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Rapid and sensitive determination of nalmefene in human plasma by gas chromatography–mass spectrometry

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Abstract

A rapid gas chromatography–mass spectrometric method for the determination of nalmefene in human plasma is described. The procedure involves protein precipitation, extraction with ethanol–chloroform mixture and derivatization with pentafluropropionic anhydride. The deuterated analog of nalmefene, 6β-naltrexol-d_7, was used as the internal standard. Quantitation was achieved on a HP-1 column (12 m×0.2 mm I.D.) with negative chemical ionization (NCI) using methane:ammonia (95:5) as the reagent gas. The standard curves were fitted using a quadratic equation with the curve encompassing a range of 0.5 to 200 ng/ml, and the intra- and inter-assay variations for three different nalmefene levels were less than 10% throughout. The limit of quantitation was found to be 0.5 ng/ml. The method described is highly specific and reproducible, and could also be applied for the determination of naltrexone and 6β-naltrexol. Application of the method to actual human plasma samples is demonstrated. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Nalmefene, 17-(cyclopropylmethyl)-4,5-α-epoxy-6-methylenemorphinan-3,14-diol, is an opioid antagonist related to naloxone and naltrexone in chemical structure but with several pharmacological advantages (Fig. 1). Naloxone is the only pure opioid antagonist available for intravenous use and has proven useful only in short-term use [1]. Naltrexone is an effective opioid antagonist with a long duration of action and was approved for the maintenance of opioid abstinence in 1983. Nalmefene, an analog of naltrexone in structure, has a significantly longer duration of action than naltrexone and other clinically used opioid antagonists [2–4]. Clinical studies indicated that intravenous nalmefene has a mean terminal β elimination half-life in plasma of 7–15 h (mean, 11 h) [5], compared to 30–60 min for naloxone [6] and 3–4 h for naltrexone [7]. For comparison to naloxone, nalmefene and naltrexone can be administered orally. Nalmefene has an estimated oral bioavailability of 40–50% [5], which is

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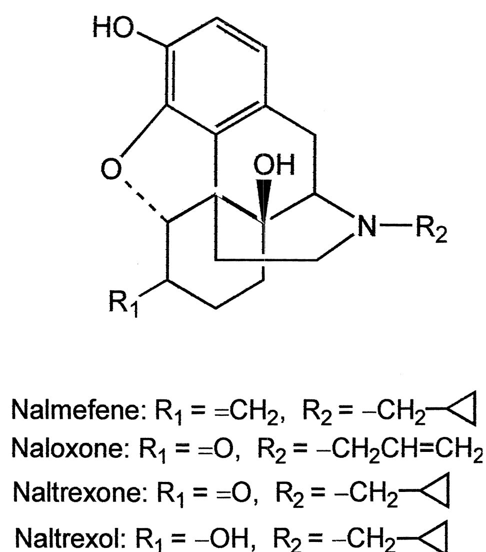

Fig. 1. Structures of nalmefene and other similar opioid antagonists.

greater than that reported for naltrexone (5–60%) [7].

Similar to naltrexone, nalmefene has demonstrated efficacy and safety in double-blind and placebo-controlled clinical trials for alcohol dependence [8,9]. However, nalmefene has the advantage over naltrexone in having no dose-dependency associated with liver toxicity [10], and a higher affinity for μ, κ and δ receptors [11], two pharmacological properties that may be advantageous in the treatment of alcoholism. Also, unlike naltrexone, nalmefene does not have biologically active metabolites, which permits a straightforward determination of any relationship between plasma concentration and efficacy or toxicity.

Two methods have been published describing the quantitation of nalmefene in plasma using either radioimmunoassay (RIA) [12] or high-performance liquid chromatography (HPLC) with electrochemical detection [13]. This article describes a sensitive and specific method for the determination of nalmefene in plasma by gas chromatography–mass spectrometry (GC–MS). Sample data obtained from GC–MS and HPLC methods are also compared.

2. Experimental

2.1. Reagents and materials

Nalmefene, purity 100% was purchased from Sigma (St. Louis, MO, USA). The internal standards, 6β-naltrexol-d_7 hydrochloride, purity 98% for GC–MS and naltrexone hydrochloride, purity 100% for HPLC were obtained from The National Institute of Drug Abuse (NIDA) (Rockville, MD, USA) and RBI (Natick, MA, USA), respectively. Pentafluoropropionic anhydride (PFPA) was from Supelco (Bellefonte, PA, USA). All other chemicals used were reagent grade and purchased from Fisher Chemical (Springfield, NJ, USA). Stock solutions of nalmefene and the internal standards were prepared using 0.01 N HCl to concentrations of 1 mg/ml. Working solutions were prepared in 0.01 N HCl at respective concentration levels of 0.2, 0.02 and 0.002 ng/ml. The stock and the working solutions were stored at 4 °C for 6 months. Carbonate buffer (pH 10.5) was prepared by dissolving 18 g of potassium bicarbonate into 1 *M* sodium carbonate solution.

Water used for preparation of standards, buffer and the mobile phase of HPLC was obtained using Milli-RO 10 Plus and Milli-Q Plus water purification system (Millipore Corp., Bedfored, MA, USA).

2.2. Instrumentation and data acquisition for GC–MS

A HP ChemStation data system was used to control the HP 5988B GC–MS system and to collect and quantitate the data. The GC–MS with a HP-1 column (12 m×0.2 mm I.D., 0.33 μm) is operated in a NCI mode using methane:ammonia (95:5) as the reagent gas. The column temperature was programmed from 80 °C (holding for 1 min) to 280 °C at rate of 30 °C/min. The samples were injected in a splitless mode and the split valve was opened 1 min after injection. The ion-source temperature was 200 °C, and the temperatures of injector and the interface between the chromatograph and the spectrometer were set at 280 °C.

The peak area of the target compound and its internal standard were measured using the HP ChemStation data acquisition system with RTE integration.

The chromatographic data were automatically processed for peak area ratios followed by the least square regression of these data using secondary degree equations. All data were calculated from curves fitted using this equation with the intercept.

2.3. Instrumentation and chromatographic conditions for HPLC

The separation was achieved using a Model 600 solvent delivery system and controller with a Model 717 Plus autosampler (Waters Corp., Milford, MA, USA) and a 5-μm particle phenyl–hexyl column, 250×4.6 mm I.D., (Phenomenex, Torrance, CA, USA) at ambient temperature. The compounds were eluted with a mobile phase of 83% 0.05 *M* monobasic potassium phosphate, which was adjusted to pH 2.5 with 85% phosphoric acid (1.0 ml/l) and *n*-butylamine (1.2 ml/l), and 17% acetonitrile at a flow rate of 1.2 ml/min. A Model 5200A Coulometric Detector (ESA Inc., Chelmsford, MA), with a guard cell set at +0.650 V and a dual analytical cell with the screening electrode set at +0.200 V and the analytical electrode at +0.600 V, was used to detect the eluted compounds. All data, including the construction of the calibration curves was processed using ChromPerfect for Windows Ver.3.5 (Justice Laboratory Software, Palo Alto, CA, USA).

2.4. Extraction and derivatization for GC–MS

To 1 ml of plasma sample were added 100 μl of naltrexol-d_7 (25 ng) and 2 ml of 5% sulfosalicylic acid. After centrifugation, the supernatant was transferred to a round bottom screw top tube, followed by the addition of 1.5 ml of 1.0 *M* carbonate buffer (pH 10.5) and 5 ml of chloroform/ethanol (80:20). The contents were mixed for 10 min. The organic phase was transferred to a 13×100 mm tube and evaporated to dryness via a vacuum centrifuge. The residue was derivatized with 100 μl of PFPA in 100 μl of ethyl acetate at room temperature for 20 min. The derivatizing mixture was transferred to glass mini vial and dried down using a vacuum centrifuge. The residue was re-dissolved in 20 μl 1% PFPA toluene solution and 2 μl was injected into the GC–MS.

2.5. Extraction for HPLC

A 1 ml of plasma sample containing 10 μl (10 ng) of internal standard naltrexone, was basified with 0.5 ml of 1.0 *M* carbonate buffer (pH ~10) and the mixture was extracted with 4.0 ml of hexane:methylene chloride (1:1). Following mixing and centrifuging for 10 min, the organic phase was re-extracted with 150 μl of 0.1 *M* acidic phosphate buffer (pH ~2). An aliquot (20–120 μl) was injected into the HPLC.

3. Results and discussion

The NCI mass spectrum of the PFPA derivatives of nalmefene is shown in Fig. 2. 6β-Naltrexol-d_7 was selected as the internal standard because of its similarity in structure and no possible existence in samples. The major fragments at m/z 631 from nalmefene and m/z 768 from 6β-naltrexol-d_7 were used for quantitation with minor ions at m/z 611 and at m/z 640 as confirming ions, respectively. Fig. 3 shows the chromatograms (SIM) of a clinical sample containing the internal standard. The retention times for nalmefene and the internal standard were 7.27 and 7.31 min, respectively. The lack of interfering peaks at or near the area of the peaks of interest demonstrates the high specificity of the method.

A series of standards containing 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 200 ng/ml of nalmefene in drug free plasma was used to construct a standard calibration curve which was run for each day's analysis. The intra-day precision was determined by using five replicates of each level of the standard curve. The results shown in Table 1 indicate an excellent precision at each concentration level with relative standard deviation (RSD%) ranging from 0.3 to 7.2. The limit of quantification for nalmefene was 0.5 ng/ml, as measured by precision and accuracy (RSD%=7.2, n=5). The inter-assay precision of the method was determined by analyzing quality control samples with each batch of samples. The quality controls were prepared at the concentration levels of 0.75, 7.5 and 75 ng/ml of nalmefene and stored at −25 °C. The results show that the assay is reproducible (RSD% less than 6) and accurate (≤8.0%

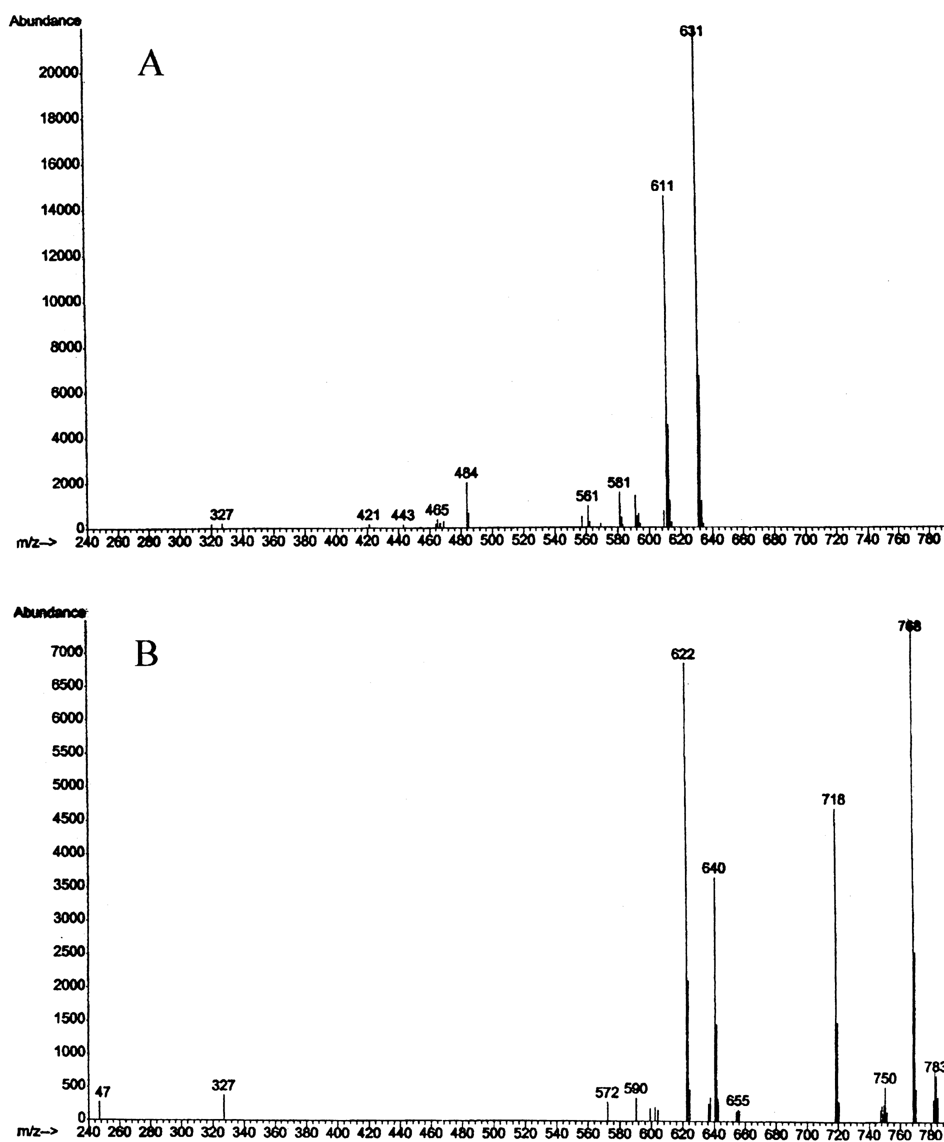

Fig. 2. Mass spectra (NCI) for PFP-nalmefene (A) and the internal standard, PFP-naltrexol-d_7 (B).

deviation from the theoretical concentration) (Table 1).

The absolute recovery of nalmefene from plasma was determined by preparing standard solutions of 0.75, 7.5 and 75 ng/ml. The internal standard was added to each. After evaporation to dryness, the residues were derivatized and injected into the GC–MS. The same concentrations were added to drug-free plasma and processed routinely, but quantitatively. The internal standard was then added to the final extract and derivatized. An aliquot was injected as above. The difference between the ratios of standards to internal standard in the processed samples vs. the unextracted standards indicated the overall extraction recovery. The percent recovery of nalmefene from plasma at the above respective concentrations was found to be 82, 85 and 81% ($n=5$ for each concentration).

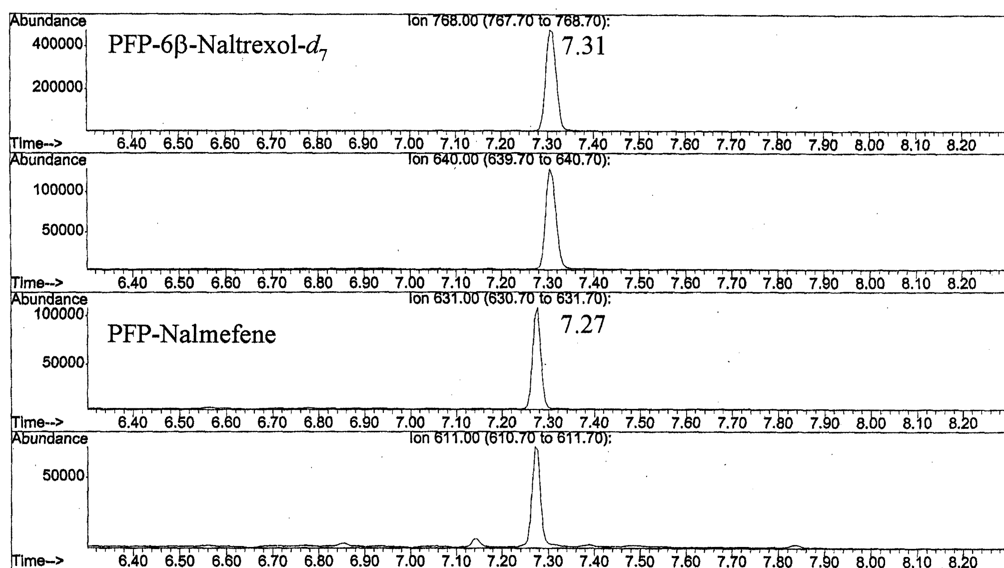

Fig. 3. Chromatograms (SIM) of PFP-nalmefene extracted from a patient sample (7.2 ng/ml measured) with the internal standard (25 ng/ml).

The stability of 32 clinical plasma samples was investigated. The initial plasma concentrations of nalmefene were compared with the results obtained from the same samples 1 month later. The samples were stored at −25 °C between the assays. The Student's *t*-test (Paired) showed that there was no significant difference between each pair of the samples, indicating that nalmefene is stable at −25 °C for at least 1 month.

Table 1
Intra- and inter-day precision and accuracy of the assay

Added (ng/ml)	Mean (ng/ml)	SD	RSD%	Accuracy
Intra-day precision				
200	200.62	0.56	0.3	100.3
100	99.23	1.04	1.0	99.2
50	49.64	0.38	0.8	99.3
25	24.94	0.96	3.9	99.8
10	10.36	0.29	2.8	103.6
5.0	5.02	0.32	6.4	100.4
2.5	2.61	0.13	4.9	104.4
1.0	1.08	0.04	3.8	108.0
0.50	0.54	0.04	7.2	108.0
n=5 at each concentration				
Inter-assay precision				
75	72.33	1.60	2.2	96.4
7.5	7.73	0.40	5.2	103.1
0.75	0.76	0.02	2.6	101.3
n=5 consecutive days with duplicate run at each concentration				

This GC–MS method was compared to a previously published procedure using HPLC with electrochemical detection [13], which was modified as described herein. A total of 27 nalmefene plasma samples were reanalyzed using the LC–EC method. Because the method had been significantly altered (e.g. different solvents and pH for the extraction and a different internal standard), the method was revalidated in terms of the intra- and inter-assay variations, and recovery. The retention times of nalmefene and the internal standard, naltrexone, were 9.9 and 4.8 min, respectively. The calibration curve ranged from 1 to 100 ng/ml using seven calibration standards. Intra-assay imprecision (RSD%) of the seven standards did not exceed 5.6 (n=8 for each standard). Inter-assay variations of the method were assessed using three levels of quality controls (in duplicate). The imprecisions (RSD%) for the low (5 ng/ml), medium (30 ng/ml) and high (75 ng/ml) nalmefene quality controls were 8.2, 5.0 and 4.4, respectively (n=11 days).

The accuracy of the LC method was determined by evaluating the precision data generated from the seven calibration levels. The accuracy ranged from

98 to 105% ($n=8$ for each level). Absolute recovery of nalmefene from plasma for the LC procedure was determined by establishing unextracted peak height ratios of nalmefene at 100, 25 and 5 ng/ml, and internal standard. Nalmefene, at the same concentrations, was then added to 1 ml of plasma and processed as described previously, but using exact quantitative transfers. The internal standard was added to the final extract and injected. The peak height ratios of the extracted nalmefene were compared to the ratios of the unextracted standards to yield recoveries of 80, 77 and 79% at the respective concentrations ($n=7$ each concentration).

Fig. 4 shows the comparison of the data from GC–MS and HPLC methods. While there was a good correlation ($r^2=0.9956$) of the sample data obtained between the two methods, the results generated from the LC–EC method were consistently lower, but still within 9% of the GC–MS values.

The development of a GC–MS procedure for plasma nalmefene provides a useful alternative to the existing published RIA [12] and LC [13] methods. While the limit of quantitation is slightly lower than for the LC method, the inherent selectivity of GC–MS eliminates the problem of interference from similar opioids such as naloxone, naltrexone and its 6β-naltrexol metabolite, which exists using the RIA method. Both the published LC method [13] and the LC procedure described herein use nalbuphine and naltrexone, respectively, as internal standards. The GC–MS procedure uses a deuterated analog of nalmefene (6β-naltrexol-d_7) for the internal standard, thus eliminating potential interference from structurally related compounds. Therefore, this method would have an advantage when other opioids are present in plasma.

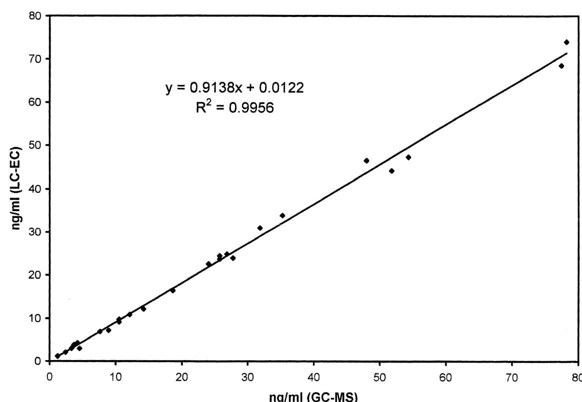

Fig. 4. Correlation of the results of 27 patient plasma samples obtained by GC–MS and LC–EC methods.

Resulting data showed a highly significant relationship between nalmefene plasma concentrations and overall severity of adverse drug reactions, specifically with GI upset and nausea [14]. Given that nalmefene has no active metabolites, the plasma nalmefene concentration may help to distinguish between drug toxicity and symptoms of alcohol withdrawal or disease. Alcoholics frequently exhibit problems with medication compliance. Therefore, stable nalmefene plasma concentrations may serve to determine if sufficient drug is circulating for therapeutic effect.

In conclusion, the method described here demonstrates a simple, sensitive and highly specific assay for the quantitation of nalmefene in plasma samples. In addition, this procedure could be adapted to the measurement of other structurally similar opioid antagonists, e.g. naltrexone and its metabolite, 6β-naltrexol, with equivalent sensitivity and specificity.

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