Journal of Chromatography, 433 (1988) 95–104
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4399

SIMULTANEOUS DETERMINATION OF FENTANYL AND ALFENTANIL IN RAT TISSUES BY CAPILLARY COLUMN GAS CHROMATOGRAPHY

SVEN BJÖRKMAN*,* and DONALD R. STANSKI

Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305 (U S A)

(Received June 27th, 1988)

SUMMARY

Fentanyl and alfentanil were determined in blood and in thirteen tissues from rats by gas chromatography, with splitless injections on a fused-silica capillary column and nitrogen-selective detection. Sufentanil was used as the internal standard for both drugs and the acetyl analogue of sufentanil was used as an external standard to quantitate extraction recoveries. The extraction solvent was isopentanol–pentane (1:49) and a back-extraction into 0.1 *M* hydrochloric acid was used for sample clean-up. The extraction recoveries from tissue homogenates averaged 88% for fentanyl, 72% for alfentanil and 87% for sufentanil. The standard curves were linear over a range of 0.5–800 ng per sample for both drugs and the coefficients of variation for eight determinations of the drugs in tissue homogenates were 2–9% at 0.8–32 ng per sample. Assays of tissues from rats treated with simultaneous intravenous infusions of fentanyl and alfentanil confirmed the high precision and sensitivity of the method, which should therefore prove feasible for studies of the tissue distribution of these opioids.

INTRODUCTION

Fentanyl (F) and alfentanil (A) are highly potent synthetic opioids used extensively in anaesthesia [1]. Their duration of action is limited chiefly by redistribution of the drugs from effect sites in the central nervous system to other tissues. Consequently, tissue assays will be of prime interest in pharmacokinetic studies on these opioids. Several assays for F in plasma or blood have been reported. Most of these utilize gas chromatography (GC) on a packed or capillary column, with nitrogen–phosphorus-selective detection (NPD) [2–6] or selected-ion monitoring [7]. None of these assays have been applied to tissue samples.

*Correspondence address: Anesthesiology Service, 112A, Veterans Administration Medical Center, 3801 Miranda Avenue, Palo Alto, CA 94304, U.S.A.

0378-4347/88/$03.50

For A, a packed column GC assay has been described that is applicable to both plasma and tissue samples [8]. The sensitivity of this assay is probably sufficient for pharmacokinetic work on A, but an assay of F was not included. Determination of F and A in plasma by high-performance liquid chromatography (HPLC) is possible [9], but their low UV absorbance severely limits the sensitivity of the assay.

Radioimmunoassays are available and extensively used for both F [10] and A [11] in plasma. Their applicability to tissue homogenates or extracts has not been investigated.

In order to perform comparative tissue redistribution studies on F and A, we have developed a GC assay for the simultaneous determination of these opioids in rat tissue. The assay uses splitless sample injection on a fused-silica capillary column and NPD. Sufentanil (S) is used as internal standard for both F and A and the acetyl analogue of sufentanil (AS) is used as an external standard for determination of extraction recoveries.

EXPERIMENTAL

Reagents and chemicals

Fentanyl citrate, alfentanil hydrochloride, sufentanil citrate and the acetyl homologue of sufentanil were kindly supplied by Janssen Pharmaceutica (Piscataway, NJ, U.S.A.). The compounds were dissolved in methanol and stock solutions were prepared by dilution with methanol or with a solution of 10^{-6} *M* decylamine hydrochloride in 10^{-3} *M* hydrochloric acid. Decylamine was purchased from Sigma (St. Louis, MO, U.S.A.) and trisodium phosphate from Mallinckrodt (St. Louis, MO, U.S.A.). Analyzed-grade methanol, pentane and isopentanol were purchased from Baker (Phillipsburg, NJ, U.S.A.). The water was double-distilled in glass. Glass-stoppered Pyrex™ centrifuge tubes were used for extractions and all glassware was rinsed with ethanol before use.

Chromatography

The gas chromatograph was a Hewlett-Packard Model 5890 equipped with a nitrogen–phosphorus detector and a split/splitless capillary inlet port. A fused-silica capillary column (25 m×0.31 mm I.D.) with a cross-linked 5% phenyl methyl silicone stationary phase was used. The carrier gas was helium (flow-rate 1.0 ml/min) and the detector was supplied with the appropriate flows of helium, hydrogen and air. The temperature of the inlet port was 350°C and that of the detector was 320°C. The oven temperature was programmed: an initial 100°C for 1.5 min was followed by a 50°C/min raise to 290°C. This temperature was held for 5 min before a second raise by 50°C/min to 300°C. This final "rinse" temperature was held for 1 min. The injections were made in the splitless mode, the purge valve being closed for 1.2 min. The detector signal was routed to a Hewlett-Packard Model 3392A integrator and to a Varian 9176 chart recorder.

Sample work-up, general procedure

A piece of rat tissue was weighed to the nearest 0.01 g and transferred to a round-bottomed glass tube. A 1.5-ml volume of 0.02 *M* hydrochloric acid was

added, as well as 0.50 ml of S (16, 160 or 1600 ng/ml) in 10^{-3} *M* hydrochloric acid solution with 10^{-6} *M* decylamine. The tissue was homogenized during cooling with ice with a Brinkmann Polytron homogenizer (Kinematica, Kriens-Luzern, Switzerland). The homogenate (or a sample of haemolysed blood) was transferred to a glass-stoppered extraction tube and 1.5 ml of 0.5 *M* trisodium phosphate and 5 ml of isopentanol–pentane (1:49) were added. The tube was vortex-mixed and centrifuged for 15 min at 2500 *g*. The organic phase was extracted with 2 ml of 0.1 *M* hydrochloric acid and discarded. The aqueous phase was washed with another 5 ml of isopentanol–pentane, alkalinized with 1 ml of 0.5 *M* trisodium phosphate solution and extracted with 5 ml of isopentanol–pentane. The organic phase was transferred to a conical tube and evaporated to near dryness on a 40°C water-bath under a stream of nitrogen. Depending on the expected amount of F and/or A in the sample, the volume of the residue was adjusted to 20–500 μl (approximately) with isopentanol. A 4-μl volume of AS in methanol was added as external standard and the tubes were ultrasonicated for 5 min. Of this solution, 1–5 μl were injected into the gas chromatograph.

Extraction recoveries from tissue homogenates

Blank tissue (1 g) was homogenized in 1.5 ml of 0.01 *M* hydrochloric acid, and 1.0 ml of 10^{-6} *M* decylamine in 10^{-3} *M* hydrochloric acid, a solution of F, A, and S in methanol (16 μl, 0.25 μg/ml, giving tissue concentrations of 4 ng/g of each compound) and 2.0 ml of 0.5 *M* trisodium phosphate were added. The sample was extracted with 5 ml of isopentanol–pentane (1:49) and the work-up proceeded as described above. After the evaporation of the final organic phase, a solution of AS in methanol (16 μl, 0.25 μg/ml) was added to the extract. The absolute recoveries of F, A and S from the homogenates were calculated relative to AS. A 1:1 mixture of the F, A and S solution and the AS solution added to a blank tissue extract served as a standard.

The procedure was also performed with homogenates representing 0.25 g of tissue spiked to concentrations of 40 or 400 ng/g of F, A and S.

Standard curves

Tissue homogenates (in 0.01 *M* hydrochloric acid), representing 0.5 g of tissue (or haemolysed blood), were spiked with F and A and worked up by the general procedure. The amounts of drugs used were: in "low" standard curves 0.50, 1.0, 2.0, 4.0 and 8.0 ng each of F and A and 8.0 ng of S; in "middle" standard curves 5.0, 10, 20, 40 and 80 ng each of F and A and 80 ng of S; in "high" standard curves 50, 100, 200, 400 and 800 ng each of F and A and 800 ng of S. The tissues used were muscle, fat, small intestine and blood.

Peak areas as given by the HP3392A integrator were generally used for quantification. In the low concentration range, however, peak-height measurements were found to give more accurate results.

Assay of spiked homogenates or blood: accuracy and precision

Tissue homogenates (muscle, fat or small intestine) or haemolysed blood spiked with various amounts of F and A (given in Table II) were assayed by the general

procedure. The absolute limit of detection of F and A in muscle homogenate was determined by assay of samples spiked with 0.01, 0.05 or 0.10 ng of F and A and 8 ng of S per 0.5 g of muscle.

Precision of the assay and the stability of F and A in tissues

Three male Charles River F344 rats were anaesthetized with halothane, and PTFE catheters were inserted into the superior vena cava and abdominal aorta. Three to four days later, F (0.15–0.30 μg/min·kg) and A (2.75–5.4 μg/min·kg) were infused simultaneously for 6 h through the venous catheter (the arterial one was used for blood sampling). Immediately on stopping the infusion, the animals were sacrified by decapitation and bled out. All organs or tissue samples (as enumerated in Table I) were dissected free, wrapped in aluminium foil and frozen on dry ice as soon as possible.

One kidney, half of the liver and a sample of abdominal wall muscle were frozen within 4 min, while the other kidney, the other half of the liver and another sample of abdominal wall muscle were wrapped in foil and kept on the bench for 1 h before freezing. The tissues were then stored at $-20°C$. Before the assay, these tissues, as well as some perirenal fat tissue, were divided into multiple samples.

Chemical stability of F and A

Stock solutions of F and A in 10^{-3} *M* hydrochloric acid with 10^{-6} *M* decylamine, that had been kept in the refrigerator for four months, were compared to freshly prepared ones by the addition of S, extraction and chromatography.

In addition, eight samples of F, A and S in isopentanol (10 ng of each in 50 μl) were prepared, assayed by GC, left on the bench for two weeks and assayed again.

RESULTS

Chromatograms

Representative chromatograms are shown in Fig. 1. The peaks due to A had a tendency to tail that was most marked with injections of pure compound or tissue extracts with high concentrations of A (where only a small proportion of the extract was injected). Co-injected ballast compounds from tissue could apparently mask the active sites that caused this tailing. The area and shape of the A peak was also highly dependent on the injection port temperature.

Varying the injection volume between 2 and 4 μl affected the peak-area ratios of F or A to S minimally, the overall coefficient of variation (C.V.) on repeated injections of the same sample (0.4 ng/μl of each compound in isopentanol) being around 2% ($n=8$). Varying the volume between 1 and 5 μl increased this C.V. to 2.6% for F and 4.1% for A ($n=13$).

Since the starting temperature of the programmed run was 100°C, or 32°C below the boiling point of isopentanol, a solvent effect was conceivably present.

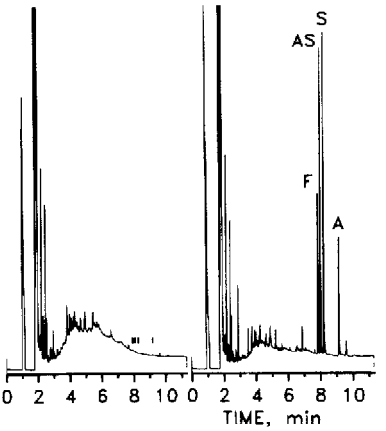

Fig. 1. Chromatograms of extracts from kidney samples (0.70 g). Left: blank kidney; the tick marks show the elution times of F, AS, S and A. Right: kidney of a rat given F (0.30 μg/min·kg) and A (2.7 μg/min·kg) by intravenous infusion. The measured tissue concentrations are 44 ng/g for F and 46 ng/g for A. The recovery of S is 84% of the 80 ng per sample originally added. AS is the external standard, 83 ng per sample.

Extraction recoveries from tissue homogenates

The recoveries of F, A and S from homogenates of various tissues are given in Table I.

Standard curves

The standard curves were linear for both drugs over the whole concentration range. Their coefficients of variation, as calculated on normalized peak-area ratios, were 3.4–7.6% (F) and 3.8–11.5% (A) in the low range, 2.6–5.6% (F) and 2.8–9.1% (A) in the middle range and 3.0–4.4% (F) and 6.1–8.2% (A) in the high range. The slopes of the standard curves were 0.156 ± 0.0073 ($n=12$, F) and 0.153 ± 0.013 ($n=12$, A), the standard deviations reflecting day-to-day variations rather than systematic differences between curves from different tissues.

Assay of spiked homogenates or blood: accuracy and precision

The results of the accuracy and precision experiments are given in Table II. The absolute limit of detection was estimated at 0.1 ng/g for both F and A in a 0.5-g sample.

Precision of the assay and the stability of F and A in tissues

The results of the assays of multiple tissue samples are given in Table III. They are from three rats given different doses of F and A and do therefore not depict the in vivo distribution of the drugs. There was no significant difference in F and A concentrations between the organs that had been kept at room temperature for 1 h and the ones that had been frozen within 4 min. The high C.V. of the assays on muscle was probably chiefly due to the visually apparent heterogeneity of the tissue, with intermingled fat and connective tissue.

TABLE I

EXTRACTION RECOVERIES OF F, A AND S FROM TISSUE HOMOGENATES

The values are means of triplicate determinations. The numbers in parentheses are extraction recoveries of F and A relative to the internal standard (S).

Tissue	Recovery (%)								
	Fentanyl			Alfentanil			Sufentanil		
	4 ng/g	40 ng/g	400 ng/g	4 ng/g	40 ng/g	400 ng/g	4 ng/g	40 ng/g	400 ng/g
Brain	82 (96)	95 (101)	92 (101)	62 (73)	80 (87)	82 (90)	84	95	92
Lung	89 (103)	89 (101)	96 (103)	67 (79)	74 (84)	85 (91)	85	88	93
Heart	97 (106)	95 (101)	99 (104)	66 (74)	86 (90)	78 (85)	90	95	91
Stomach	81 (104)	79 (100)	80 (100)	66 (86)	67 (86)	69 (86)	78	78	79
Small intestine	80 (102)	86 (103)	84 (100)	62 (80)	72 (86)	68 (81)	79	84	84
Caecum	87 (98)	86 (100)	87 (98)	66 (74)	71 (82)	68 (77)	88	87	88
Liver	81 (103)	90 (102)	88 (96)	59 (75)	68 (77)	70 (77)	79	88	91
Spleen	95 (104)	96 (102)	94 (101)	71 (78)	82 (88)	83 (88)	92	94	93
Pancreas	84 (99)	84 (99)	89 (99)	53 (62)	66 (77)	74 (82)	86	86	90
Testes	85 (102)	92 (102)	92 (101)	66 (80)	84 (92)	79 (88)	83	91	90
Kidney	85 (99)	87 (101)	88 (100)	63 (74)	71 (82)	68 (81)	86	87	88
Fat	87 (100)	90 (100)	90 (100)	81 (94)	75 (82)	78 (87)	86	91	90
Muscle	78 (106)	84 (105)	91 (104)	57 (77)	71 (88)	78 (89)	74	80	87
Blood	92 (97)	92 (99)	96 (101)	82 (86)	83 (90)	87 (92)	96	94	95
Mean	86 (101)	89 (101)	90 (101)	66 (78)	75 (85)	76 (85)	85	88	89
S.D.	±5.7 (±3.2)	±4.9 (±1.6)	±5.0 (±2.2)	±8.1 (±7.5)	±6.7 (±4.6)	±6.7 (±4.9)	± 5.9	± 5.3	± 4.1
Water	93 (98)	93 (98)	94 (103)	87 (93)	85 (91)	86 (93)	94	94	91

TABLE II

ACCURACY AND PRECISION OF ASSAYS ON SPIKED TISSUE HOMOGENATES

Tissue	Sample size (g)	Drug	Amount added (ng per sample)	Amount found (mean ± S.D.) (ng per sample)	C.V. (%)	*n*
Muscle	0.5	F	0.80	0.82 ± 0.036	4.4	8
	0.5	A	0.80	0.84 ± 0.057	6.8	8
	0.5	F	32	33.0 ± 1.167	3.5	10
	0.5	A	32	31.2 ± 1.150	3.7	10
Fat	0.5	F	1.6	1.67 ± 0.104	6 2	8
	0.5	A	1.6	1.70 ± 0.105	6 2	8
	0.5	F	32	31.6 ± 0.685	2.2	8
	0.5	A	32	31.4 ± 1.268	4.0	8
Small intestine	0.5	F	1.0	1.02 ± 0.039	3.8	8
	0.5	A	1.0	1.06 ± 0.096	9.0	8
	0.5	F	32	31.8 ± 0.930	2.9	8
	0.5	A	32	33.3 ± 1.346	4.0	8
Blood	0.4	F	0.80	0.79 ± 0.057	7 3	8
	0.4	A	0.80	0.82 ± 0.014	1.7	8
	0.2	F	12	11.8 ± 0.342	2.9	8
	0.2	A	12	11.9 ± 0.325	2.7	8

TABLE III

ASSAYS OF TISSUE SAMPLES FROM RATS GIVEN INFUSIONS OF F AND A

Tissue	Drug	Found concentration (mean ± S.D.) (ng/g)	C.V. (%)	*n**	Recovery of S (%)
Liver	F	16.0 ± 0.96	6.0	5	69 ± 2.9
	A	201 ± 8.02	4.0	5	69 ± 2.9
Liver**	F	17.1 ± 0.97	5.7	5	71 ± 2.9
	A	222 ± 11.98	5.4	5	71 ± 2.9
Kidney***	F	15.5 ± 0.59	3.8	4	72 ± 2.9
	A	103 ± 4.10	4.0	4	72 ± 2.9
	F	23.9 ± 0.88	3.7	4	73 ± 3.3
	A	141 ± 7.74	5.5	4	73 ± 3.3
Muscle	F	12.7 ± 2.65	21	7	55 ± 4.5
	A	31.1 ± 4.02	13	7	55 ± 4.5
Muscle**	F	14.8 ± 2.05	14	8	58 ± 4.9
	A	33.7 ± 4.29	13	8	58 ± 4.9
Fat	F	115 ± 3.96	3.4	8	80 ± 3.0
	A	212 ± 12.55	5.9	8	80 ⊥ 3.0

*Number of samples per organ.
**This part of the tissue was left for 1 h at room temperature.
***Pooled data from organs frozen within 4 min and organs left at room temperature, results from two animals.

The mean ($\pm$S.D.) absolute recovery of internal standard (S) from the homogenization mixture was $69 \pm 9.5\%$ for all the samples in Table III ($n=41$).

Chemical stability of F and A

The concentrations of F and A in the stock solutions that had been kept refrigerated for four months were 101 and 103%, respectively, of the concentrations in the freshly prepared solutions.

Keeping the isopentanol solutions of F, A and S at room temperature for two weeks changed the peak-area ratios F/S from 1.204 ± 0.024 to 1.165 ± 0.026 (mean $\pm$ S.D., $n=8$), a 3.3% decrease ($P<0.02$), and the peak-area ratios A/S from 1.683 ± 0.044 to 1.660 ± 0.035, a 1.4% decrease ($P>0.2$). Addition of external standard to these solutions on day 0 or 14 showed that the absolute amounts of F, A and S had decreased by less than 3% over two weeks.

DISCUSSION

The extraction recoveries of F and A relative to S were remarkably consistent between different tissue homogenates (Table I), which indicates that standard curves prepared from all these homogenates would have similar slopes. Further experiments on four selected tissues, muscle, fat, small intestine and blood, confirmed this notion. These tissues are readily obtained in large quantities and do also play important parts in the in vivo disposition of F [12,13]. Samples from other tissues were run using standard curves made up in muscle or fat homogenate.

The facile extraction of F from tissue homogenates with organic solvents is consistent with the results of experiments using tritiated F [12–14]. For A, our results are in agreement with those of Woestenborghs et al. [8].

The application of the terms accuracy and precision to tissue assays requires some caution. The data in Table II obviously only refer to assays of spiked homogenates, and the C.V. values given express variance due to pipetting, extraction and chromatography. In contrast, the C.V. values in Table III also include variance from the weighing and homogenization of the tissues, as well as from the natural heterogeneity of the organs or tissues themselves. This distinction is not always observed in the literature on tissue assays, where the C.V. of assays of homogenates are often taken to represent the precision of the assay as a whole. Similarly, some tissue homogenate is inevitably lost in the homogenizer, which decreases the absolute recovery of the drugs, as compared to experiments where spiked homogenates are prepared directly in the extraction tubes. The use of AS as an external standard allows routine monitoring of the recovery of internal standard.

The accuracy and sensitivity of the assay for F is comparable to those of published capillary column GC assays for F in blood or plasma [5,6]. The assay for A in plasma and tissues described by Woestenborghs et al. [8] utilizes a packed column, and the minimum detectable amount of A in tissues is 2 ng/g, with a standard error of the mean of 9.6% at 12.5 ng/g. Clearly, our use of a capillary column with splitless injection gave a considerably higher sensitivity and precision. With an extraction procedure similar to ours, these authors had problems

with emulsions forming in the organic phase and resorted to column extractions on Clin Elut™. The use of a nearly saturated solution of trisodium phosphate instead of dilute sodium hydroxide for alkalinization of the homogenates did in our case suppress the formation of emulsions, and high recoveries of drugs were readily obtained from all tissues.

As lipophilic bases, F, A and S can be expected to adsorb to glass surfaces. Procedures for avoiding this include silanization of the glassware [3,4] and preparation of dilute F stock solution in human plasma [3]. The use of a competing hydrophobic amine, decylamine, in all stock solutions (except those in methanol) proved effective in our assay, as previously in an assay for quinacrine, a drug very prone to adsorption [15]. In addition, to avoid activation or contamination of the glass surfaces, the glassware was never washed with strong acids or detergent, but only with distilled water, methanol and ethanol.

F, A and S are chemically stable in solution and no breakdown of F or A was observed over 1 h in samples of liver and kidney, the two most important sites of drug metabolism. This is in keeping with the findings [14,16] that homogenates of these and other tissues will not metabolize F without the addition of suitable co-factors. However, metabolism that quickly exhausted the supply of these co-factors may have taken place after the sacrifice of the animal but before the organ could be frozen. The practical implication of the results is therefore only that the dissection of the rats can be done without unreasonable hurry. In practice it takes about 20 min from the decapitation of the rat until all samples are collected and frozen.

Tissue concentrations of F have previously been estimated by radiotracer techniques [12,13]. In keeping with these reports, F was found to partition extensively into tissue. The tissue concentrations of F shown in Table III and Fig. 1 were found in rats whose concomitant steady-state blood concentrations were 1.2–2.6 ng/ml. For A, partitioning into tissue is much less extensive, since the concomitant blood concentrations were 63–132 ng/ml. A complete tissue distribution study on F and A will be published elsewhere.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Mrs. Joanna Field for advice and assistance with animal surgery and handling, Mrs. Sandra Harapat for practical assistance in the laboratory and Miss Sunny Pinneau for typing and editorial assistance with the manuscript. This work was supported by the National Institute on Aging, Grants R01-AGO3104 and R01-AGO4594, the Anesthesia/Pharmacology Research Foundation and the Veterans Administration Merit Review.

REFERENCES

1 L.E. Mather, Clin. Pharmacokin., 8 (1983) 422–446.
2 H.H. Van Rooy, N.P.E. Vermeulen and J.G. Bovill, J. Chromatogr., 223 (1981) 85–93.
3 T.J. Gillespie, A.J. Gandolfi, R.M Maiorino and R.W. Vaughan, J. Anal Toxicol., 5 (1981) 133–137.

4 J.A. Phipps, M.A. Sabourin, W Buckingham and L. Strunin, J. Chromatogr., 272 (1983) 392-395.
5 S.R. Kowalski, G.K Gourlay, D.A. Cherry and C.F. McLean, J. Pharmacol. Methods, 18 (1987) 347-355.
6 R.J.H. Woestenborghs, D R. Stanski, J.C Scott and J.J.P. Heykants, Anesthesiology, 67 (1987) 85-90.
7 S.N. Lin, T.P.F. Wang, R.M Caprioli and B.P.N. Mo, J Pharm. Sci., 70 (1981) 1276-1279.
8 R Woestenborghs, L. Michielsen and J Heykants, J. Chromatogr., 224 (1981) 122-127.
9 K. Kumar, D.J. Morgan and D.P. Crankshaw, J. Chromatogr., 419 (1987) 464-468.
10 M Michiels, R Hendriks and J. Heykants, Eur. J. Clin. Pharmacol., 12 (1977) 153-158.
11 M Michiels, R. Hendriks and J. Heykants, J. Pharm. Pharmacol., 35 (1983) 86-93.
12 R. Hess, A. Herz and K. Friedel, J. Pharmacol. Exp. Ther., 179 (1971) 474-484.
13 C.C. Hug and M.R. Murphy, Anesthesiology, 55 (1981) 369-375.
14 K.A. Lehmann, G. Moseler and D. Daub, Anaesthesist, 30 (1981) 461-466
15 S. Bjorkman and L.O. Elisson, J. Chromatogr., 420 (1987) 341-348.
16 K.A. Lehmann, C. Weski, L. Hunger, C. Heinrich and D. Daub, Anaesthesist, 31 (1982) 221-227