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The combined use of high-performance liquid chromatography and radioimmunoassay for the bioanalysis of nicomorphine and its metabolites

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Abstract: Methods have been developed for the determination of nicomorphine using reversed-phase HPLC with UV detection; for the simultaneous assay of morphine and mononicotinoylmorphine by a coupled normal-phase HPLC–radioimmunoassay method; and for conjugates of morphine and mononicotinoylmorphine by radioimmunoassay. The methods have been evaluated and applied to a pharmacokinetic study of nicomorphine administered intramuscularly.

Keywords: *Nicomorphine; morphine; mononicotinoylmorphine; pharmacokinetics; reversed-phase high-performance liquid chromatography; normal-phase high-performance liquid chromatography; coupled radioimmunoassay–HPLC methods.*

Introduction

Nicomorphine (Vilan®, I in Fig. 1) is the 3,6-dinicotinoyl ester of morphine and may be described as a morphinomimetic prodrug having an approximate equipotency with morphine, although with a quicker onset and a longer duration of action [1]. It may also have fewer side-effects than morphine [2]. The two ester groups of nicomorphine make it highly lipophilic, though subject to both chemical and enzymatic hydrolysis and biotransformation. Opiate receptor binding studies have shown that the central analgesic activity of nicomorphine can be attributed to its two main metabolites, 6-mononicotinoylmorphine (II in Fig. 1) and morphine (III in Fig. 1) [3]. Although the compound is frequently used clinically, no pharmacokinetic and/or metabolic studies in man have been described. However a pharmacokinetic study in the rat, using radioactive labelled nicomorphine, has shown that nicomorphine rapidly disappears from the blood and enters the brain, with the concomitant appearance of 6-mononicotinoylmorphine and morphine in the blood and the brain [4]. To investigate the pharmacokinetics of nicomorphine in man using non-radiolabelled nicomorphine, an analytical method that determines nicomorphine, 6-mononicotinoylmorphine, morphine, and the conjugates of the two latter compounds, all in plasma at concentrations of 1–100 ng/ml, is required.

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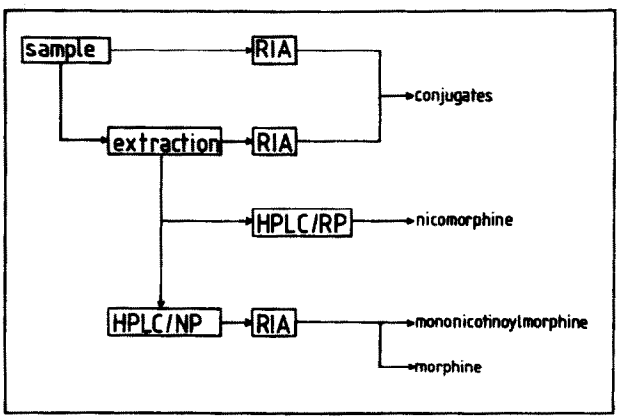
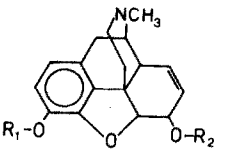
Of the methods available for the determination of morphinomimetics at such low concentrations electrochemical detection in combination with reversed-phase chromatography has been described [5, 6]. However, nicomorphine itself is not electrochemically active, and due to interferences by other drugs, the assay of its two main metabolites, both of which are electrochemically active, is not possible by this method. Radioimmunoassay (RIA) has been frequently described for the analysis of morphinomimetics [7], but its low specificity has been regarded as a disadvantage. The combination of high-performance liquid chromatography (HPLC) with radioimmunoassay may, however, provide the necessary specificity.

The present paper describes the development of three methods: a reversed-phase assay of nicomorphine using HPLC and UV detection, normal-phase HPLC coupled to radioimmunoassay for the determination of 6-mononicotinoylmorphine and morphine, and an RIA method which permits the estimation of the conjugates of the two latter compounds. Preliminary data describing the pharmacokinetics of intramuscular nicomorphine in patients are presented.

Scheme 1





I (nicomorphine) $R_1 = R_2 =$ nicotinoyl : -CO- 

II $R_1 = H$, $R_2 =$ nicotinoyl .

III (morphine) $R_1 = R_2 = H$.

IV (pindolol) :

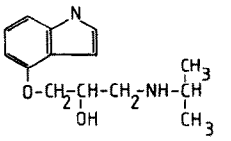


Figure 1
Chemical structures of nicomorphine (I), 6-mononicotinoylmorphine (II), morphine (III) and pindolol (IV).

Experimental

Apparatus

The liquid chromatograph consisted of a double piston reciprocating Pye Unicam LC-3 XP pump, a Rheodyne 7010 injection valve with a 100-microlitre sample loop, a UV detector (Pye Unicam LC–UV), a linear potentiometric recorder (Goerz, Servogor, type R542), and an electronic integrator (Autolab IV) controlling a fraction collector (Gilson type mini MTDC). Stainless steel columns (i.d. 4.6 mm, length 100 mm) were used. The wavelength of detection was 265 nm. ^{125}I radioactivity was measured in a liquid scintillation counter (Packard Tricarb type 3255).

Materials

De-ionized water was used in all experiments. The radioimmunoassay was performed using Abuscreen® (Roche, Nutley) kits with ^{125}I-morphine as the radioligand. The scintillation fluid was Hydrocount (Baker, The Netherlands). Extraction columns, (Extube^{tm}, Clin-Elut CE1003) were obtained from Analytichem International through Jones Chromatography, (Mid Glamorgan, UK). Hypersil and Hypersil ODS (Shandon) were used as column supports for HPLC. Nicomorphine HCl, 6-mononicotinoylmorphine HCl and morphine HCl.$3H_2O$ were obtained through Nourypharma (Oss, The Netherlands). Pindolol, used as an internal standard in HPLC, was a gift from Sandoz, (Basel, Switzerland). Morphine-3-glucuronide was obtained from Sigma (USA). All other chemicals were of analytical grade.

Solutions

Standard solutions of the drugs and metabolites were prepared in methanol and further diluted with a phosphate buffer solution (pH = 7.0, 50 mM). A standard solution of morphine-3-glucuronide was prepared directly in the phosphate buffer. Aliquots of these standard solutions were added to blank pooled plasma samples to establish calibration curves. Plasma samples were diluted at least 1:1 since a higher protein content was found to have an adverse influence on the RIA.

Extraction

Aliquots of the plasma samples (0.1–1.0 ml) were added to the top of an extraction column, followed by an equal volume of a freshly prepared buffer solution (sodium bicarbonate, 1 M, adjusted to pH 8.9 with sodium hydroxide). After 3 min, 2 6-ml lots of a mixture of chloroform and 2-propanol (90:10, v/v) were applied to the extraction column, and the compounds of interest were eluted into glass-stoppered centrifuge tubes. The organic extract was evaporated in a water bath at 50°C.

Storage

Plasma samples and dried residues, obtained after extraction or HPLC and reconstituted in phosphate buffer (pH 7.0, 50 mM) or in methanol, were stored at −23°C.

Chromatography

HPLC columns were packed using a tetrachloromethane–methanol slurry mixture (95:5 v/v), with methanol as the packing fluid. After washing the column and the precolumn with aqueous methanol (1:1 v/v) or with chloroform, the columns were conditioned with

the mobile phase. For the determination of nicomorphine in plasma of patients and in fresh human blood, reversed-phase chromatography was used with Hypersil ODS as the column packing and with a mobile phase consisting of a mixture of 2-propanol and water (3:7 v/v), containing 0.35 mM sodium dodecyl sulphate, 0.05 M sodium acetate and 0.15 M acetic acid. The flow rate was 0.45 ml/min. Samples were extracted as described above and the residue dissolved in the mobile phase. A 100-μl portion was injected on to the HPLC column.

For the determination of the metabolites, normal-phase chromatography coupled off-line to a radioimmunoassay was used. Here, the column packing was Hypersil 5 μm, with a mobile phase of chloroform–methanol (93:7, v/v) containing 0.01 M ammonium acetate. The flow rate was 0.65 ml/min. The extracted and evaporated samples were dissolved in the mobile phase containing 5 μg/ml of the internal standard. UV detection of this internal standard (IV in Fig. 1) was necessary to control the stability of the chromatographic system, as only the 6-mononicotinoyl morphine and morphine fractions were collected.

Radioimmunoassay

All samples were diluted to contain not more than 4 ng of morphine and 6 ng of either 6-mononicotinoylmorphine or morphine-3-glucuronide in 100 μl. Aliquots of these samples (100 μl) were pipetted into glass stoppered centrifuge tubes (13 × 80 mm) and subjected to RIA. Antibody solution (200 μl) was carefully pipetted into each solution avoiding contact with the glass wall before mixing with the bulk of the solution. Then 200 μl of the radioligand solution were added to each tube, followed by mixing on a vortex mixer and incubation for 15 min at room temperature. A 0.5 ml aliquot of a saturated solution of ammonium sulphate was then added, and the mixture was further incubated for 15 min to complete precipitation. The tube was centrifuged in a swinging bucket rotor (Sorvall type HS-4) for 15 min at 1732 ***g*** in a Sorvall RC-5 high speed centrifuge at 20°C. After centrifugation, 0.5 ml of the supernatant was carefully removed, avoiding the transfer of small precipitate particles and mixed with 9 ml of scintillation fluid. The sample was then counted using a liquid scintillation counter. The radioimmunoassay was carried out in duplicate and a logit–log transformation was used to calculate the cross-reactivity of the compounds under investigation.

Radioimmunoassay for the study of the conjugates

An aliquot of each plasma sample was diluted with phosphate buffer as described above. To 100 μl of this solution the radioimmunoassay reagents were added and further treated as above. The difference between the value found in this assay and the value obtained after extraction of the same sample, followed by RIA, was taken as the amount of conjugate in the sample.

Results and Discussion

The scheme describes the method developed for the analysis of nicomorphine, its metabolites and their conjugates. HPLC–radioimmunoassay combinations have been described previously for the analysis of LSD [8] and for Δ^9-tetrahydrocannabinol [9], while more recently a combined reversed-phase HPLC method and radioimmunoassay has been described for the assay of morphine, codeine and their metabolites in biological fluids [10]. In the present study, however, it was found that the components of reversed-phase eluents affect the radioimmunoassay in an irreproducible way, so normal-phase

chromatography was used instead. In addition it was found that nicomorphine could not be determined by radioimmunoassay as it did not show cross-reactivity with the morphine antibody of the Abuscreen kit.

Liquid–liquid extraction at pH 8.9 of nicomorphine and its metabolites from plasma has been described [4]. In the present study the extraction procedure was based on liquid–liquid chromatography, with a diatomite earth as column support, buffer pH 8.9 as the stationary phase and chloroform–isopropanol as the mobile phase. The three compounds could be easily recovered without any decomposition. Evaporation of the organic fluid using a gentle stream of nitrogen did not result in any loss of the solutes. However, storage of the dried residue for several days did result in substantial loss: in the case of morphine this was 33% after 1 day and as high as 90% after a 5-day period. Possibly the extremely small amounts of finely divided morphine obtained after extraction favour its evaporation. The residues were therefore dissolved in methanol or phosphate buffer (pH 7, 50 mM) and stored at −23°C. Under these conditions no loss of solutes was observed. The recovery and reproducibility of the column extraction were determined by spiking blank plasma samples with different amounts of morphine or 6-mononicotinoylmorphine. Using RIA the recovery of 1.5 ng of these solutes was 98.7% (R.S.D. 6.1%, $n = 5$), while using HPLC the recovery of 10.0 μg morphine was 94.2% (R.S.D. 3.5%, $n = 5$). The difference in precision was probably due to the method of analysis. The recovery and reproducibility after collecting fractions eluted from the HPLC column and assaying with RIA was, for 1.5 ng morphine, 102.7% (R.S.D. 5.2%, $n = 6$). Similar results were found for the recovery and reproducibility of 6-mononicotinoylmorphine. The precision of the retention times of 6-mononicotinoylmorphine, morphine and the internal standard pindolol was measured by repeated injections of solutions of these compounds. From the result (R.S.D. less than 0.6%) it was concluded that the fraction collection could be performed reproducibly.

To improve the selectivity of the radioimmunoassay, normal-phase chromatography was chosen as the separation mode. The advantage of this compared with reversed-phase chromatography is the compatibility of the eluent with the radioimmunoassay. Normal-phase chromatography has been previously described for the separation of opium alkaloids [11]. However, in this study chloroform, methanol and ammonium acetate were selected as constituents of the mobile phase, as these are either more readily evaporated or do not interfere with the radioimmunoassay. The large dependence of the capacity factor on the concentration of methanol and ammonium acetate indicates that careful preparation of the mobile phase is necessary. By using an internal standard control over the collection of fractions of the eluent was achieved: possible contamination of the radioimmunoassay was avoided by selecting a non-opioid as internal standard (pindolol, IV in Fig. 1).

Nicomorphine was assayed using a reversed-phase ion-pair HPLC method with sodium dodecyl sulphate as the ion-pair reagent and either methanol or propanol as the organic modifier. Such ion-pair containing mobile phases have been used for the reversed-phase separation of catecholamines [12], butyrophenones and diphenylalkylpiperidines [13] and amino acids [14]. They allow easy adjustment of selectivity [15], result in high column efficiencies, and are usually used at pH 3–5. Such conditions are favourable for the chromatography of hydrolytically labile compounds such as nicomorphine (Table 1), since hydrogen- and hydroxyl-ion catalysed degradation is often at a minimum at pH 3–5 [16]. Unfortunately, these reversed-phase systems are incompatible with radioimmunoassay techniques, because of the presence of the surface

Table 1
Chemical and enzymatic hydrolysis

	$t_{1/2}$ (min)	
	Nicomorphine	6-Mononicotinoylmorphine
Buffer pH = 7.4, 37°C	366	3700
Fresh human blood, 37°C	30	240
P_2 suspensions of rat brain homogenate, 25°C	807	>6000

active pairing ion and/or an alcohol. In addition, evaporation to dryness is not possible due to the thermally labile character of the compounds under investigation. No attempt was made to evaluate freeze-drying procedures. Figure 2 shows chromatograms of a normal-phase separation and a reversed-phase separation.

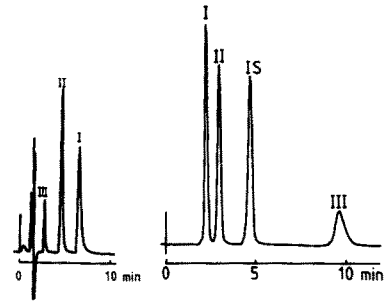


Figure 2
Separation of test mixtures of nicomorphine and its metabolites. Roman numerals as in Fig. 1. Left: reversed-phase separation; right: normal-phase separation.

The limits of detection of HPLC with electrochemical detection are similar to the limits of detection of the radioimmunoassay (0.2 ng and 0.1 ng respectively for morphine), but radioimmunoassay was chosen as the detection method in this work since it did not appear to be subject to interferences from other drugs, which are generally administered with nicomorphine (e.g. diazepam, droperidol, bupivacaine, adrenaline, pancuronium, thiopentone sodium and methotrimeprazene). As previously described, neither radioimmunoassay nor HPLC with electrochemical detection could be applied to the determination of nicomorphine. Using UV spectrometry the detection limits for this compound at 210 and 265 nm were 2 and 8 ng respectively, corresponding to a detection limit of 4 and 16 ng/ml in plasma. Because of spectral interferences the 265 nm was the better wavelength in practice.

The precision and the linearity of the HPLC method for the determination of nicomorphine in plasma were determined by injecting 100 µl samples containing 20–170 ng of nicomorphine. The regression line of peak height vs injected amount was linear up to 170 ng, with a correlation coefficient, r, of 0.9998. The precision was measured by repeated ($n = 5$) injections of samples containing 165 ng nicomorphine and was found to be 3.5% R.S.D.

The commercially available radioimmunoassay for morphine (Abuscreen®) has been applied not only to the determination of morphine [7, 17–19], but also to the determination of its conjugates [17] and of hydrocodone and hydromorphone [18]. Because of the low selectivity of the antibody for morphine derivatives modified at the 3-

or 6-positions [10] the use of the radioimmunoassay in the determination of the metabolites of nicomorphine was studied. From the transformed calibration curves for morphine, 6-mononicotinoylmorphine and morphine-3-glucuronide the relative cross-reactivities were calculated to 1.00, 0.93, 0.89 respectively on a molar basis. This cross-reactivity makes it possible to measure the conjugates directly in plasma, provided that the results can be corrected for the presence of morphine and 6-mononicotinoylmorphine. This can be done by performing an extraction step and applying the radioimmunoassay to the residue thus obtained. The results of the assay show that the concentrations of the conjugated products are 10–20 fold higher than the concentration of morphine or 6-nicotinoylmorphine. However, the identity of the conjugates was not further investigated. Dilution of plasma samples was necessary during the direct assay of the conjugates to avoid exceeding the upper limit of linear calibration. Diluting all plasma samples at least 1:1 also avoided the interference of plasma proteins (Fig. 3). The precision of the radioimmunoassay was determined by assaying 1.5 ng of morphine and 3.0 ng of 6-mononicotinoylmorphine added to 100 microlitres of plasma and was found to be 4.1% (n = 5) and 5.2% (n = 4) respectively (R.S.D.). The precision was greatly affected by the formation of a solid pellet during centrifugation: the whirling of particles from the pellet into the supernatant sometimes caused very high errors. Addition of a second antibody might reduce this interference, but a second antibody was not available in the RIA kit used in this work.

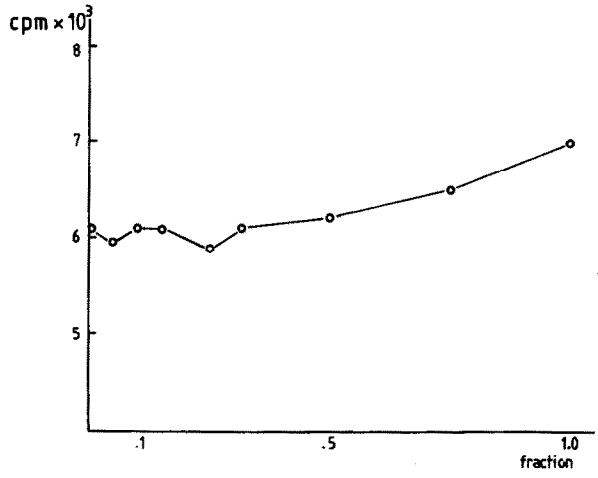


Figure 3
Dependence of the immunoassay response on the plasma fraction in the sample.

Plasma levels of nicomorphine and its metabolites after intramuscular administration of a 10 mg dose of nicomorphine to 5 patients were investigated. Nicomorphine could not be detected in the first blood sample taken 10 min after administration (Fig. 4). Although it is a labile compound, with a half-life in fresh human blood of about 30 min at 37°C (Table 1), it is also lipophilic (log P = 2.17, Table 2). It is therefore highly probable that it was not detected because of rapid distribution to tissues and organs. This view is supported by the rapid disappearance of the even less stable diacetylmorphine ($t_{½}$ = 8 min, fresh dog plasma, 25°C) after intravenous injection [20, 21]. Plasma levels of 6-mononicotinoylmorphine could be determined up to 60 min after administration. The plasma elimination half-life was 15 min (mean value for 3 patients), while a maximum

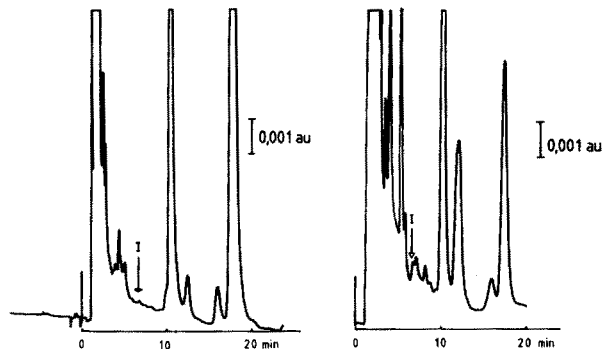


Figure 4
Chromatograms of extracts of a blank plasma sample (left), and of a plasma sample taken 10 min after injection (right). Reversed-phase separation. Arrow indicates the position of nicomorphine (I).

Table 2
Partition coefficients. Octanol–water, determined by HPLC [23]

Compound	log P
Morphine	0.45
6-Mononicotinoylmorphine	1.44
Nicomorphine	2.17

plasma level could be observed *ca.* 10–20 min after administration. This indicates that the appearance of the monoester in plasma is not an instantaneous process. As expected, the concentration in plasma of the polar metabolite morphine is higher than that of the monoester. The mean plasma elimination half-life of morphine was 2.1 h (n = 3), and was calculated using a two-exponential model to describe the plasma concentration-time profile. Here also a maximum plasma level was observed after *ca.* 20–30 min, showing that the decomposition of nicomorphine through the monoester to morphine is a gradual process. Figure 5 shows the time course of the plasma concentration of morphine and of the monoester. Figure 6 shows the concentration of the conjugates vs time, the concentrations being, as expected, 10–20 fold higher. The mean plasma elimination half-life of these compounds amounted to 2.8 h (n = 5).

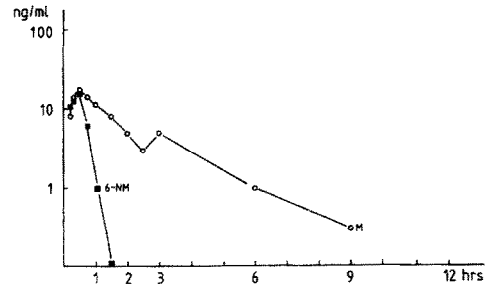


Figure 5
Time course of the metabolites after intramuscular administration of 10 mg nicomorphine. 6-NM — 6-monoester of nicomorphine, M — morphine.

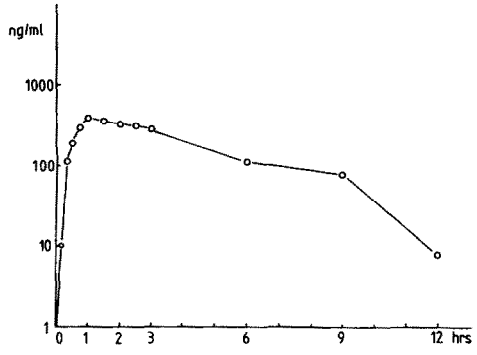


Figure 6
Time course of the conjugates of the metabolites after intramuscular adminstration of 10 mg nicomorphine.

Conclusion

Although the pharmacokinetic parameters of nicomorphine itself cannot be established, the analytical method developed provides information on the plasma levels of its two active metabolites and their conjugates. However, in order to evaluate the pharmacodynamic profile of nicomorphine in relation to its kinetics, the measurement of brain tissue concentrations [4] of the three compounds is essential. Alternatively a theoretical study of the rapid distribution of the prodrug nicomorphine to a receptor compartment, followed by first-order decomposition to active metabolites, might be related to its (lesser) side-effects. Such studies have already been performed on other prodrugs [22].

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References

[1] K. L. Zirm and A. Pongratz, *Arzneim. Forsch.* **9**, 511–513 (1959).
[2] J. W. M. Pinckaers, G. M. M. Nijhuis and R. Dirksen, in *Anesthesiologie und Intensivmedizin*, Band 153, *Schmerzbehandlung-Epidurale Opiatanalgesie* (J. B. Brückner, Ed.), pp. 16–24, Springer, Berlin (1982).
[3] M. W. Lobbezoo, H. H. van Rooij, I. van Wijngaarden and W. Soudijn, *Europ. J. Pharmacol.* **82**, 207–211 (1982).
[4] W. Lindner, M. Raab and K. Schaupp, *Arch. Pharm.* **314**, 328–337 (1981).
[5] J. E. Wallace, S. C. Harris and M. W. Peek, *Anal. Chem.* **52**, 1328–1330 (1980).
[6] R. D. Todd, S. M. Muldoon and R. L. Watson, *J. Chromatogr.* **232**, 101–110 (1982).
[7] P. Y. Grabinski, R. F. Kaiko, T. D. Walsh, K. M. Foley and R. W. Woude, *J. Pharm. Sci.* **72**, 27–30 (1983).
[8] P. J. Twichett, S. Fletcher, A. T. Sullivan and A. C. Moffat, *J. Chromatogr.* **150**, 73–84 (1978).
[9] P. L. Williams, A. C. Moffat and L. J. King, *J. Chromatogr.* **155**, 273–283 (1978).
[10] P. E. Nelson, S. M. Fletcher and A. C. Moffat, *J. Forens. Sci. Soc.* **20**, 195–202 (1980).
[11] S. H. Hansen, Å. M. Hansen and B. Poulsen, *Arch. Pharm. Sci. Ed.* **8**, 181–186 (1980).
[12] J. P. Crombeen, J. C. Kraak and H. Poppe, *J. Chromatogr.* **167**, 219–230 (1978).
[13] H. H. van Rooij, R. L. Waterman and J. C. Kraak, *J. Chromatogr.* **164**, 177–185 (1979).
[14] J. C. Kraak, K. M. Jonker and J. F. K. Huber, *J. Chromatogr.* **142**, 671–688 (1977).
[15] C. M. Riley, E. Tomlinson and T. M. Jefferies, *J. Chromatogr.* **185**, 197–224 (1979).
[16] E. R. Garrett, in *Advances in Pharmaceutical Science* (H. S. Bean, A. H. Beckett and J. E. Carless, Eds), Vol. 2, pp. 1–94. Academic Press, London (1967).
[17] M. R. Murphy and C. C. Hug, *Anesthesiology* **54**, 187–192 (1981).
[18] I. L. Honigberg and J. T. Stewart, *J. Pharm. Sci.* **69**, 1171–1173 (1980).
[19] D. H. Catlin, *J. Pharm. Exp. Ther.* **200**, 224–235 (1977).
[20] E. R. Garrett and T. Gürkan, *J. Pharm. Sci.* **68**, 26–31 (1979).
[21] E. R. Garrett and T. Gürkan, *J. Pharm. Sci.* **69**, 1116–1134 (1980).
[22] P. R. Byron, R. E. Notari and Mei-Ying Huang, *Int. J. Pharmaceutics* **1**, 219–231 (1978).
[23] T. L. Hafkenscheid and E. Tomlinson, *J. Chromatogr.* **264**, 47–62 (1983).

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