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Sensitive method for the determination of pentamorphone in serum by liquid chromatography–mass spectrometry with thermospray interface

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ABSTRACT

A liquid chromatographic–mass spectrometric method for the determination of 14-β-*n*-pentylaminomorphinone (pentamorphone) and 14-β-*n*-pentylaminocodeinone (PAC) as internal standard is developed. Concentration levels in serum were calculated by the ratio of the peak areas of pentamorphone to PAC *versus* the concentration of pentamorphone. Peak areas were measured using selected-ion-recording of the pseudo-molecular ions of pentamorphone and PAC (*m/z* 369 and *m/z* 383, respectively). Aliquots (50 μl) of sample were injected on a C_{18} μBondapak column following solid-phase extraction The lowest limit of quantitation observed was 43 pg/ml The sensitivity, accuracy and reproducibility of the method were demonstrated to be satisfactory for application in pharmacokinetic study of pentamorphone.

INTRODUCTION

The search for analgesic compounds with enhanced efficacy and improved safety in the treatment of acute postoperative pain has led to the discovery of potent drug products. Pentamorphone (14-β-*n*-pentylaminomorphinone, Fig. 1), a morphine derivative, is one such product which is 500–700 times more potent than morphine. Quantification of such products at projected therapeutic human doses of < 1.0 μg/kg [1] presents a challenge to the analytical chemist developing cold assay methods to routinely monitor their pharmacokinetic behavior.

The use of liquid chromatography–mass spectrometry (LC–MS) as an analytical tool, first reported in 1973 [2], in quantification of drugs and/or metabolites in biological fluids is gaining widespread recognition [3]. One of the main advantages of LC–MS over the conventional gas chromatography–mass spectrometry (GC–MS) is that polar, non-volatile or thermally stable drug products do not have to be derivatized. This eliminates the need to compensate for incomplete recoveries during quantification. The development of thermospray (TSP) LC–

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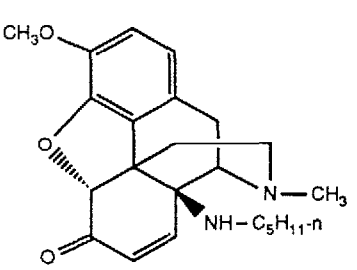
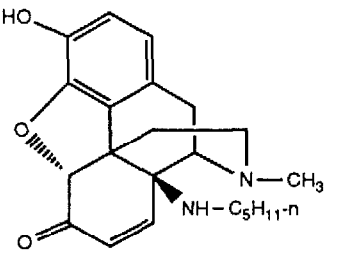


14-ß-n-Pentylaminocodeinone



14-ß-n-Pentylaminomorphinone

Fig. 1 Structure of 14-*β*-*n*-pentylaminomorphinone and 14-*β*-*n*-pentylaminocodeinone (internal standard)

MS, a soft form of ionization that produces pseudo-molecular ion species with little fragmentation, has significantly improved the sensitivity, reliability and applicability of LC–MS in the pharmaceutical industry. Detection limits of < 100 pg/ml in blood serum, using TSP-LC–MS technique coupled with selected-ion recording (SIR), have been reported by several authors [4,5].

In this study we have utilized a positive-ion TSP-LC–MS technique on a VG Trio2 mass spectrometer (VG Masslab, Manchester, U.K.) using SIR to develop a cold assay method for pentamorphone from spiked serum with a limit of detection (L.O.D.) of < 50 pg/ml based upon 3 ml of serum. 14-*β*-*n*-Pentylaminocodeinone (PAC, Fig. 1), a precursor of pentamorphone in its synthesis process, was used as an internal standard.

EXPERIMENTAL

Chemicals and reagents

Pentamorphone and PAC were synthesized at Anaquest (Murray Hill, NJ, U S.A.). All compounds were shown to be pure by high-performance liquid chromatographic (HPLC) analysis and direct probe MS. Acetonitrile, methanol, water (HPLC grade), acetic acid and phosphoric acid were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Monobasic sodium phosphate, ammonium acetate and triethylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.).

Instrumentation

LC–MS was performed on a system consisting of a Waters 600 MS pump, a Waters U6K manual injector (Waters, Milford, MA, U.S.A.), a VG Trio2 mass spectrometer and a VG thermospray interface. Data were collected and processed on a DEC PDP 11/73 computer (Digital Equipment, Maynard, MA, U.S.A.) with a VG RSX11M operating system. Quantification was based on the ratio of

integrated peak areas of selected ions m/z 369 $[M+1]^+$ for pentamorphone and m/z 383 $[M+1]^+$for PAC. The chromatographic column used was a Waters μBondapak C_{18} (15 cm × 4.6 mm I.D.) column. The mobile phase consisted of ammonium acetate (0 05 mol/l, pH 3.7 with glacial acetic acid)–acetonitrile (70:30, v/v). Triethylamine (2 ml/l) was added to enhance the chromatography. The flow-rate was 1 ml/min. The capillary temperature for the thermospray interface was set to 300°C for optimum ionization of pentamorphone The MS source temperature was set to 230°C. Electrode and multiplier voltages were optimized for maximum sensitivity to pentamorphone. Typical values were 260 and 650 V, respectively.

Sample collection and storage

Drug-free human and canine serum was obtained from Biological Specialty (Lansdale, PA, U.S.A.) and stored at −20°C until used.

Dog blood samples for pharmacokinetic studies were collected at specific time points following a 10 μg/kg intravenous bolus dose into 10-ml tubes containing sodium heparin (Becton Dickinson, Rutherford, NJ, U.S.A.). Samples were centrifuged at 1200 *g* for 10 min to separate the plasma and stored at −20°C until analyzed.

Extraction procedure

Blank human serum was allowed to thaw at ambient temperature and then centrifuged at 1200 *g* for 10 min to remove particulate matter. Ten 3-ml aliquots of the serum were spiked with 30 μl each of a 2000 ng/ml stock solution of PAC (internal standard; final concentration 20 ng/ml). These aliquots were then spiked with stock pentamorphone solution in varied volumes to cover a concentration range from 0.04 to 67 ng/ml. Stock solutions of pentamorphone and PAC were prepared in the LC–MS mobile phase. Spiked serum samples were then vortex-mixed for 15 s and loaded onto 3-ml Bond-Elut (Analytichem, Harbor City, CA, U.S.A.) solid-phase-extraction (SPE) columns preconditioned with 3 ml of methanol and 2 × 3 ml of HPLC-grade water. The SPE columns were then washed with 2 × 3 ml of HPLC-grade water and eluted with 2 × 1 ml methanol into 100 mm × 16 mm culture tubes. The eluent was evaporated to dryness under nitrogen at 60°C, reconstituted with 200 μl of LC–MS mobile phase, vortexed for 10 s and centrifuged at 1200 *g* for 7 min prior to analysis.

Canine serum samples containing pentamorphone, collected from pharmacokinetic studies, were spiked with 30 μl of the 2000 ng/ml PAC stock solution (internal standard) and extracted in the same manner as described above. A 50-μl aliquot was injected into the liquid chromatograph for all samples.

RESULTS AND DISCUSSION

Glass *et al.* [1] have concluded from their study that the potent analgesic

TABLE I

DETECTION LIMITS FOR PENTAMORPHONE USING VARIOUS ANALYTICAL METHODS

Method	Limit of detection (ng/ml)
(1) HPLC–UV detection (226 nm)	100
(2) HPLC–electrochemical detection	1
(3) HPLC–dansyl chloride fluorescence derivatization	20
(4) Positive-ion electron-impact GC–MS PFBCl derivatization	10
(5) GC–electron-capture detection PFBCl derivatization	10
(6) GC–nitrogen-phosphorus detection PFBCl derivatization	10
(7) HPLC–radiochemical detection[a]	0.05
(8) TSP-HPLC–MS	0 04

[a] Tritium-labeled pentamorphone

activity of pentamorphone limits the therapeutic range for postoperative pain to between 0.05 and 0.2 μg/kg. At such low dose levels it was extremely difficult to quantitate the pharmacokinetic activity of the drug by conventional methods of analysis. Table I shows the detection limits achieved in our laboratory for pentamorphone using a variety of available techniques. Prior to the method reported herein, the best restult for a cold serum assay for pentamorphone was 1 ng/ml using HPLC with electrochemical detection. This concentration range is far above the sensitivity required to quantitate pentamorphone at the expected therapeutic concentration levels. Also, the 3-pentafluorobenzoyl (PFB) derivative of pentamorphone using a positive-ion electron-impact GC–MS technique with SIR failed to produce the desired detection limits below 10 ng/ml. Philips *et al.* [6], in their comparative study between SIR and multiple-reaction monitoring (MRM) using tandem mass spectroscopy (MS–MS), have reported detecting pentafluoropropionic anhydride (PFP) derivatives of morphine at 1 ng/ml using MRM. However, they were unable to detect the PFP derivatives of morphine below 50 ng/ml using the traditional SIR-GC–MS technique.

Specificity

The key advantage to using the TSP-LC–MS technique is that it eliminates the need to derivatize pentamorphone. In addition, a relatively simple extraction procedure produces clean serum samples that minimize the build-up of back-pressure caused by the deposition of protein molecules on the column frits after multiple injections. The thermospray was tuned on the ammonium acetate and triethylamine ions at m/z 77 and 101, respectively with the source temperature set at 230°C and the probe temperature at 280°C. The probe temperature was further optimized for the sensitivity of pentamorphone at m/z 369 with direct loop injections of 200 ng/ml stock solution. It was observed that thermospray capillary tubes producing spray plumes extending between 1 and 1.2 m at ambient temper-

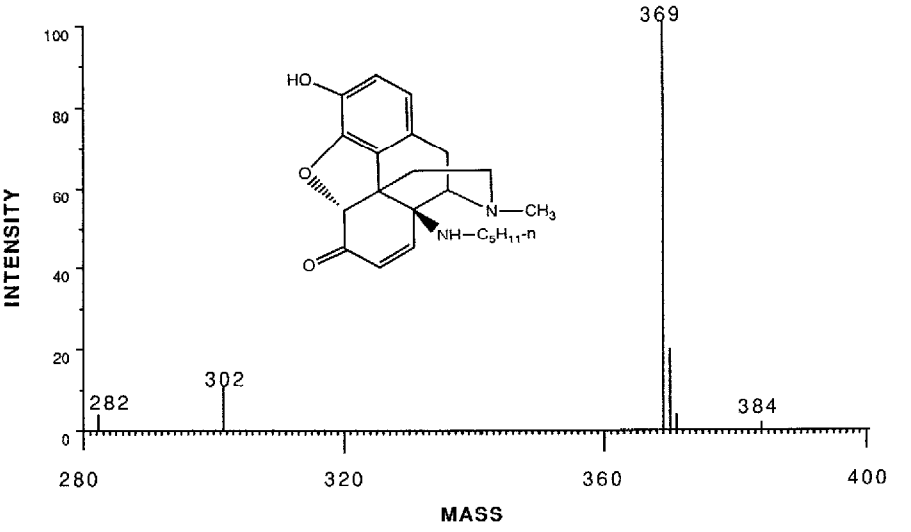


Fig 2 Full scan spectrum of pentamorphone, m/z 369, $[M+1]^+$.

atures produced the ideal ionization conditions at the optimal temperature. Optimal sensitivity was achieved at probe temperature between 290 and 300°C. In order to maintain optimal sensitivity, it was essential to clean the source every 4–6 h of continuous operation. The chromatographic total ion current profile of the mixture of pentamorphone and PAC gave retention times of 4.1 and 8.2 min, respectively. Figs. 2 and 3 show the mass spectra of pentamorphone and PAC with their $[M+1]^+$ ion appearing as base peaks at m/z 369 and 383. These

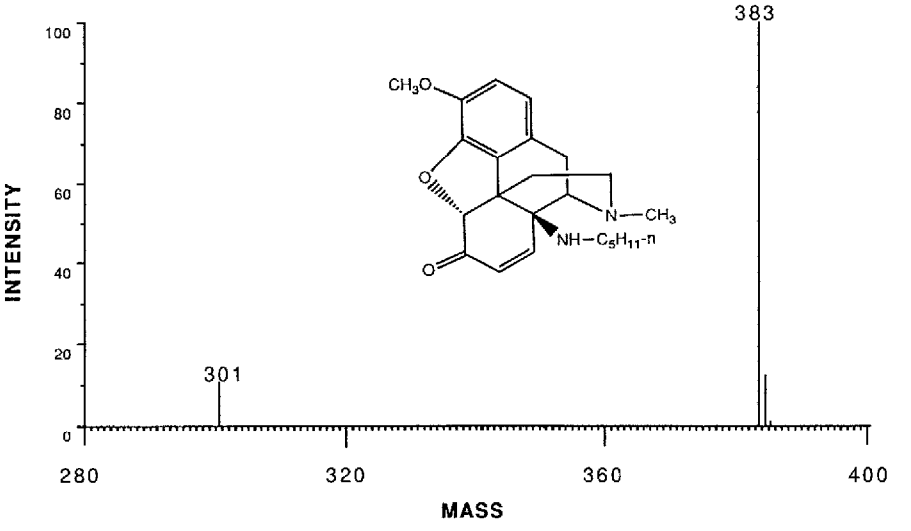


Fig 3 Full scan spectrum of PAC, m/z 383, $[M+1]^+$

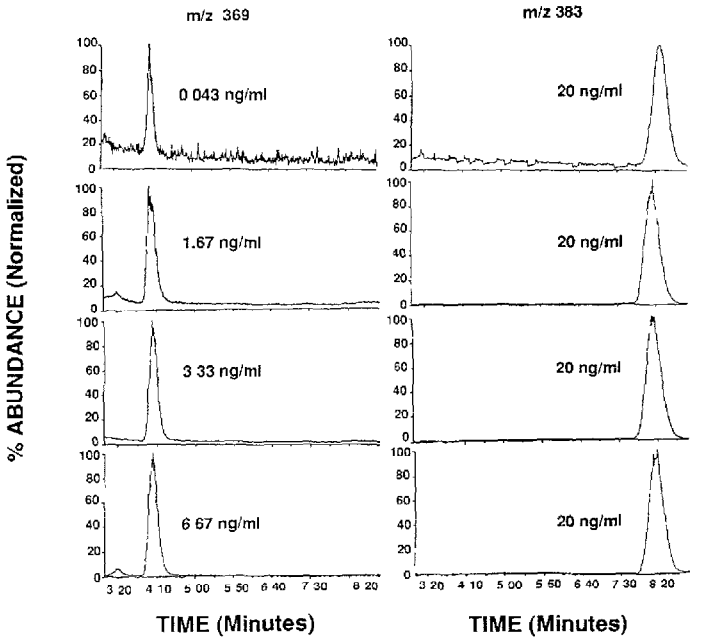


Fig 4 SIR profiles of *m/z* 369 and 383 at four different concentrations.

two ions were selectively used in the SIR mode to quantitate pentamorphone in spiked serum standards and in the canine serum samples extracted from the pharmacokinetic studies.

Linearity and quantifiable detection levels

A series of spiked standards were prepared with pentamorphone concentrations ranging from 0.04 to 67 ng/ml. All of these samples were also spiked with 30 μl of a 2000 ng/ml stock solution of PAC as internal standard and subjected to the extraction procedure outlined in the paper. Duplicate injections of 50-μl volumes of each samples were made and peak areas of pentamorphone and PAC were averaged. Area ratios of pentamorphone to PAC *versus* concentration of pentamorphone were then used to construct the standard curves. Fig. 4 shows the SIR profile of *m/z* 369 and 383 at four concentration levels between 0.04 and 13.3 ng/ml A signal-to-noise ratio of better than 5:1 for the 0.043 ng/ml standard is observed. Fig 5 shows the SIR profiles for *m/z* 369 and 383 for the serum blank. Several consecutive standard curves ranging in concentration from 0.04 to 200 ng/ml in both serum and mobile phase were generated, with correlation coefficients of greater than 0.993, over a period of five weeks. Table II shows the inter-day system reproducibility results of spiked serum standards.

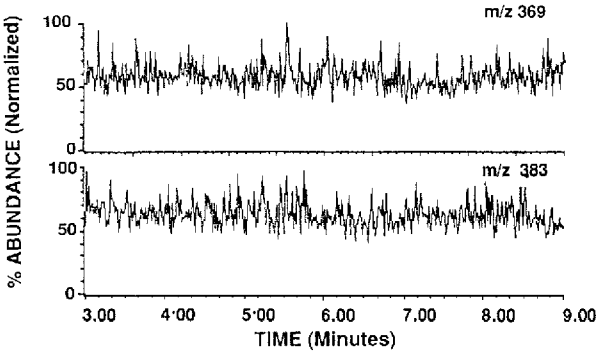


Fig 5. SIR profiles of *m/z* 369 and 383 for serum blank

TABLE II

INTER-DAY SYSTEM REPRODUCIBILITY TEST WITH SPIKED SERUM STANDARDS

Concentration added (ng/ml)	Peak-area ratio	Concentration found (ng/ml)	Error (%)
Day 1			
33 9	1 14	34 3	1.17
20.3	0 678	20 4	0.49
13.5	0.424	13.1	−3 00
6 77	0 204	6 14	−9.40
3 39	0 137	4 13	21.8
1 69	0 0642	1 94	14 9
0 14	0 00406	0 14	0
Mean			3 71
Day 2			
26 1	0 604	23 5	−10 0
13 1	0 340	13 2	0 70
6 53	0 193	7 55	15 6
3 26	0 0765	3.05	−6 40
0 54	0 0118	0 54	0
Mean			−0 02
Day 3			
28 0	0 882	26 3	−6 00
14 0	0 507	14 9	6 50
7.00	0 258	7 32	4 50
3 50	0 128	3 36	−4.10
1 40	0 063	1 38	−1 70
0 70	0 041	0.706	0 90
Mean			0 02

TABLE III

EXTRACTION EFFICIENCY OF PENTAMORPHONE FROM SERUM

LC–MS		HPLC–radiochemical detection	
Concentration (ng/ml)	Recovery[a] (%)	Concentration (ng/ml)	Recovery[a] (%)
0.70	80.0	0 05	75.0
1 40	76.0	0 10	76 3
3.50	73 1	0 50	90.0
7.00	80 2	1.00	87 0
14 0	81 5	10 0	86 0
Mean	78.2		82.9
S D	3 50		6.76

[a] Based upon unextracted standards prepared in mobile phase.

Extraction efficiency

To further validate our analytical procedure with regard to solid-phase extraction efficiency, a series of serum standards spiked with pentamorphone were analyzed for their solid-phase extraction efficiency using the TSP-LC–MS method. These results were compared to the extraction efficiency measured by the HPLC–radiochemical detection method for pentamorphone containing a tritium label on the *n*-pentyl side-chain. Table III shows the absolute efficiency of the extraction procedure in terms of percentage recovery of pentamorphone with the two detection methods at five concentration points. The mean recoveries of the two detection methods are in good agreement with each other. Quantification of the absolute recovery was based upon unextracted standards of pentamorphone prepared in the HPLC mobile phase. In addition, extraction efficiency of the drug product using different volumes of serum was also determined. Three 1-, 3- and 5-ml aliquots each of serum were spiked with a known amount of pentamor-

TABLE IV

RECOVERY OF PENTAMORPHONE FROM VARIOUS AMOUNTS OF SERUM

Serum volume (ml)	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Percentage of theoretical
1 0	10 5	11.2	107
3.0	3 5	3 6	103
5 0	2.1	2 2	105

[a] Based on separate 1-ml extracted serum standards.

phone and subjected to the same solid-phase extraction procedure. Recovery efficiency was calculated based on separate 1-ml extracted serum standards. Table IV shows the results of the mean concentrations of pentamorphone recovered for each volume category. These data indicate that recovery is not a function of serum volume between 1 and 5 ml. However, for very low amounts of the drug product (<0.1 ng/ml), larger serum volumes could greatly enhance the detection signal by virtue of the increase in the absolute concentration of the drug in the final reconstituted volume of 200 μl prior to the analysis.

Stability

The stability of pentamorphone both in mobile phase and serum is not known at this time. During the course of this study, mobile phase and serum standards were freshly prepared for every experiment. Blood samples for pharmacokinetic study were extracted and analyzed immediately. Further studies are in progress to determine the stability of pentamorphone in mobile phase and in serum.

Applications

This method has been successfully applied to the pharmacokinetic study of pentamorphone in dogs dosed intravenously at 10 μg/kg and blood levels monitored up to 2 h after dosing. The serum concentration ranged from 37.0 ng/ml at 2 min to 0.36 ng/ml at 120 min after dosing. A representative profile of pentamorphone concentration *versus* time is shown in Fig. 6. These results illustrate the potential application of this TSP-LC–MS method to assay pentamorphone in human serum samples. Due to the low dose level in humans, it is recommended

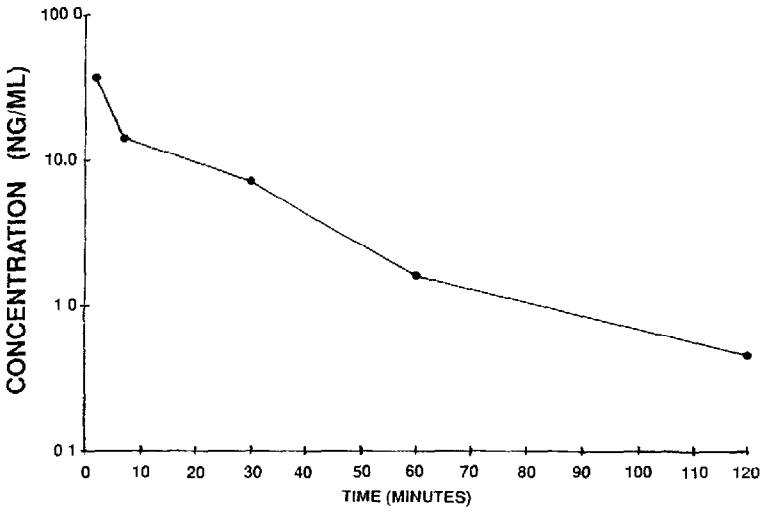


Fig 6 Concentration *versus* time profile of pentamorphone in dog serum dosed at 10 μg/kg

that a minimum of 10 ml of blood sample be drawn for every time point in the pharmacokinetic study. Larger sample volumes containing higher absolute amounts of drug will facilitate detection of very low concentrations of pentamorphone over longer time periods.

CONCLUSION

The data presented here further demonstrate the applicabilty of TSP-LC–MS in the quantification of potent drug products at very low concentrations in serum In this cold assay method we have successfully achieved a limit of quantitation of 43 pg/ml for pentamorphone. The conventional methods for quantitation of pentamorphone in biological samples have involved complicated extraction and derivatization processes. The detection limits of the multiple methods examined were either irreproducible or unacceptable for the study involved. We have also demonstrated that the extraction efficiency of pentamorphone from serum is independent of concentration and sample volume The reproducibility of the thermospray results without derivatization of the drug product and with minimal sample preparation has allowed LC MS to emerge as a sensitive and selective analytical tool for pentamorphone in serum.

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