ELSEVIER

Journal of Chromatography B, 769 (2002) 227–233

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Very sensitive and specific determination of sufentanil in human serum applying liquid chromatography–two stage mass spectrometry

J. Martens-Lobenhoffer*

Institute of Clinical Pharmacology, Otto-von-Guericke-University Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany

Received 16 July 2001; received in revised form 13 November 2001; accepted 21 November 2001

Abstract

Sufentanil is the most potent synthetic opioid currently in use. Extremely low serum concentrations have to be determined for therapeutic drug monitoring and to support brain death diagnosis by excluding opioid induced coma. The described method utilizes a HPLC–MS–MS system with an electrospray ion source and an ion-trap mass spectrometer. The serum samples were extracted under basic conditions with toluene–2-propanol (10:1, v/v). Chromatographic separation was achieved on a RP-18 70×2 mm column with a 0.02% trifluoroacetic acid in a water–acetonitrile gradient as mobile phase. The limits of detection and quantification are 3 and 10 pg/ml, respectively. At the limit of quantification, the intra-day relative standard deviation of the assay is 12.6% and the inter-day relative standard deviation is 14%.

Keywords: Sufentanil

1. Introduction

Sufentanil is the most potent synthetic opioid currently in use in anesthesia. In comparison to fentanyl, sufentanil has a more rapid onset and a shorter duration of action [1]. It is about 5–7-times more active but otherwise not significantly different in clinical response to fentanyl in various surgical interventions [2–4]. In a certain way, the high activity of sufentanil is a disadvantage since the determination of relevant serum concentrations are very difficult to achieve. Therapeutic serum concentrations are in the range of 0.5 to 10 ng/ml for anaesthesia, where artificial respiration is applied [5]. For analgesic purposes the serum concentrations are about a factor of 10 lower. Concentrations beyond these levels have to be determined for a secure exclusion of opioid induced coma in support of brain death diagnosis. Most of the pharmacokinetic studies were conducted using a radioimmunoassay [6]. Chromatographic methods for the determination of sufentanil in human serum or plasma utilizing gas chromatography–mass spectrometry (GC–MS) [7,8] or GC–nitrogen–phosphorus detection (NPD) [9] were also described. The later was criticized for not being selective enough and not being validated properly [10]. Sophisticated liquid–liquid extraction procedures [7,8] or as an alternative, solid-phase

*Tel.: +49-391-6713-060; fax: +49-391-6713-062.

E-mail address: jens.martens-lobenhoffer@medizin.uni-magdeburg.de (J. Martens-Lobenhoffer).

1570-0232/02/$ – see front matter
PII: S1570-0232(01)00569-4

extraction with ion-exchange resins [8] make the sample preparation laborious for this methods. To our knowledge, high-performance liquid chromatography (HPLC) assays for sufentanil were, until now, only applied to non-biological systems containing the pure drug [11–13] in relatively high concentrations.

In this paper an extremely sensitive assay for sufentanil in human serum is described. For maximum sensitivity and selectivity, an ion trap mass spectrometer working in the two-stage mode (MS–MS) equipped with an electrospray ionization (ESI) interface to the HPLC system is utilized. Due to the selectivity achieved with this detector, a relatively easy sample preparation with a one-step liquid–liquid extraction and a fast HPLC method is sufficient to detect sufentanil without interferences in concentrations as low as 3 pg/ml.

2. Experimental

2.1. Instrumentation

The HPLC part of the analytical system consists of an Agilent 1100 system (Waldbronn, Germany) comprising a degasser, a binary pump, an autosampler and a thermostatted column compartment, controlled by an Agilent 1100 control module. The chromatographic separation of the analytes took place in a Machery–Nagel Nucleosil CC 100-5 C_{18} HD 70×2 mm column (Düren, Germany). The analytes were detected by a ThermoFinnigan LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with an ESI source. Data were collected and analyzed by the ThermoFinnigan Xcalibur software package, revision 1.1.

2.2. Chemicals

Sufentanil and the internal standard (I.S.) fentanyl were obtained as pure substances in form of their citrate salts from US Pharmacopeia (Rockville, MD, USA) and Sigma (Steinheim, Germany), respectively. Trifluoroacetic acid was obtained from Riedel-de Haën (Seelze, Germany), acetonitrile in Ultra-Gradient-Grade from Baker (Gross-Gerau, Germany). Ultra pure water was produced in our laboratory by a Barnstead EASYpure UV system (Werner, Leverkusen, Germany). Toluene, 2-propanol and cyclohexane were obtained in analytical grade from Merck (Darmstadt, Germany).

2.3. Sample collection

Blood samples of about 5 ml were drawn form the cubital vein into glass containers without additives. Serum was obtained by separation of the blood cells by centrifugation at 2400 *g* for 10 min. Serum samples were either analyzed immediately or were frozen at −20 °C until analysis.

2.4. Calibration samples

A stock solution of sufentanil was prepared by dissolving 14.97 mg sufentanil citrate salt in 100 ml water, resulting in a concentration of 100 μg/ml of the free base. This stock solution was diluted with water in two steps by a factor of 10000 to get calibration solution A (10 ng/ml) and by another factor of 10 to get calibration solution B (1 ng/ml). These solutions remain stable for at least 4 months while stored in an refrigerator at 5 °C. Volumes of 10, 20 and 50 μl of solution B and 10, 20 and 50 μl of solution A were added up to 1 ml with blank human serum to obtain calibration samples in a concentration range of 10 to 500 pg/ml.

2.5. Quality control samples

Quality control samples were prepared from drug-free human serum in three concentration levels (10, 100 and 500 pg/ml). A sufficient number of 1 ml samples were portioned and frozen at −20 °C until analysis.

2.6. Sample preparation

To 1 ml serum 100 μl of the I.S. solution (10 ng/ml fentanyl in water) and 100 μl of 1 *M* NaOH were added. This mixture was extracted twice by 2 ml toluene together with 0.2 ml of 2-propanol for 20 min on a roller-shaker. Phase separation was achieved by centrifugation at 4000 *g* for 5 min. The combined organic phases were evaporated in vacuum. The residue was dissolved in 10 μl cyclohexane and 200 μl 1 *M* HCl. After intense shaking for 10 s and phase separation by centrifugation for 2

min at 4000 *g* the aqueous phase was forwarded to the HPLC system.

2.7. Chromatographic conditions and MS detector settings

The chromatographic separation of the analytes was accomplished by gradient elution starting with 0.02% trifluoroacetic acid in water–acetonitrile (85:15). The percentage of acetonitrile was raised in the first 0.4 min to 50% and was held constant up to 6.5 min. Finally, it was turned back in 0.4 min to the initial value of 15%. After the end of the run at 7 min a column reequilibration time of 3 min before the next injection was necessary. The flow-rate was set to 0.4 ml/min at the start of the run and was slowed down to 0.15 ml/min at 2.4 min. At 6.3 min, the flow-rate was raised back to the initial value of 0.4 ml/min. With a column temperature of 30 °C, the retention times of the I.S. fentanyl and sufentanil were 4.8 and 5.3 min, respectively.

The settings for the ESI ion source were as follows: the capillary temperature was set to 250 °C, the capillary voltage was set to 3 V and an ion-spray-voltage of 0.2 kV was applied. The sheath gas and the auxiliary gas flow-rates were set to 40 and 10 units (about 1.1 and 3 l/min), respectively. A divert valve directs the HPLC-flow in the first 3.5 min of the chromatographic run to the waste container and afterwards to the ion source. During the first 3.5 min it is possible to save gas by turning the sheath gas and the auxiliary gas to lower values. The ESI source was working in the ''positive mode'', producing positive charged ions in the form of $[H^+]$ adduct ions. From the ions generated in this way, those with mass-charge ratios of m/z 337 and 387 were trapped, representing the I.S. fentanyl and sufentanil, respectively. These trapped ions were further fragmented by collision induced dissociation. The ion m/z 337 dissociated at a relative collision energy of 30% [this corresponds to 1.5 V peak-to-peak resonance excitation radio frequency (RF) voltage] into its main fragments with relative masses of 188, 216 and 281. The ion m/z 387 dissociated at a relative collision energy of 24% to m/z 238 and 355. The chromatographic traces of these fragment ions were monitored in the selected reaction monitoring mode of the mass spectrometer. All samples were injected twice and the quantification results were averaged.

3. Results and discussion

3.1. Sample preparation

The extraction of human serum with toluene–2-propanol under basic conditions lead to high recoveries for the analytes, but also for lot of other lipophilic substances. To get rid of these substances which interfere with the chromatographic separation and the ion-forming process in the ESI source, the acidic extracts have to be washed with cyclohexane in a last preparation step. The overall sample preparation process yields sufficiently clean extracts with analyte recoveries of 75% for sufentanil and 74% for the I.S. fentanyl. The extraction yield was determined by comparing extracts from spiked serum with extracts from blank serum, spiked with the analytes after the extraction in concentrations corresponding to 100% extraction yield.

3.2. Chromatography and mass spectrometry

The limitations of the mobile phase of the chromatographic system, which are set by the requirements of the ESI source of the mass spectrometer, lead to quite difficult conditions. The mobile phase has to have a low buffer concentration at a pH value where the analytes exist in ionized form (achieved with 0.02% trifluoroacetic acid, pH≈2.5) and the percentage of the organic modifier has to be about 50% for low surface tension and good evaporatability. To meet these conditions, the analytes have to be injected in a mobile phase with only 15% acetonitrile to be separated form highly polar interferences and then the ratio of acetonitrile has to be raised to 50% to refocus the analytes and to get an ESI-compatible mobile phase. The flow-rate is slowed down to 0.15 ml/min in the elution time window to optimize the ion-forming conditions in the ESI source, and speed up to 0.4 ml/min at all other times to reduce the overall cycle time of the chromatographic separation to an acceptable value.

Small matrix effects on the ionization efficiencies of sufentanil and the I.S. fentanyl were observed, but both analytes were affected by the matrix in similar manner and therefore the quantification was not compromised. This is proven in patients were the original sample and a sample diluted with blank serum were extracted. Although the areas of the I.S.

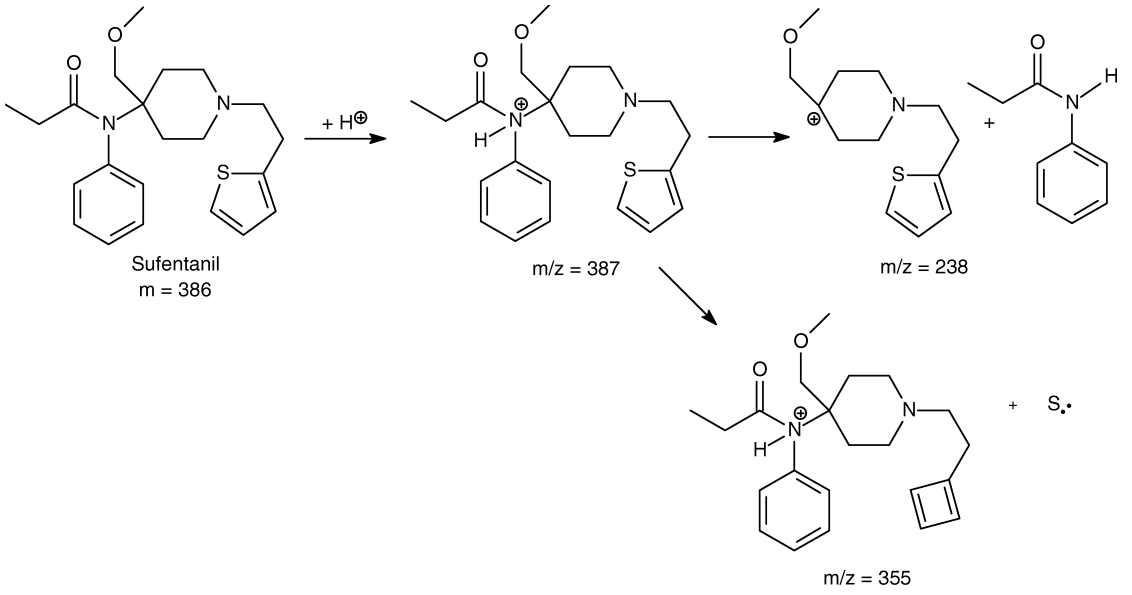

Fig. 1. Proposed dissociation path of sufentanil in an ESI-MS–MS experiment.

in the two samples can differ significantly, the quantification of sufentanil yields the same result in both samples (with respect to the dilution factor). Furthermore, the validation of the method was carried out with different lots of blank human serum with no significant consequences for precision and accuracy.

The very soft ionization process in the ESI source

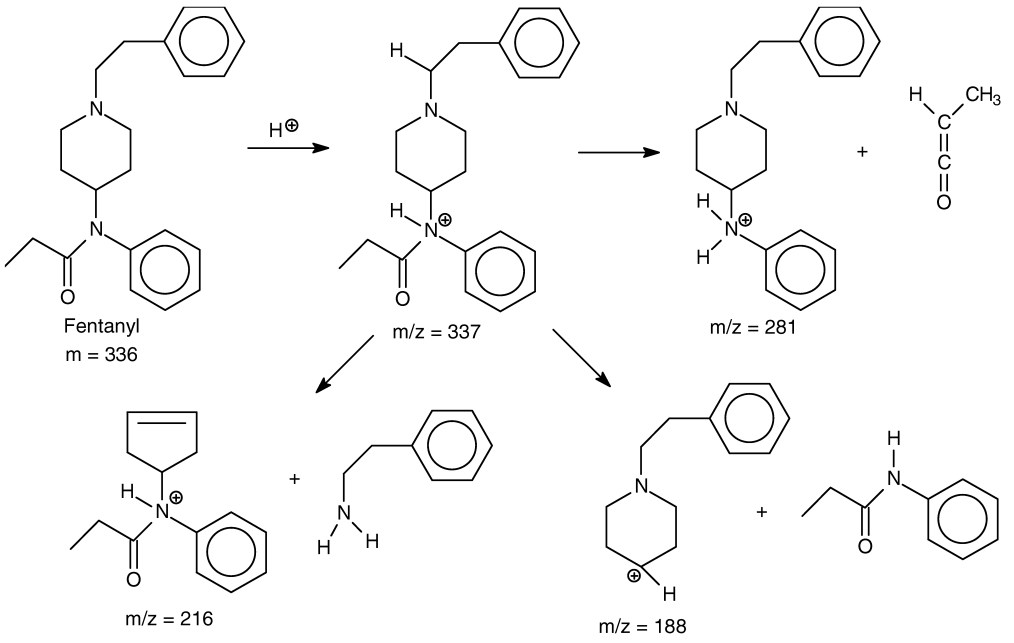

Fig. 2. Proposed dissociation path of the I.S. fentanyl in an ESI-MS–MS experiment.

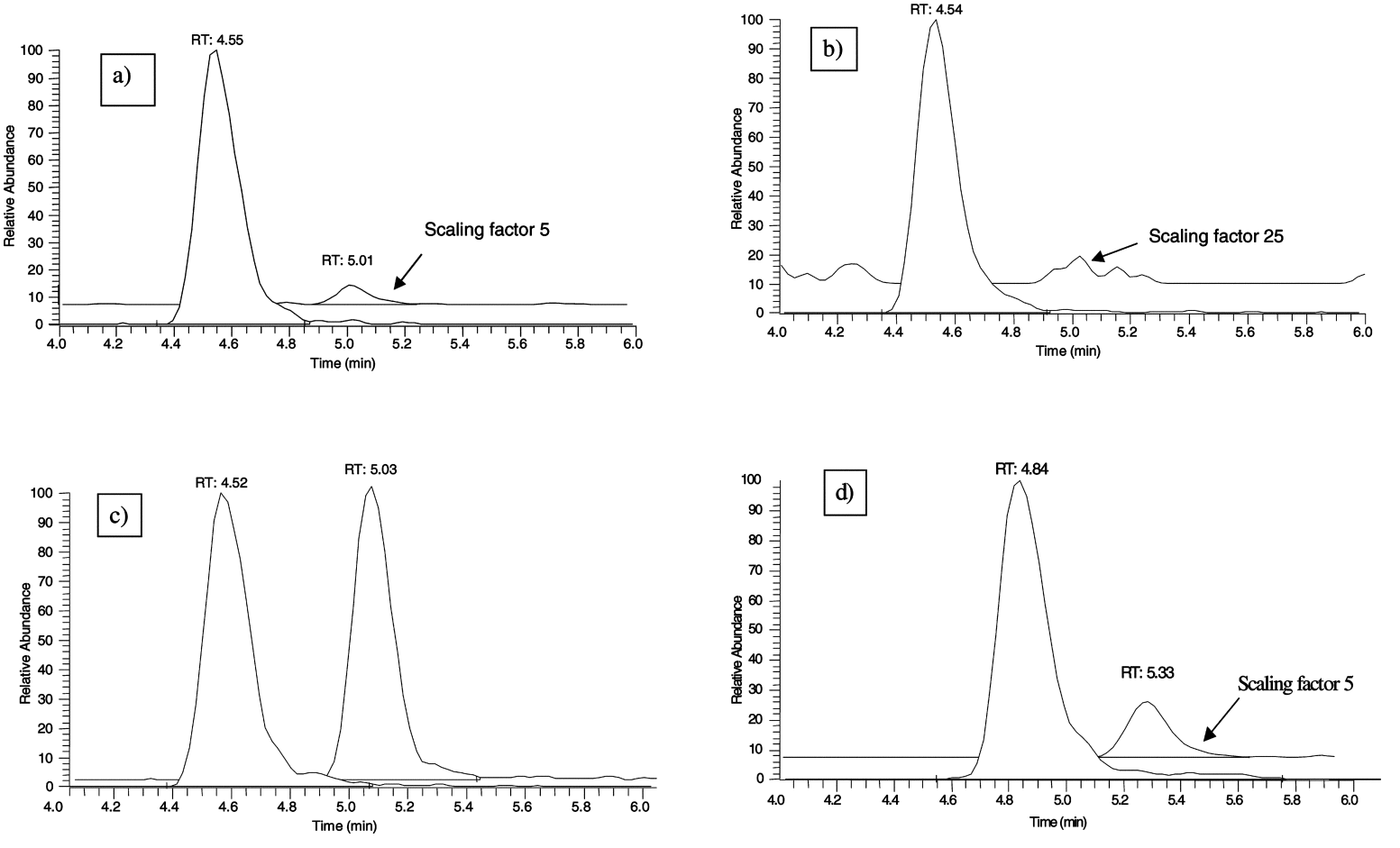

Fig. 3. Chromatograms obtained by the described method. The lower chromatographic trace corresponds to the I.S. fentanyl (m/z 337 trapped, product ions m/z 188, 218 and 281 observed), and the upper trace to sufentanil (m/z 387 trapped, m/z 238 and 355 observed). Chromatograms: (a) calibration sample 10 pg/ml, (b) blank human serum, (c) calibration sample 500 pg/ml, (d) patient sample containing 720 pg/ml, diluted factor 10 with blank human serum.

Table 1
Intra- and inter-day precision and accuracy

Concentration (pg/ml)	Intra-day			Inter-day		
	n	RSD (%)	Accuracy (%)	*n*	RSD (%)	Accuracy (%)
10	9	12.6	−11.6	6	14.0	0.69
20	9	3.22	5.14			
100				5	9.92	−5.71
500	10	7.73	3.98	6	6.33	−9.73

produces the $[M+H]^+$ quasimolecular ions of the analytes. No sodium or other adducts or the formation of dimers are observed. The quasimolecular ion with the mass of m/z 387 represents sufentanil. After trapping, the collision induced dissociation results in two main product ions (Fig. 1): the one with the mass 355 (relative intensity 75%) represents probably the loss of the sulfur atom and the other with the mass 238 (relative intensity 100%) may be formed by the loss of neutral *N*-propa-1-on-aniline. The conditions for the dissociation of the quasimolecular ion m/z 337 of the I.S. fentanyl are quite similar (Fig. 2): the most prominent product ion may be again formed by the loss of neutral *N*-propa-1-on-aniline (m/z 188, relative intensity 100%). Less intense signals are probably from the piperidino-ring-opening with the loss of the neutral alkyl-nitrogen moiety, leaving an ion with the mass of m/z 216 (relative intensity 12%) and the neutral loss of methylketene by cleavage of the anilinonitrogen-acid amide bonding (m/z 281, relative intensity 6%). Evidence for this proposed dissociation pattern is gained by the further fragmentation of the product ions in MS^3 experiments, in which the observed fragment ions confirm the assumed structures of the product ions (data not shown). In the chromatograms, the traces of the product ions produced from the quasimolecular ions are added up with regard to their parent ions to enhance the signal-to-noise ratio.

Chromatograms obtained under the described conditions are depicted in Fig. 3. Neither endogenous substances nor the main metabolites of sufentanil, *N*-desalkylsufentanil and *O*-desmethylsufentanil [1], which are present in patient samples (Fig. 3d), do interfere with the peaks of the analytes. The S/N ratio of sufentanil is about 10 in the 10 pg/ml calibration sample and therefore well above the limit of detection.

3.3. Calibration function and limits of detection and quantification

For calibration, the quotients of the peak areas of sufentanil and the I.S. fentanyl are correlated with the concentrations of sufentanil. The calibration function is linear in the range of 10 to 500 pg/ml. The parameters of the calibration function ($n=6$) are: slope$=1.967\cdot10^{-3}$ ($\pm3.161\cdot10^{-5}$), intercept$=1.265\cdot10^{-3}$ ($\pm7.10\cdot10^{-3}$) and correlation coefficient $r=0.9995$. The limit of detection is about 3 pg/ml, where the S/N ratio is 3. The limit of quantification is 10 pg/ml. This concentration is equal to the lowest calibration level where the repeatability results in an RSD of 12.6% (see Section 3.4)

3.4. Intra-day precision, inter-day precision and accuracy

All samples have to be injected twice and the quantification results have to be averaged. This is necessary due to the well known quantification problems of an ESI ion source [14] and helps to improve the precision and accuracy to acceptable values. The validation data regarding intra-day as well as inter-day precision and accuracy are summarized in Table 1. The inter-day precision and accuracy were derived from the results of the quality control samples used in normal sequences. All values are satisfying the requirements for a validated method [15].

4. Conclusion

The described method is easy, robust and relatively quick. Due to the LC–MS equipment, which is nowadays standard in many laboratories, the method

is sensitive and selective enough to determine concentration as low as 10 pg/ml. It is therefore capable to monitor serum concentrations in the awakening phase after a sufentanil narcosis (especially useful in cases of delayed elimination of sufentanil) and to support brain death diagnosis by secure exclusion of an opioid induced coma. The method has been applied successfully to patient samples (for example, see Fig. 3d).

References

[1] C. Rosow, Pharmacotherapy 4 (1984) 11.
[2] A. Wilhelm, H. Dieleman, Pharm. World Sci. 16 (1994) 7.
[3] I. Thomson, R. Hudson, M. Rosenbloom, R. Meathaerall, Can. J. Anaesth. 34 (1987) 227.
[4] J. Bovill, P. Warren, J. Schuller, H. van Wezel, M. Hoenefeld, Anesth. Analg. 63 (1984) 1081.
[5] M. Schulz, A. Schmoldt, Pharmazie 52 (1997) 895.
[6] M. Michiels, R. Hendriks, J. Heykants, J. Pharm. Pharmacol. 35 (1983) 86.
[7] R. Woestenborghs, P. Timmerman, M.-L. Cornelissen, F. van Rompaey, E. Gepts, F. Camu, J. Heykants, D. Stanski, Anesthesiology 80 (1994) 666.
[8] C. Dufresne, P. Favetta, C. Paradis, R. Boulieu, Clin. Chem. 47 (2001) 600.
[9] S. Weldon, D. Perry, R. Cork, A. Gandolfi, Anesthesiology 63 (1985) 684.
[10] J. Heykants, R. Woestenborghs, P. Timmerman, Anesthesiology 65 (1986) 112.
[11] P. Roos, J. Glerum, J. Meilink, L. Zwang, Pharm. World Sci. 18 (1993) 139.
[12] K. Movig, M. Langen, A. Egberts, J. Pharm. Biomed. Anal. 21 (1999) 845.
[13] J. Lambropoulos, G. Spanos, N. Lazaridis, J. Pharm. Biomed. Anal. 23 (2000) 421.
[14] W.M.A. Niessen, J. Chromatogr. A 856 (1999) 179.
[15] W. Lindner, I.W. Wainer, J. Chromatogr. B 707 (1998) 1.