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Determination of mirfentanil hydrochloride (A-3508·HCl) in human plasma by liquid chromatography–mass spectrometry*

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Abstract: A method for the quantitation of mirfentanil hydrochloride (A-3508·HCl) in human plasma is presented for the first time, using LC–MS with single ion monitoring. The drug is extracted with a C-18 solid-phase cartridge and the extract is analysed using a 3 cm C-18 column connected to the ion source of a mass spectrometer via a thermospray interface. The intense ion produced by the protonated molecular ion at *m/z* 377 is detected by the mass spectrometer in positive-ion mode. The range of quantitation is 0.4–100 ng ml^{-1} from a 0.5 ml plasma sample. Results of assay validation are given. The method was used to analyse samples from a human pharmacokinetic study following intravenous administration of mirfentanil hydrochloride.

Keywords: *Mirfentanil hydrochloride; LC–MS; pharmacokinetics; plasma; assay validation.*

Introduction

Mirfentanil hydrochloride (A-3508·HCl) is currently being investigated as a narcotic analgesic [1–6]. Several assays for this class of compounds have been reported; they include HPLC [7], GC [8–12] and RIA [13, 14]. To date there have been no reported analytical assays capable of quantifying sub-ng ml^{-1} concentrations of mirfentanil in human plasma, which is required for studying the terminal phase of elimination following intravenous infusion of this drug.

This paper describes a sensitive and specific method for the analysis of mirfentanil hydrochloride in the range 0.4–100 ng ml^{-1} from a 0.5 ml plasma sample using HPLC combined with mass spectrometry (LC–MS). The method was used to analyse mirfentanil hydrochloride in a pharmacokinetic study in human subjects. Data from one subject are shown here to demonstrate the applicability of the method for clinical study samples.

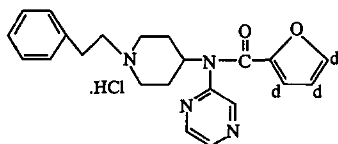

Figure 1
Structure of mirfentanil hydrochloride. The molecular weight of native mirfentanil base is 376. The hydrogen atoms at the three positions indicated (d) were substituted with deuterium for the internal standard.

Experimental

Chemicals

Mirfentanil hydrochloride, [N-(1-phenethyl-4-piperidinyl)-N-(2-pyrazinyl)furan-2-carboxamide hydrochloride], A-3508·HCl (lot no. 3494-159) (Fig. 1) was supplied by Anaquest, (Murray Hill, NJ, USA); the tri-deuterated internal standard was synthesized by Anaquest. All other chemicals were AR grade, and all solvents were HPLC grade.

Standards and solutions

A stock solution of mirfentanil hydrochloride in methanol–water (1:1, v/v) was used to prepare a series of calibration standards in plasma ranging from 0.4 to 100 ng ml^{-1}. The internal standard was prepared as a working solution at 1.0 μg ml^{-1} in methanol–water.

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Chromatography

A Waters Model 510 pump was equipped with a Rheodyne Model 7125 manual injector (100 μl fixed sample loop volume), a Scientific Systems Model LP-21 pulse dampener and C-18 column (3 μm particles, 3.3 cm × 4.6 mm i.d., Supelco no. 5-8978); a Rheodyne 0.22 μm in-line filter was used to protect the analytical column. The mobile phase consisted of methanol–water–triethylamine–tetrahydrofuran (250:300:0.5:2, v/v/v); the apparent pH of this mixture was adjusted to 5.5 with acetic acid, and it was passed through a 0.2 μm Nylon filter under vacuum before use. It was pumped at 1.5 ml min^{-1}.

Mass spectrometry (MS)

The effluent from the LC column led directly to the heated thermospray vaporizer of a Vestec Model 201B LC–MS (Houston, TX, USA). The thermospray tip incorporated a 0.004 μm diameter orifice in a replaceable sapphire disc. The vapour temperature was maintained at 290–320°C, and the tip heater and ion source block were at 250–300°C. The lens stack was kept at 110–130°C. The mass spectrum of reference compound is shown in Fig. 2. Selected $(M + H)^+$ ions passing through the HP 5970 quadrupole activated an electron multiplier at 2600–2800 V, the signals from which were amplified and monitored by a Technivent Vector-One data system. The sweep width was 0.1 amu, and the signals were integrated for 30 ms. Peak areas of *m/z* 377 (mirfentanil) and 380 (internal standard) were measured. The instrument was calibrated using PEG-300 and the thermospray was tuned to maximize the signal for the solvent ion.

Extraction procedure

A 0.5 ml aliquot of heparinized plasma clarified by centrifugation was placed in a test tube with 50 μl (= 50 ng) of internal standard working solution and mixed thoroughly. Solid-phase cartridges (1 ml size, C-18 packing, Cat. no. 7020-1, J.T. Baker), were activated by drawing methanol, followed by water, through them prior to use. The plasma samples were transferred to the cartridges and gently pulled through by vacuum. The cartridges were then washed successively with water (2 ml), followed by methanol–water (1:1) (1 ml), after which the packing was dried by drawing air for a few seconds. The analytes of interest were eluted with 1 ml of methanol and collected into glass tubes. Following evaporation of the solvent by a stream of nitrogen in a 40°C water bath, the extracts were dissolved in 100 μl of mobile phase and a 50 μl aliquot was injected onto the LC–MS. Extracts could be stored dry in the refrigerator without apparent degradation before analysis.

Recovery

Recovery of mirfentanil hydrochloride and the internal standard from plasma was estab-

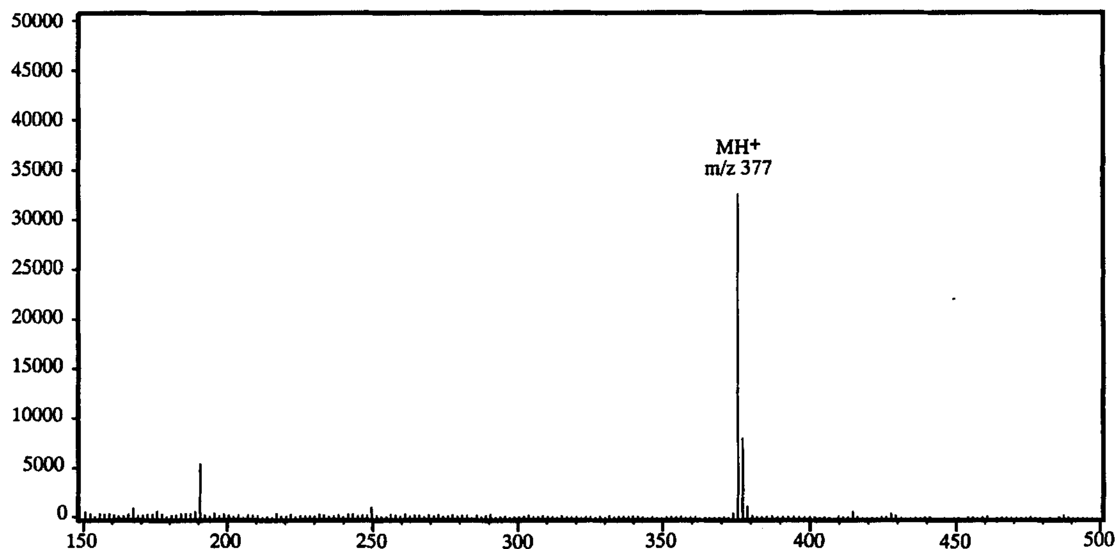

Figure 2
Thermospray positive ion LC–MS spectrum of reference mirfentanil hydrochloride. The base peak is at *m/z* 377 (100%). The ordinate scale is ion intensity.

lished by comparing peak areas of the analytes from extracted calibration samples to those of corresponding non-extracted solutions spiked into blank plasma extracts injected directly onto the LC–MS system. Three replicates of each recovery sample (at 0.4, 1.0, 20 and 100 ng ml^{-1} of mirfentanil hydrochloride concentrations) were processed.

Stability

Short term stability studies consisted of the following experiments: room temperature (benchtop) stability in plasma; three cycles of freeze–thaw stability in plasma; and stability of extracts awaiting analysis.

Four sets, low (1 ng ml^{-1}), medium (10 ng ml^{-1}), high (50 ng ml^{-1}) and very high (500 ng ml^{-1}), of spiked plasma samples were used to study short-term stability of mirfentanil hydrochloride. The first set consisted of controls which were analysed at the beginning of the study. The second set of plasma samples was thawed and left on the benchtop at room temperature for 6 h before extraction. The third set was taken through three freeze–thaw cycles before analysis. In addition, sample extracts ready for analysis were allowed to stand at room temperature for 12 h to simulate the time and conditions that extracts may be subjected to before analysis.

Calculations

The peak area ratios (y) of the mirfentanil ion at m/z 377 to the internal standard ion at m/z 380 and the concentrations (x) of the calibration standards were fitted by weighted ($1/x^2$) linear least squares regression to the equation $y = a + bx$, where a is the y-intercept and b is the slope of the calibration line. Peak area ratios of unknown samples were converted to concentrations of mirfentanil hydrochloride using these computer-generated parameters. Samples containing concentrations of mirfentanil hydrochloride that exceeded the calibration range by more than 10% were diluted with blank matrix and re-analysed.

Validation

The method was validated in plasma by analysing, in triplicate, samples fortified with mirfentanil hydrochloride at 0.4, 1.0, 2.0, 5.0, 20, 50 and 100 ng ml^{-1}. Calibration standards at these concentrations were prepared from different weighings of drug on three separate days. Triplicate samples from four control pools were included on each day. These samples, which were selected to span the calibration range, were stored at −20°C. Other samples similarly prepared were used to determine long term stability and the day-to-day acceptance of subsequent clinical study analysis runs.

Results

The intense protonated molecular ion due to reference mirfentanil was observed when the LC–MS was operated in the positive ion thermospray mode (Fig. 2). Use of the filament or discharge to promote ionization was found to be unnecessary as good sensitivity was obtained by thermospray ionization alone with this mobile phase.

The tri-deuterated mirfentanil hydrochloride used as the internal standard was found to possess similar chromatographic and mass spectral characteristics to the native compound; it gave the desired protonated molecular ion at m/z 380. Mass chromatograms of blank plasma extracts showed no endogenous peaks that interfered with the analysis of mirfentanil hydrochloride or the internal standard.

Mirfentanil hydrochloride and the internal standard eluted with a retention time of about 1 min as sharp peaks of approximately 10 s width at half height (Fig. 3). This provided sufficient separation from the solvent front. Samples could be injected about every 2 min, keeping the total run time for an analysis set relatively short. The peaks were subsequently integrated with keyboard-operated cursors. The injection of half of the extract (equivalent to 0.25 ml of plasma) resulted in a detection limit of about 0.1 ng ml^{-1}. However, the lower limit of quantitation was established at 0.4 ng ml^{-1} (RSDs $< 20\%$) because of the difficulty of reliably integrating very small peaks.

The method was validated and found to be linear over the range 0.4–100 ng ml^{-1} for plasma. Weighted ($1/x^2$) linear regression analysis of the three days of validation gave a coefficient of determination (r^2) of 0.9959 or greater for mirfentanil hydrochloride in plasma. This weighting of the regression line was justified because it provided the smallest residuals. The mean value ($n = 3$) for the y-intercept was 0.00390, and for the slope was 0.00986 in plasma. Precision and relative accuracy were evaluated by treating the peak

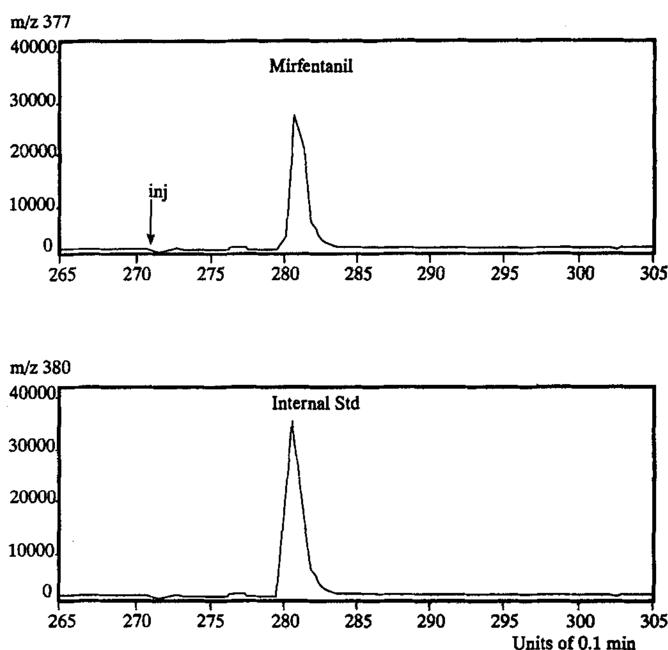

Figure 3
Mass chromatograms of human plasma extract. Plasma (0.5 ml) was fortified with mirfentanil hydrochloride at 100 ng ml^{-1} and tri-deuterated internal standard and extracted as described in the text. The ordinate scale is ion intensity.

area ratios of the calibration standards as unknowns and entering them into the derived equation for the least-squares regression line to obtain 'amount found' values. The mean absolute deviation between 'amount found' and 'amount added' over the entire calibration range was 3.26% (Table 1). The ruggedness of the assay procedure was evaluated by analysing calibration standards in triplicate (intra-assay) on three separate days (inter-assay). The mean intra-day precision for triplicate samples over the entire calibration range was 6.1% (n = 21), and the mean inter-day precision was 5.7% (n = 7).

Additionally, in order to provide a determination of absolute accuracy and precision of the method, the four pools of control plasma samples, spiked at 1.0, 10.0, 50.0 and 500 ng ml^{-1} concentrations (the 500 ng ml^{-1} was diluted 1:10 with blank plasma before analysis) were analysed in triplicate with each day of analysis during validation and in duplicate with subsequent study sample analyses. The mean concentrations found during the three days of validation were 93.7–105% of their theoretical concentrations, and the RSDs were less than 11% at all concentrations (Table 2), again demonstrating the accuracy and ruggedness of the assay.

The mean recovery for mirfentanil hydrochloride and its tri-deuterated internal standard in plasma was 98.2 and 103%, respectively (data not shown for the sake of brevity).

After 6 h at benchtop conditions, changes in mirfentanil hydrochloride concentrations were less than 8.5% at 500, 50, 10 and 1 ng ml^{-1}, compared with their respective controls (n = 3). After three freeze–thaw cycles, these changes were less than 10%. It was concluded that mirfentanil hydrochloride is stable and is not significantly affected by these conditions, since the found concentrations were all within the precision and accuracy of the assay that had been determined during validation. Similarly, extracts stored for 12 h at room temperature showed changes from theoretical values of less than 8.5%, demonstrating good stability of sample extracts awaiting analysis.

Extended term stability of mirfentanil hydrochloride in plasma at −20°C was assessed by analysing samples which had been stored for a period of approximately 6 months. The data from analyses throughout that period indicate no evidence of decomposition (less than 10%) of mirfentanil hydrochloride in plasma at −20°C (data not shown).

The concentration–time profile of mirfentanil in heparinized plasma from a volunteer

Table 1
Summary of mirfentanil hydrochloride standard curve data for validation

		Amount mirfentanil hydrochloride found (ng ml^{-1})							Intercept	Slope	r^2
	Day 1	0.437	1.09	2.07	5.27	21.7	48.6	100	0.00202	0.01028	0.9971
		0.567*	0.935	1.94	5.45	19.5	50.0	97.0			
		0.375	0.902	1.84	5.10	21.6	47.5	92.4			
	RSD	11	10	5.9	3.3	5.9	2.6	4.0			
	Day 2	0.442	0.899	2.07	5.55	20.1	46.6	93.7	0.00568	0.01032	0.9967
		0.360	0.968	2.22	5.36	21.0	46.5	93.2			
		0.781*	1.01	2.04	4.94	21.2	47.5	99.9			
	RSD	14	5.8	4.6	5.9	2.8	1.2	3.9			
	Day 3	0.403	1.31*	1.96	5.07	19.6	46.8	92.3	0.00401	0.00897	0.9959
		0.356	1.00	1.76	5.34	21.1	46.6	103			
		0.442	0.935	2.32	5.68	20.4	49.4	95.3			
	RSD	11	4.8	14	5.7	3.7	3.3	5.7			
Mean		0.402	0.967	2.02	5.31	20.7	47.7	96.3	0.00390	0.00986	0.9966
SD ($n - 1$)		0.0388	0.0643	0.175	0.239	0.822	1.31	3.88			
RSD (%)		9.6	6.6	8.6	4.5	4.0	2.7	4.0			
Amount added (ng ml^{-1})		0.4	1	2	5	20	50	100			
Absolute deviation (%)		0.5	3.3	1.2	6.1	3.4	4.6	3.7			

*Standard dropped from calculations; outlier.

Table 2
Quality control validation data for mirfentanil hydrochloride in plasma

	Very high (500 ng ml^{-1})	High (50 ng ml^{-1})	Medium (10.0 ng ml^{-1})	Low (1.0 ng ml^{-1})
Day 1	528	46.5	10.2	0.908
	530	40.9	8.72	1.04
	497	44.3	9.27	0.957
Day 2	511	44.5	9.52	0.914
	497	44.7	9.78	0.936
	537	47.1	10.9	3.42*
Day 3	540	51.6	9.65	1.23
	531	53.0	9.81	1.00
	553	49.1	10.4	0.933
Mean	525	46.9	9.81	0.990
SD (n − 1)	19.3	3.84	0.640	0.107
RSD (%)	3.68	8.19	6.53	10.8
% Theor.	105	93.7	98.1	99.0

*QC dropped from calculations; outlier according to Dixon criterion.

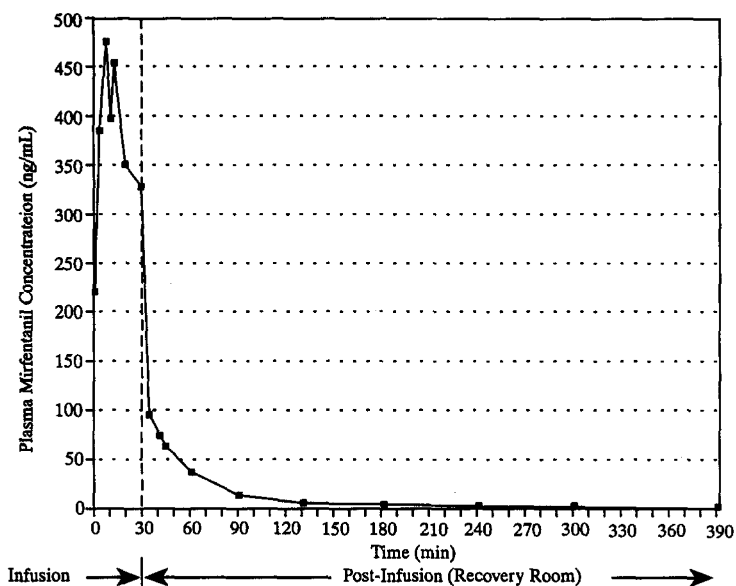

Figure 4
Plasma mirfentanil hydrochloride concentrations (ng ml^{-1}) in subject no. 101 during the infusion and post-infusion period.

given an infusion of 25 μg kg^{-1} min^{-1} for 30 min, is shown in Fig. 4 to demonstrate the applicability of the method to clinical samples. After i.v. dosing, mirfentanil hydrochloride disappeared rapidly from plasma, and was not measurable 6 h after ending infusion.

Discussion

The 3 cm C-18 chromatographic column allowed efficient separation and high sample throughput. A column could be used for about 300 analyses before peak shape deteriorated. Reversal of the direction of column flow or flushing with methanol in most cases restored the peak shape. Tetrahydrofuran in the mobile phase was used to improve the peak shape and reduce tailing.

The vaporizer tip required cleaning on average every 300 samples due to deposit build-up; this was quickly done (less than 1 h) because the gate-valve design of the pumping system allowed the diffusion pumps to operate undisturbed during the venting procedure. Thermospray ionization took place in the ion source. This 'soft' ionization produced $(M + H)^+$ ions, which represented the base peak. This is in contrast to the findings elsewhere in

EI–MS where molecular ions were of low intensity or absent [6]. The quadrupole settings and the vaporizer potentiometer that controlled the amount of heat applied to the vaporizer capillary were used to tune the thermospray to give the maximum response. These were critical for achieving maximum sensitivity of the LC–MS. The response of the instrument sometimes varied during the course of a day's run due to changes in the thermospray ionization efficiency. Nevertheless, it was generally found that when the signal was reduced, the noise was also reduced, and the deuterated internal standard effectively compensated for changes in response.

The assay was highly specific because of four synergistically contributing factors: (1) the extraction on solid-phase cartridges provides a measure of separation from plasma components; (2) the HPLC system is efficient at further chromatographically separating interferences; (3) the deuterated internal standard was used to exactly pinpoint and verify the retention time of native mirfentanil in each analysis; and (4) mass spectrometry in selected ion mode is one of the most specific of all detector systems available: only mass fragments at m/z 377 in positive ion mode are detected as mirfentanil. The combination of all four factors makes the likelihood of interference from other drugs or biological contaminants extremely remote. The superior specificity of this method is an advantage over the RIA methods [13, 14] used for other compounds in the fentanil class, where cross-reactivity with metabolites was noted.

This method was shown to be suitable for the analysis of samples from pharmacokinetic studies in humans. It is anticipated that the results obtained from these clinical trials with mirfentanil hydrochloride will be presented elsewhere.

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References

[1] J.R. Bagley, R.L. Wynn, F.G. Fudo, B.M. Doorley, H.K. Spencer and T. Spaulding, *J. Med. Chem.* **32**, 663–671 (1989).
[2] J.R. Bagley, B.M. Doorley, M.H. Ossipov, V.V. Knight, T.P. Jerussi, M.J. Benvenga, J.A. Wilhelm, S.J. Waters, T. Spaulding, R.L. Wynne, F.G. Rudo and P.S.A. Glass, *Drugs of the Future* **15**, 798–800 (1990).
[3] R.L. Wynn, J.R. Bagley, H.K. Spencer and T.C. Spaulding, *Drug Dev. Res.* **22**, 189–195 (1991).
[4] J.R. Bagley, L.V. Kudzma, N.L. Lalinde, J.A. Colapret, B.S. Huang, B.S. Lin, T.P. Jerussi, M.J. Benvenga, B.M. Doorley, M.H. Ossipov, T.C. Spaulding, H.K. Spencer, F.G. Rudo and R.L. Wynn, *Med. Res. Rev.* **11**, 403–436 (1991).
[5] R. Hess, G. Stiebler and A. Herz, *Eur. J. Clin. Pharmacol.* **4**, 137–141 (1972).
[6] A.F. Casey, and M. Huckstep, *J. Pharm. Pharmacol.* **40**, 605–608 (1988).
[7] K. Kumar, D.J. Morgan and D.P. Crankshaw, *J. Chromatogr.* **419**, 464–465 (1987).
[8] H.H. van Rooy, N.P.E. Vermeulen and J.G. Bovill, *J. Chromatogr.* **223**, 85–93 (1981).
[9] R. Woestenborghs, L. Michielsen and J. Heykants, *J. Chromatogr.* **224**, 122–127 (1981).
[10] J.A. Phipps, M.A. Sabourin, W. Buckingham and L. Strunin, *J. Chromatogr.* **272**, 392–395 (1983).
[11] T.J. Gillespie, A.J. Gandolfi, R.M. Maiorino and R.W. Vaughn, *J. Anal. Toxicol.* **5**, 133–137 (1981).
[12] S.R. Kowalski, G.K. Gourlay, D.A. Cherry and C.F. McLean, *J. Pharmacol. Methods* **18**, 347–355.
[13] M. Michiels, R. Hendriks and J. Heykants, *Eur. J. Clin. Pharmacol.* **12**, 153–158 (1977).
[14] M. Michiels, R. Hendriks and J. Heykants, *J. Pharm. Pharmacol.* **35**, 86–93 (1983).

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