Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, Nos 6–8, pp. 887–893, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00

Analysis of naltrexone urinary metabolites*

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Abstract: A reversed-phase HPLC method using ion-pair formation has been developed for the simultaneous determination of naltrexone and three urinary metabolites. The extraction of the free and conjugated metabolites was studied by liquid–solid procedures using styrene–divinylbenzene copolymers (Amberlite XAD-2) and bonded octadecyl silica supports (ODS-silica). Optimum recovery was obtained with ODS-silica extraction using 25% acetonitrile in a 5 mM diammonium phosphate buffer pH 2.1 as elution solvent. The chromatographic behaviour of naltrexone metabolites and naloxone (internal standard) was examined by varying the mobile phase composition. Increments of both the diammonium phosphate buffer concentration and the percentage of organic solvent in the eluent decreases the retention of compounds in a non-linear manner. Increments of the dodecyl sulphate (counter-ion) concentration, increases the retention time. The method was applied to determine the urinary levels of major naltrexone metabolites in a volunteer receiving a 50 mg oral dose. This is the first method reported which permits the simultaneous quantitative determination of naltrexone and its metabolites, 6β-naltrexol, naltrexone glucuronide and 6β-naltrexol glucuronide, in urine.

Keywords: *Naltrexone; naltrexone metabolites; reversed-phase liquid chromatography.*

Introduction

Naltrexone (NTX) is an orally effective opiate antagonist used in maintenance treatment for opiate dependence. The purpose being to prevent readdiction of ex-opiate addicts who have successfully undergone detoxication [1, 2].

The main NTX metabolites in man are its conjugate with glucuronic acid (GL NTX), 6β-naltrexol (NTXOL) and its conjugate with glucuronic acid (GL NTXOL) [3–5]. A series of analytical methods has been developed to detect NTX and its metabolites in biological fluids in order to study the pharmacokinetics, metabolism and excretion [6–11] and also to validate the compliance of patients submitted to maintenance programmes with this drug [12]. The analytical methods described to date allow the determination of the major NTX metabolites in their free form, but not when glucuronoconjugated because the conjugates are not readily extracted by organic solvents and cannot be detected directly by gas chromatography (GC), the primary methodology used. GC has the major drawback of requiring the preparation of derivatives, which may be multiple

*Presented at the "International Symposium on Pharmaceutical and Biomedical Analysis", September 1987, Barcelona, Spain.

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and unstable [7, 10, 13]. The structural similarity between such derivatives also makes GC separation difficult [8, 10, 11].

A reversed-phase liquid chromatography method to detect the main NTX urinary metabolites has been developed, which allows direct detection of both free and glucuronoconjugate compounds, and simplifies their determination. Simultaneous extraction of free and conjugated metabolites is accomplished by solid–liquid procedures. Both the extraction and the chromatographic separation conditions have been optimized to obtain optimum yield and selectivity.

Experimental

Extraction of urine samples

An automatic 12 sample centrifugal processor (PREP™ I, Du Pont Company, USA) was used. Columns were filled either with 50–105 μm bonded octadecyl silica particles (ODS-silica, Waters Assoc., USA) or with 100–200 μm styrene–divinylbenzene copolymers (Amberlite XAD-2, Serva Feinbiochemica, FRG). Before introducing samples, the columns were washed with methanol and water.

Urine samples (3 ml) were made alkaline with 3 M ammonium sulphate buffer pH 9.3 (4 ml) and passed through the columns. With ODS-silica, two washing steps were used: firstly 5 mM ammonium sulphate buffer pH 9.3 (50 ml/12 columns), and secondly distilled water (8 ml/12 columns). Only water (15 ml/12 columns) was used for Amberlite XAD-2. The elution solvents (24 ml/12 columns) were 25, 15 or 10% acetonitrile in 5 mM diammonium phosphate buffer pH 2.1 (v/v) for ODS-silica (procedures 1, 2 and 3, respectively), and 50% methanol in distilled water (v/v) for Amberlite XAD-2 (procedure 4). Eluates were directly analysed by high-performance liquid chromatography (HPLC).

Comparison of recoveries by each extraction procedure was carried out either with normal urine samples spiked with NTX (Du Pont Company, USA) and naloxone (NALOX, internal standard; Naloxone®, Laboratorios Abelló, Spain) or with urine collected from patients receiving NTX (Trexan®, Du Pont Company, USA) in the Department of Toxicomanies of the "Hospital Ntra. Sra. del Mar" (Barcelona, Spain). Data are presented as mean ± standard deviation.

High-performance liquid chromatography

Separation was carried out through 5 μm reversed-phase columns (Spherisorb ODS-1, 20 × 0.4 cm and Spherisorb ODS-2, 15 × 0.4 cm; Tracer Analítica, Spain) fitted to a Series 400 flow delivering system, an ISS-100 automatic injector and an LC-75 variable wavelength detector adjusted to 210 nm (all from Perkin Elmer, USA).

The mobile phase was a diammonium phosphate buffer pH 2.1 (adjusted with phosphoric acid) containing dodecyl sulphate (counter-ion) and acetonitrile. The flow rate was 1.0–1.5 ml min^{-1}.

Identification of the 6β-naltrexol peak

Urine samples (3 ml) adjusted to pH 6.7–7 with 0.2 M phosphate buffer were hydrolised with β-glucuronidase (40 μl of a 200 U ml^{-1} solution; Boehringer Mannheim, FRG) for 3 h at 55°C, and subjected to the ODS-silica extraction after being made alkaline. Selected HPLC fractions were collected corresponding to the u.v. detection of NTXOL. The fractions were made alkaline and extracted with ethyl acetate (5 ml). The

organic phase was evaporated and the residue stored in a dessicator until derivatisation, which was done by adding 20 μl of a mixture of *N*-methyl-*N*-trimethylsilylheptafluorobutiramide, trimethylsilylimidazole and trimethylclorosilane (100:2:5, v/v/v) and heating at 80°C for 5 min.

Trimethylsilyl (TMS) derivatives were analysed by gas chromatography–mass spectrometry (GC/MS) (5890 model GC coupled to a 5970 model mass spectrometric detector; Hewlett–Packard, USA). Identification of metabolites was done in the scan mode after detection by selective ion monitoring: m/z 559, 544 and 372 for NTXOL- tris-TMS; m/z 557 and 542 for NTX-tris-TMS and m/z 485 for NTX-bis-TMS derivatives.

Application to excretion studies

Urine samples were collected for 24 h from a healthy male receiving 50 mg NTX orally. Each fraction was analysed by HPLC after extraction by the ODS-silica procedure. NALOX was added (12 μg ml^{-1} urine) as internal standard. Quantitation was done by comparison with a curve produced from NTX (0, 1, 3, 10, 30 and 100 μg ml^{-1}); the same molar extinction coefficient for all metabolites was assumed.

Results

The recoveries obtained for NTX and NALOX added to urine samples extracted by the different procedures are reported in Table 1. Values ranged from 21.2 to 28.4% with Amberlite XAD-2 (procedure 4), and from 71.6 to 90.3% with ODS-silica using the less polar elution solvent (procedure 1). The 25% organic:aqueous mixture also gave the best results for NTXOL. The NTXOL recoveries for procedures 2, 3 and 4 were 79.8 ± 3.5, 43.4 ± 2.0 and 35.1 ± 7.2% ($N = 3$) as compared with procedure 1 (taken as 100%). Extractions of GL NTXOL by procedures 2 and 3 were 95.6 ± 2.3 and 64 ±8.5% ($N = 5$) as compared to procedure 1.

The effect of the HPLC mobile phase components on retention of NTX metabolites and NALOX was studied. The effects of diammonium phosphate buffer concentration, acetonitrile percentage and dodecyl sulphate concentration are shown in Fig. 1. A chromatographic profile of NTX metabolites, under optimal conditions, is shown in Fig. 2.

Quantitation of NTX metabolites was based on an NTX standard curve using NALOX as internal standard. Concentration (C) of NTX followed the equation ($r = 0.995$): $C = 11.48R + 0.54$, where R is the area ratio between NTX and the internal standard,

Table 1
Recoveries (percentage) for naltrexone and naloxone

Urine levels	Procedure 1 X	SD	Procedure 2 X	SD	Procedure 3 X	SD	Procedure 4 X	SD
	$N = 5$		$N = 5$		$N = 5$		$N = 4$	
NTX (30 μg ml^{-1})	90.3	5.3	75.3	8.5	46.9	3.1	28.4	1.3
NTX (3 μg ml^{-1})	86.5	5.6	68.4	7.2	50.5	2.9	27.8	7.1
	$N = 10$		$N = 10$		$N = 10$		$N = 9$	
NALOX (12 μg ml^{-1})	71.6	6.7	62.9	7.4	40.8	3.3	21.2	3.4

Procedures: 1, ODS-silica extraction using 5 mM phosphate buffer pH 2.1 with 25% of acetonitrile as elution solvent; 2, with 15% of acetonitrile; 3, with 10% of acetonitrile. Procedure 4: Amberlite XAD-2 extraction with 50% methanol in distilled water as elution solvent.

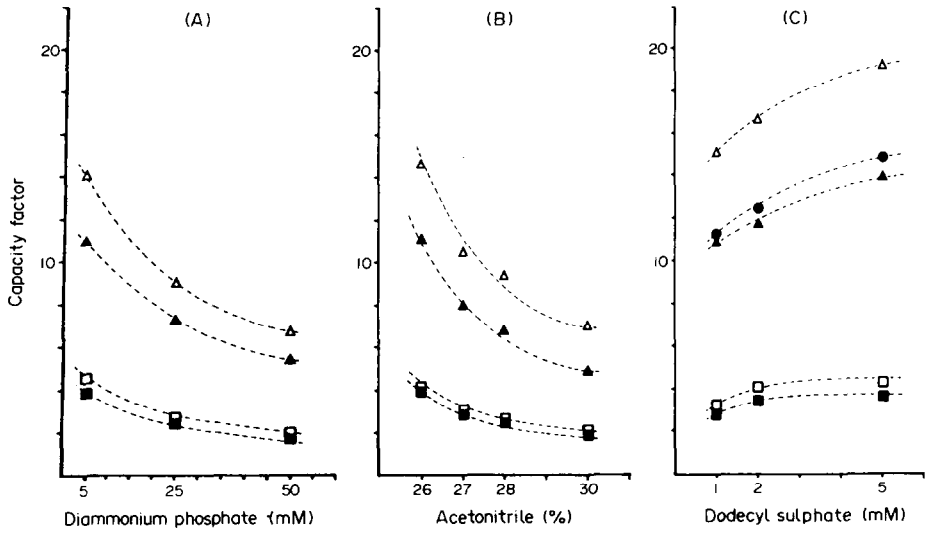

Figure 1
Effect of the concentration of buffer (A), acetonitrile (B) and dodecyl sulphate (C) in the mobile phase on the capacity factors of naltrexone metabolites (△, naltrexone; ▲, 6β-naltrexol; □, naltrexone glucuronide; ■, 6β-naltrexol glucuronide) and naloxone (●). Mobile phase: 5 mM diammonium phosphate pH 2.1 for Figs B and C; 5 mM and 1 mM dodecyl sulphate for A and B, respectively; and 30% acetonitrile for A and C. Flow rate: 1.5 ml min^{-1} for A and B; 1.0 ml min^{-1} for C. Columns: Spherisorb ODS-2 for A and B; Spherisorb ODS-1 for C.

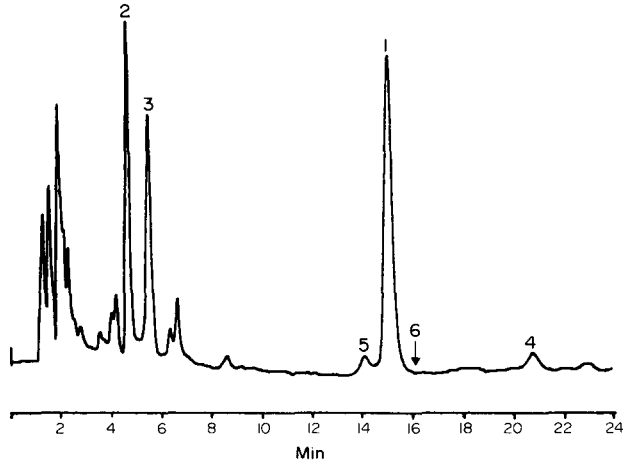

Figure 2
Chromatogram of a naltrexone positive urine. Peaks: 1, 6β-naltrexol; 2, 6β-naltrexol glucuronide; 3, naltrexone glucuronide; 4, naltrexone; 5, possibly 2-hydroxy-3-*O*-methylnaltrexol (another naltrexone metabolite); 6, naloxone. Mobile phase: 5 mM diammonium phosphate buffer pH 2.1 with 5 mM dodecyl sulphate and 30% acetonitrile. Flow rate: 1.0 ml min^{-1}. Column: Spherisorb ODS-1.

NALOX. The coefficients of variation for the analysis of NTX in urine were 6 and 7.1% at a level of 3 and 30 $\mu g\ ml^{-1}$, respectively ($N = 10$).

When the total amounts of NTX metabolites are measured in 24 h urine samples, 79% of the dose is recovered. Individual values for the metabolites are 1.5, 10.8, 43.1 and 23.6% for NTX, GL NTX, NTXOL and GL NTXOL, respectively, with the excretion pattern shown in Fig. 3.

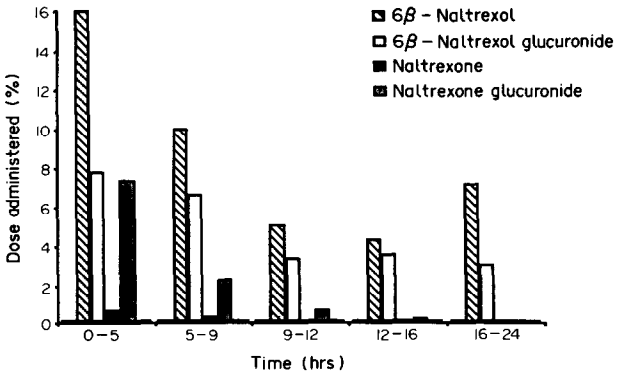

Figure 3
Urinary excretion pattern of naltrexone and metabolites shown as percentages of the administered dose in a volunteer receiving 50 mg naltrexone orally.

Discussion

The major metabolites of NTX are NTXOL and the glucuronoconjugates of this metabolite and of the unchanged drug [3–5]. Many techniques described in the literature measure the concentration of the conjugated fraction by an indirect procedure comparing hydrolysed and non-hydrolysed urine samples [6–11]. HPLC offers a direct approach for quantitative measurements. A prerequisite is reliable extraction of metabolites with different polar characteristics from the biological matrix. Satisfactory results cannot be obtained with conventional liquid–liquid procedures, but solid–liquid methods have been useful in the extraction of various other glucuronides, either by the use of bonded octadecyl silica supports [14, 15] or with styrene–divinylbenzene copolymers [16]. Manipulation of eluent polarity and pH allows optimisation for the compounds under study.

Previous results [17] obtained in the isolation of morphine metabolites by means of Amberlite XAD-2 demonstrated that a compromise was needed regarding solvent polarity in order to obtain both a high yield and a reasonably selective extraction; a methanol:water [1:1] mixture was selected when analysing for structurally related morphine-type compounds. Similarly, results obtained by Svensson *et al.* [14] in extracting opiate compounds using ODS-silica, indicated the usefulness of mixtures of aqueous solvents with acetonitrile to obtain good recoveries. In the present work, ODS-silica gave higher yields for the extraction of NTX and its metabolites than did Amberlite XAD-2 (Table 1). Increasing the acetonitrile concentration from 10 to 25% when using ODS-silica decreases the eluent polarity and helps to dissolve the free amine NTX metabolites adsorbed onto the extraction resin. In contrast, the higher water solubility of GL NTX and GL NTXOL allows nearly maximal extraction using only 15% acetonitrile in the elution solvent. In this case, the less polar solvent (25% acetonitrile) does not

significantly improve the recovery of conjugates. The final choice as to the amount of acetonitrile (15 or 25%) when extracting actual samples will depend upon the appearance of compounds interfering with chromatography, especially near the retention times for GL NTX and GL NTXOL.

The composition of the HPLC mobile phase used to analyse NTX metabolites was optimised for a reverse-phase separation using ion-pair formation as initially described for morphine-related compounds [14]. Acidity in all cases has been carefully adjusted to pH 2.1 in order to ionise the piperidinic nitrogen but not the glucuronic acid group (pKa = 3.2; [14]) when present. A positive charge is necessary for the formation of ion-pairs.

When the molar concentration of ammonium phosphate is increased, the retention of the compounds under study is reduced non-linearly (Fig. 1A) with tailing peaks. This trend is consistent with the occupancy of active column sites for ion-pair reagents of the same charge as the sample [18]. The ammonium ions occupy the active column sites and their positive charge excludes molecules with the same charge, thus reducing retention times of positively charged compounds. Also, the strong influence of buffer ionic strength on the retention of glucuronoconjugates, but not on other interfering peaks, is readily observable. Optimum separation of conjugates from interfering substances was obtained with a buffer concentration of 5 mM.

The influence of organic solvent concentration on retention characteristics is as expected for a conventional reverse-phase separation. A non-linear reduction in capacity factors is seen for all compounds when acetonitrile content is increased (Fig. 1B). The relative retention between NTX and NTXOL is not greatly affected by changes in organic solvent content.

The effect of increasing counter-ion concentration is also consistent with previous findings [18, 19]. Retention by the non-polar column is increased when molarity of the counter-ion is increased (Fig. 1C). The NTXOL–NALOX resolution is considerably improved by increasing the counter-ion concentration. Optimal separation (resolution factor, Rs 1.53) is obtained with 5 mM dodecyl sulphate, compared with Rs 1 for 2 mM and Rs 0.68 for 1 mM.

The identity of NTXOL was verified by GC/MS of the TMS derivative. The mass spectrum obtained (main peaks at m/z 372 and 559) is comparable to that reported in the literature [11]. The identity of GL NTX and GL NTXOL was verified by comparison of selected hydrolysed and non-hydrolysed urines. The decrease of the glucuronide peaks and the corresponding increase of the free analogous peaks can be seen in hydrolysed samples. In the chromatographic profile shown in Fig. 2, a peak probably due to 2-hydroxy-3-*O*-methylnaltrexol (another NTX metabolite) is observed in addition to the peaks from NTX, NTXOL, GL NTX and GL NTXOL.

The applicability of this technique to the study of urinary excretion will contribute to an improved understanding of the metabolic disposition of NTX, see Fig. 3. Our total recovery of the dose (79%) is slightly higher than that found by other authors and can be improved by longer collection times. The total relative excretion of each metabolite is consistent with the published data [3–6]. The availability of pure samples of NTXOL, GL NTX and GL NTXOL to prepare individual standard curves will undoubtedly improve the reliability and the accuracy of further quantitative studies.

In summary, a liquid chromatographic method for the simultaneous determination of the main NTX metabolites has been developed which is suitable for studying the urinary excretion profiles of NTX.

Acknowledgements — The authors thank M. Mestres and Ll. San for their cooperation and the Du Pont Company for the gifts of naltrexone standard.

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[*Received for review 24 September 1987*]