carrier gas flow in the capillary column enables a direct inlet of the capillary column into the ion source without disturbing the high vacuum system. Attempts were made to perform the analysis with the column connected directly to the ion source. This however resulted in poor reproducibility with a decreasing sensitivity and short lifetime of the filament. The ion source became severely contaminated after some injections and frequent rinsing was therefore necessary. When the filament was switched off during the entrance of the solvent and later turned on during the chromatographic analysis these problems were eliminated. Using this procedure, we performed several hundreds of analyses without a decrease in sensitivity and with excellent reproducibility during several weeks. It was sufficient to tune the instrument once a month which makes this procedure very well suited for routine analysis.

As mentioned earlier the chromatographic behaviour of cotinine is excellent in our system. The mass spectrometer was employed in the EI mode at 70 eV. Fig. 1 shows the peaks of the molecular ion of cotinine and trideuterated cotinine from an urine sample at a concentration of 20 ng/ml. No interfering peaks were observed in the urine samples studied. The mass spectrometer was also employed in the CI mode with isobutane as the reaction gas. Fig. 2 shows the peaks of cotinine and trideuterated cotinine in urine, using CI.

Quantitative analysis

Calibration graphs. The calibration graphs for cotinine in toluene solution using capillary GC-TSD with an automated cold on-column injector gave virtually linear calibration plots of the peak area ratio or peak height ratio *vs.* concentration. The calibration graph of the peak area ratio passed through the origin with a correlation coefficient of 0.999 ($n = 34$, $p = 0.0001$) in the concentration range investigated (10–510 ng/ml and peak area ratio 0.06–2.5) of cotinine in toluene. The slope was 0.004 and the intercept was 0.03 for peak area measurements.

The calibration graphs for cotinine in urine using capillary GC and SIM in the EI mode and the CI mode were constructed as follows. Different amounts of cotinine were added to aqueous solutions or to urine and the entire work-up was performed as described above. For each concentration, duplicate determinations with duplicate injections were made and the average values of the peak area or peak height were plotted. In the case of EI the average value of the ratio of the signal of the $m/e = 176$ molecular ion to that of the molecular ion of the internal standard, $m/e = 179$, was

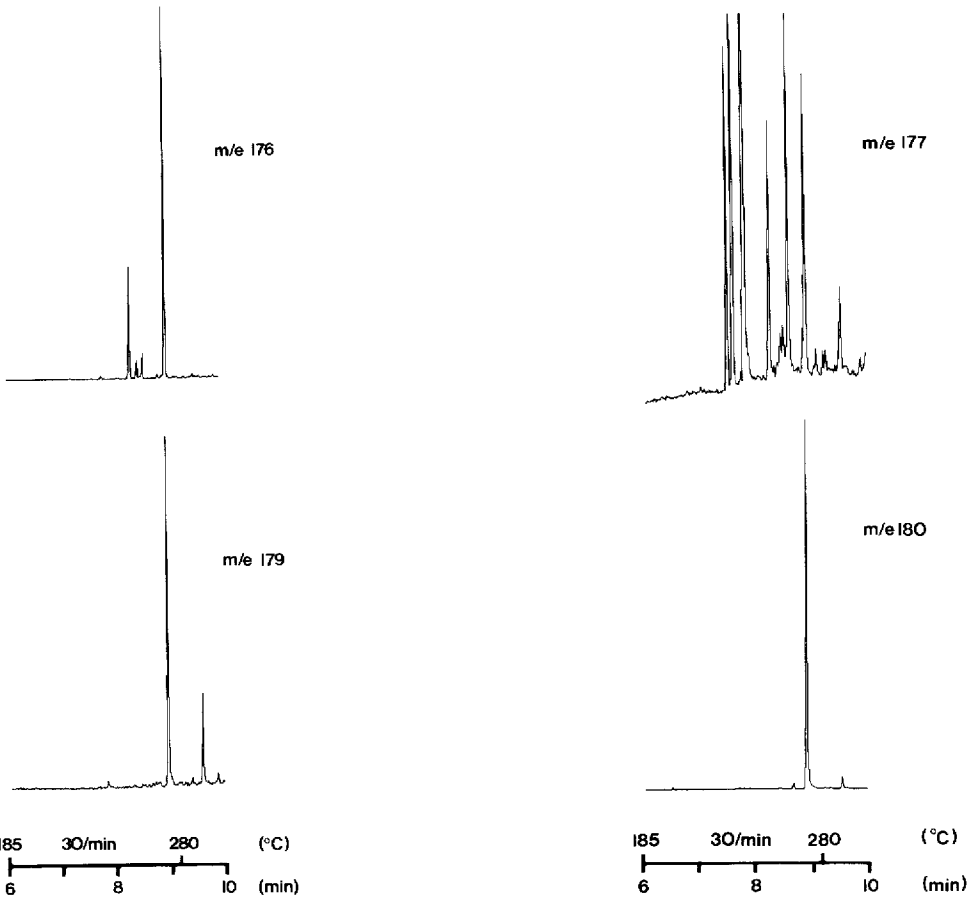

Fig. 1. Selected ion monitoring of urine samples using the EI ionization mode. The upper chromatogram shows the molecular ion of cotinine (176) from an urine sample from a 5-year-old asthmatic boy, whose parents both smoked *ca.* 20 cigarettes each evening. The peak corresponds to a concentration of *ca.* 40 ng per ml of urine, the lower chromatogram shows the molecular ion of trideuterated cotinine (179) used as an internal standard. Column: fused silica coated with SE-54 bonded stationary phase (25 m × 0.32 mm I.D.), film thickness 1.0 μm. Inlet pressure of the carrier gas (helium); 0.2 kg/cm^2. Splitless injection (1 min) of 1 μl toluene. Temperature programming: isothermal at 95°C (3 min), raised at 5°C/min to 110°C, then at 30°C/min to a final temperature of 280°C which was maintained for 5 min.

Fig. 2. Selected ion monitoring of cotinine in a spiked urine sample corresponding to a concentration of 0.5 ng/ml of cotinine, using CI with isobutane as the ionization gas and the M + 1 ion for detection. Upper: molecular ion (M + 1) of cotinine (177). Lower: molecular ion (M + 1) of trideuterated cotinine (180). Chromatographic conditions as in Fig. 1.

plotted. The calibration graph for the concentration range investigated (0–20 ng/ml and peak height ratio 0.007–1.72) of cotinine in urine and peak height ratio measurements gave a correlation coefficient of 0.998 ($n = 5, p = 0.0001$). The slope was 0.085 and the intercept was −0.004. No noticeable difference was found between aqueous solutions and urine and no significant difference between peak area or peak height ratio measurements. The use of CI with isobutane gave a linear calibration plot

in the concentration range investigated (0.2–2 ng/ml and peak height ratio 0.01–0.055) of cotinine in urine. The correlation coefficient was 0.996 ($n = 6, p = 0.0001$) for the average peak area ratio six concentrations with duplicate injections. The slope and the intercept were 0.02 and 0.01 respectively. The response curve was linear for the GC–MS mode in the ranges 1–100 ng/ml (EI) and 0.2–10 ng/ml (CI) and in the case of GC-TSD in the range 5–50 000 ng/ml for an injected volume of 1 μl.

The detection limit determined from the intercept according to Miller and Miller[29] was 2.2 ng/ml of urine with GC–MS using the EI mode and 0.20 ng/ml in the CI mode using isobutane as the reagent gas. For the calibration plots above we used urine from one of our non-smoking colleagues having a very low estimated exposure to tobacco smoke. In no urine tested so far was the concentration of cotinine found to be zero. For water solutions no cotinine was found, resulting in a practically zero intercept. The detection limit in aqueous solutions is therefore at least one decade lower than in human urine.

Recovery. The overall recovery was studied by working-up ten urine samples spiked with cotinine to a concentration of 25 ng/ml. Ten urine samples of the same concentration were then worked up by the same procedure but with no internal standard present. In the final step of the work-up procedure before the evaporation of the dichloromethane solution, 1 ml of dichloromethane containing the same amount of internal standard was added. The separately prepared solutions yielded a ratio between cotinine and internal standard of 100 $\pm$ 5% and 98 $\pm$ 5.2% for the peak height ratio comparison and 100 $\pm$ 5 and 102 $\pm$ 4.6% for the peak area ratio comparison. The values are given with a 95% confidence range.

The procedure of extraction of cotinine from urine samples to the dichloromethane solution was investigated and the recovery was *ca.* 100%. The recovery in the evaporation step was also studied. After working-up ten urine samples, 10 μl of a toluene solution containing 2500 ng/ml of cotinine were added to the dichloromethane solution in the final step before evaporation of the solvent. The following samples were then compared with a solution containing 250 ng/ml. For a confidence range of 95%, the recovery in the evaporation step was 98.8 $\pm$ 3% based on peak areas $n = 10$. Standard solutions of cotinine and internal standard were injected, and the peak height and peak area ratio were established. The temperature-controlled vacuum desiccator, designed at our laboratory, made it possible to evaporate 25 samples containing 1 ml dichloromethane within 1 h and to study the possible evaporation losses in detail. No artefact and no carry over was found during this procedure.

Accuracy and precision. The usefulness of a deuterium-labelled internal standard is shown by the very good precision in the analysis even at the low ng/ml level. When analysing twelve different standard samples in urine at concentrations of 5 and 20 ng/ml respectively with the entire work-up procedure followed by GC with multiple ion detection (MID) during 2 weeks, the relative standard deviations of the ratio between the area of the cotinine and the trideuterated cotinine molecular ion peak were 5.2 (100 $\pm$ 3%) and 3.5% (100 $\pm$ 2%) respectively. When analysing urine from six non-smoking colleagues spiked to a concentration of 5 ng/ml (the blank was subtracted), the relative standard deviation of the peak height ratio between cotinine and trideuterated cotinine was 5% (100 $\pm$ 5%). The values in brackets are given with a 95% confidence range.

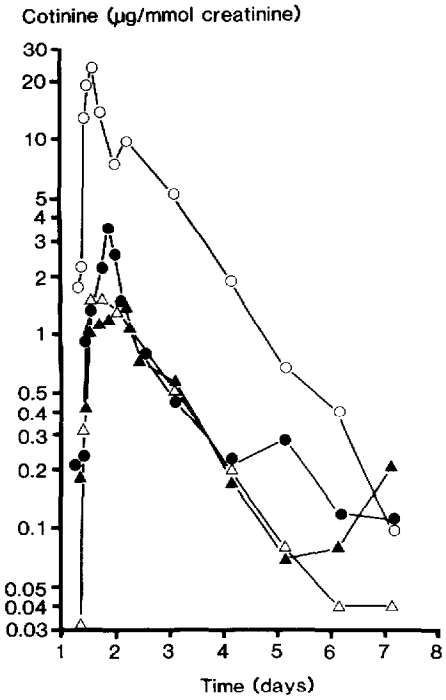

Fig. 3. Elimination curves of cotinine in urine from four male persons, one active smoker (○) and three passive smokers (△, ▲, ●). The active smoker consumed 20 cigarettes during 1.5 h in the presence of the three non-smokers while driving a car with closed windows and normal ventilation.

Applications

Four male persons were placed in a car, which was driven for 1.5 h. One person was an active smoker who consumed 20 cigarettes during this hour and the other three persons were non-smokers. The three non-smokers were told not be exposed for tobacco smoke for 1 week before and 1 week after this test was applied. The cotinine concentrations in urine, using GC-SIM and EI, *versus* creatinine concentrations were monitored for all four persons and the results are plotted in Fig. 3. This figure shows *ca.* 20 times higher cotinine levels for the active smokers *versus* the three non-smokers. Actually, person 1 visited a public restaurant on day 5 for less than 2 h and person 3 was in an apartment on day 6 where another person smoked a few cigarettes. From the elimination curve it is possible to estimate the half-time of cotinine in urine. It was found to be *ca.* 18 h for both active smokers and non-smokers, in agreement with findings by other workers[12,14]

CONCLUSIONS

The method described for the determination of cotinine in human urine based upon high resolution gas chromatography–mass fragmentography makes it possible to determine cotinine in a more complex matrix than is the case with GC-TSD. No special clean-up procedure is necessary and several samples can be worked up simultaneously. Very selective and sensitive determinations of cotinine at the

sub ng/ml level are possible. The use of trideuterated cotinine as the internal standard gave accurate and precise determinations. The combination of high resolution chromatography and selective detection is an appropriate choice for the analysis of cotinine in biological matrixes at the low ng/ml level. Mass fragmentography is well suited to this task.

ACKNOWLEDGEMENTS

We are indebted to Professor Staffan Skerfving, head of the department of Occupational Medicine, for his interest in this work. We also gratefully acknowledge the skilful technical assistance of Miss Anita Hultberg, and the Swedish Work Environment Fund for financial support. We thank Dr. Antti Manninen, NORDION instruments Oy Ltd. (Finland) for supplying dedicated capillary columns and Dr. Margareta Curvall for deuterium-labelled standards. We also gratefully acknowledge Dr. Sven Jackobsson (Apotekarnas Compositae, Stockholm, Sweden) for valuable discussion, especially concerning automatization of the filament on/of procedure for the ion source.

REFERENCES

1 Y. Saint-Jalm and P. Moree-Testa, *J. Chromatogr.*, 198 (1980) 188–192.
2 I. Schmeltz and D. Hoffmann, *Chem. Rev.*, 77 (1977) 295.
3 R. F. Severson, K. L. McDuffie, R. F. Arrendale, G. R. Gwynn, J. F. Chaplin and A. W. Johnson, *J. Chromatogr.*, 211 (1981) 111.
4 R. A. Greenberg, *New England J. Med.*, 310 (1984) 1075.
5 E. N. Pattishall, G. L. Strope, R. A. Etzel, R. W. Helms, N. J. Haley and F. W. Denny, *Am. J. Dis. Child.*, 139 (1985) 1101.
6 S. Matsukura, T. Taminato, N. Kitano, Y. Seino, H. Hamada, M. Uchihashi, H. Nakajima and Y. Hirata, *New England J. Med.*, 311 (1984) 828.
7 M. J. Jarvis, A. D. McNeill, M. A. H. Russell, R. J. West, A. Bryant and C. Feyerabend, *Lancet*, i (1987) 1324–1325.
8 M. J. Jarvis, M. A. H. Russell, C. Feyerabend, J. R. Eiser, M. Morgan, P. Gammage and E. M. Gray, *Br. Med. J.*, 291 (1985) 927.
9 M. J. Jarvis, M. A. H. Russell and C. Feyerabend, *Thorax*, 38 (1983) 829.
10 A. Weber and T. Fischer, *Int. Arch. Occup. Environ. Health*, 47 (1980) 209.
11 D. T. Darby, J. E. McNamee and J. M. van Rossum, *Clin. Pharmacokin.*, 9 (1984) 435.
12 L. C. Johnson, H. Letzel and J. Kleinschmidt, *Int. Arch. Environ. Health*, 56 (1985) 99.
13 N. J. Wald, J. Boreham, A. Bailey, C. Ritchie, J. E. Haddow and G. Knight, *Lancet*, i (1984) 230–231.
14 R. Hopkins, L. E. Wood, M. I. Biol and N. M. Sinclair, *Clin. Pharmacol. Ther.*, 36 (1984) 788.
15 K. Husgafvel-Pursiainen, *Doctoral thesis*, ISBN 951-801-585-6, Helsinki, 1987.
16 P. N. Lee, *Toxicol. Lett.*, 35 (1987) 157.
17 K. Überla, *Int. Arch. Occup. Environ. Health*, 58 (1987) 421.
18 P. Daenens, L. Laruelle, K. Callewaert, P. de Schepper, R. Galeazzi and J. van Rossum, *J. Chromatogr.*, 342 (1985) 79.
19 D. A. Machacek and N. Jiang, *Clin. Chem.*, 32 (1986) 979.
20 C. Feyerabend and M. A. H. Russell, *Analyst (London)*, 105 (1980) 998.
21 M. Curvall, E. Kazemi-Vala and C. R. Enzell, *J. Chromatogr.*, 232 (1982) 283–293.
22 G. Stehlik, J. Kainzbauer, H. Tausch and O. Richer, *J. Chromatogr.*, 232 (1982) 295.
23 R. A. Davies, *J. Chromatogr. Sci.*, 24 (1986) 134.
24 J. Dow and K. Hall, *J. Chromatogr.*, 153 (1978) 521.
25 J. A. Thompson, Ming-Shan Ho and D. R. Petersen, *J. Chromatogr.*, 231 (1982) 53.
26 G. C. Norbury, *J. Chromatogr.*, 414 (1987) 449.
27 A. Biber, G. Scherer, I. Hoepfner, F. Adlkofer, W-D. Heller, J. E. Haddow and G. J. Knight, *Toxicol. Lett.*, 35 (1987) 45.
28 A. Zlatkis, W. Bertsch, H. A. Lichtenstein, A. Tishbee, F. Shunbo, H. M. Liebich, A. M. Coscia and N. Fleischer, *Anal. Chem.*, 45 (1973) 1973.
29 J. C. Miller and J. N. Miller, *Statistics for Analytical Chemistry*, Wiley, Chichester, 1984.