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Enantioselective high-performance liquid chromatographic method for the determination of methadone and its main metabolite in urine using an AGP and a C_8 column coupled serially

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Abstract

A simple and sensitive method for the enantioselective high-performance liquid chromatographic determination of methadone and its main metabolite, EDDP, in human urine is described. (−)-(*R*)-Methadone, (+)-(*S*)-methadone, (+)-(*R*)-EDDP, (−)-(*S*)-EDDP and imipramine as an internal standard are detected by ultraviolet detection at 200 nm. The enantiomers of methadone and EDDP were extracted from human urine by a simple liquid–liquid extraction procedure. The extracted sample was reconstructed in mobile phase and the enantiomers of methadone and EDDP were quantitatively separated by HPLC on a short analytical LiChrospher RP8 column coupled in series with a chiral AGP column. Determination of all four enantiomers was possible in the range of 0.03 to 2.5 μ*M*. The recoveries of methadone enantiomers and EDDP enantiomers added to human urine were about 90% and 80%, respectively. The method was applicable for determination of methadone enantiomers and the enantiomers of its main metabolite in urine samples from methadone maintenance patients and patients suffering from severe chronic pain.

Keywords: Enantiomer separation; Methadone; 2-Ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine

1. Introduction

Methadone is commercially available as a racemic mixture of *R*- and *S*-enantiomers. The analgesic and abstinence relieving effects are due to the *R*-enantiomer [1]. Differences in pharmacokinetic parameters (elimination half-life, volume of distribution and clearance) between the *R*- and *S*-enantiomer have been demonstrated [2]. The main metabolite of methadone is the cyclical *N*-demethylated metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), which also contains a chiral centre (see Fig. 1). Measured as the racemic EDDP, this metabolite was only found in a small amount in serum but in urine a considerable amount is seen [3,4].

More recently highly sensitive analytical methods have been developed for chiral separation of methadone enantiomers in plasma and serum [5–10]. However, none of these methods measured methadone metabolites.

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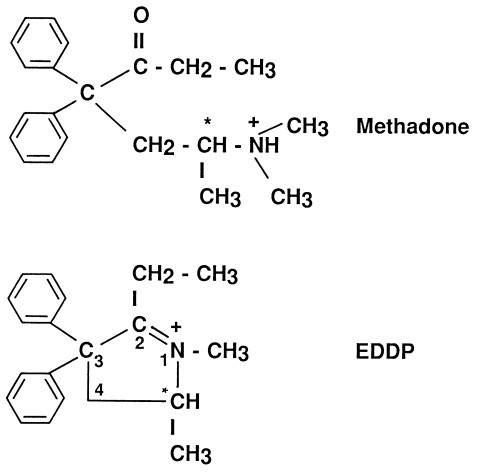

Fig. 1. Structures of methadone and its main metabolite EDDP. *=Chiral centre.

With the purpose of examining the stereoselectivity of the metabolism of methadone, a simple and sensitive method was needed for the determination of the enantiomers of methadone and EDDP in urine. Two capillary electrophoretic analytical methods [11,12] and a liquid chromatography–mass spectrometry (LC–MS) method [13] for simultaneous quantitative determination of enantiomers of both methadone and EDDP have recently been published, but since this equipment is not available in our laboratory we needed a method based on high-performance liquid chromatography (HPLC) or gas chromatography (GC). A previously described HPLC method using UV detection [7] has successfully been used for several years in pharmacokinetic studies of methadone in serum [2]. However, the method was not optimized for determination of EDDP enantiomers since the peaks of EDDP appeared not to be separated from the (−)-*R*-methadone peak in this HPLC system. This paper describes a simple and economic method based on a C_8 analytical column coupled in series with a chiral AGP column for quantitative determination of the enantiomers of EDDP and methadone. The method shows a sufficient selectivity for enantioselective measuring of methadone and EDDP in urines from addicts and sufficient sensitivity for enantioselective measuring methadone and EDDP in pain patients treated with low doses (5 mg i.v.) of methadone.

2. Experimental

2.1. Chemicals

rac-Methadone and imipramine hydrochloride (pharmacopoeias grade) were supplied by the hospital pharmacy, Bispebjerg Hospital. (−)-(*R*)-Methadone hydrochloride (Levomethadone hydrochloride) was a gift from Hoechst (Frankfurt/Main, Germany). (+)-(*R*)- and (−)-(*S*)-EDDP perchlorate salt was a gift from RTI (NC, USA). EDDP perchlorate salt was purchased from Sigma (St. Louis, MO, USA). Stock standard solutions were prepared in ethanol–water (50:50, v/v). Acetonitrile was of LiChrosolv grade and *n*-hexane was of Uvasol grade from Merck (Darmstadt, Germany). Dimethyloctyladlmine (DMOA) was obtained from K & K Labs. (Cleveland, OH, USA). All other chemicals were of analytical-reagent grade.

2.2. HPLC conditions

The HPLC system consists of a Shimazu LC-6A HPLC pump with a UV–VIS ChromPack detector operating at 200 nm, Perkin-Elmer ISS-101 sampler, Turbochrom 3 software (Perkin-Elmer).

The reversed-phase guard column from Chrompack and the analytical column LiChrospher RP8 endcapped, 5 μm (30×2 mm I.D.) (Knauer, Bad Homburg, Germany) were coupled in series with a Chiral-AGP chiral column (100×4 mm I.D.) from ChromTech (Norsborg, Sweden).

The mobile-phase was 10 m*M* sodium phosphate buffer (pH 5.0)–acetonitrile–DMOA (860:140:0.5) with final pH 6 and a flow-rate of 0.9 ml/min. The percentage of acetonitrile was lowered as the column became older (see Section 4). The mobile phase was degassed with helium for 5 min before use.

2.3. Procedures

A 1.0-ml aliquot of 1 *M* sodium carbonate buffer (pH 10.0) containing imipramine was added to 3.0 ml of urine. (For analyzing urine samples from addicts 1.0 ml urine was used, see Table 1). The sample was extracted with 6.0 ml of *n*-hexane by horizontal shaking for 15 min. After centrifugation for 5 min at 1300 *g* and cooling in a dry-ice acetone

Table 1
Reproducibility of replicate analysis of EDDP and methadone enantiomers added to human urine

Added (μmol/l)[a]	Measured (μmol/l)[a]		C.V. (%)	
	(+)-*R*	(−)-*S*	(+)-*R*	(−)-*S*
EDDP				
0.06	0.026	0.029	16.1	19.5
0.25	0.12	0.13	7.1	9.4
3.0	1.7	1.7	9.4	8.9
8[b]	4.2[b]	4.5[b]	1.5[b]	4.8[b]
18[b]	9.2[b]	9.7[b]	20[b]	6.2[b]
Methadone				
0.06	0.032	0.031	9.3	6.1
0.25	0.14	0.14	3.5	4.2
3.0	1.6	1.6	2.0	1.6
3.0[c]	1.6[c]	1.6[c]	3.1[c]	6.8[c]
8[c]	4.2[c]	4.3[c]	1.9[c]	2.9[c]

[a] Mean values from five duplicate samples at each concentration.
[b] =1 ml extracted (a calibration curve of 1 to 25 μmol/1 was used).
[c] =1 ml extracted (a calibration curve of 0.4 to 10 μmol/1 was used).

bath for 10 min, the organic phase was decanted into new glass tubes, 20 μl dimethylformamide (DMF) was added after which the glasses were evaporated to dryness at room temperature under nitrogen. The residue was dissolved in 100 μl of mobile phase with 15% acetonitrile and 70 μl was injected into the HPLC system.

Calibration samples were prepared by adding known amounts of racemic methadone and EDDP to blank urine. Quantification was achieved by comparing the peak-height ratios of unknown samples with those obtained for the calibration samples. Three quality control samples were analyzed on each day of analysis.

3. Results

Fig. 2 shows chromatograms obtained from analysis of urine samples. The separation of the enantiomers was satisfactory, the resolution being not less than 1.3. The assay linearity for the enantiomer was determined by performing linear regression analysis on the plot of the peak-height ratios of either (+)-(*R*)-EDDP, (−)-(*S*)-EDDP, (−)-(*R*)-methadone or (+)-(*S*)-methadone to the internal standard (y) versus concentration (x) in the range 0.02 to 2.5 μ*M* (concentration range for each EDDP and methadone enantiomer). The line of best fit $n=10$, $R^2=0.999$ for (+)-*R*- and (−)-*S*-EDDP and (−)-*R*- and (+)-*S*-methadone was $y=4.19x$, $y=3.46x$, $y=1.93x$ and $y=1.70x$, respectively.

The reproducibility was determined by analyzing spiked urine samples at random on different days (Table 1). The lower limit of quantitation was 0.03 μmol/l for both the methadone and EDDP enantiomers (coefficient of variation<20%).

The selectivity was checked by comparing results from analysis of urine samples from addicts and pain patients with the described method and the results obtained by a racemic GC method [14]. Only urine samples from addicts found positive for ketobemidone in a thin-layer chromatography (TLC) test had to be omitted due to interfering peaks. The results from comparison of the remaining samples from 21 addicts and five pain patients are shown in Fig. 3. The equations of the linear regression analysis shows an equation close to the identity line with a correlation coefficient of 0.996 and 0.984 for methadone and EDDP, respectively. The calculated *S*/*R* enantiomeric and EDDP/methadone ratios from the addict samples are shown in Table 2.

4. Discussion

Combination of an analytical column with a chiral AGP column provided a satisfactory separation of the methadone and EDDP enantiomers with R_s values of 1.3, 6.8 and 2.1 for (+)-*R*-EDDP and (−)-*S*-EDDP, (−)-*S*-EDDP and (−)-*R*-methadone, and (−)-*R*-methadone and (+)-*S*-methadone, respectively. The selective separation could be attained for analysing more than 1000 samples, whereas the separation of the (−)-(*S*)-EDDP enantiomer from (−)-(*R*)-methadone became unsatisfactory after the injection of only 300 samples using the AGP column alone. As previously described the use of an analytical column prior to the AGP column seemed to extend the AGP column lifetime, when regeneration procedure and mobile phase modifications were used [7]. With the C_8 analytical column the selectivity was improved compared with former use of a

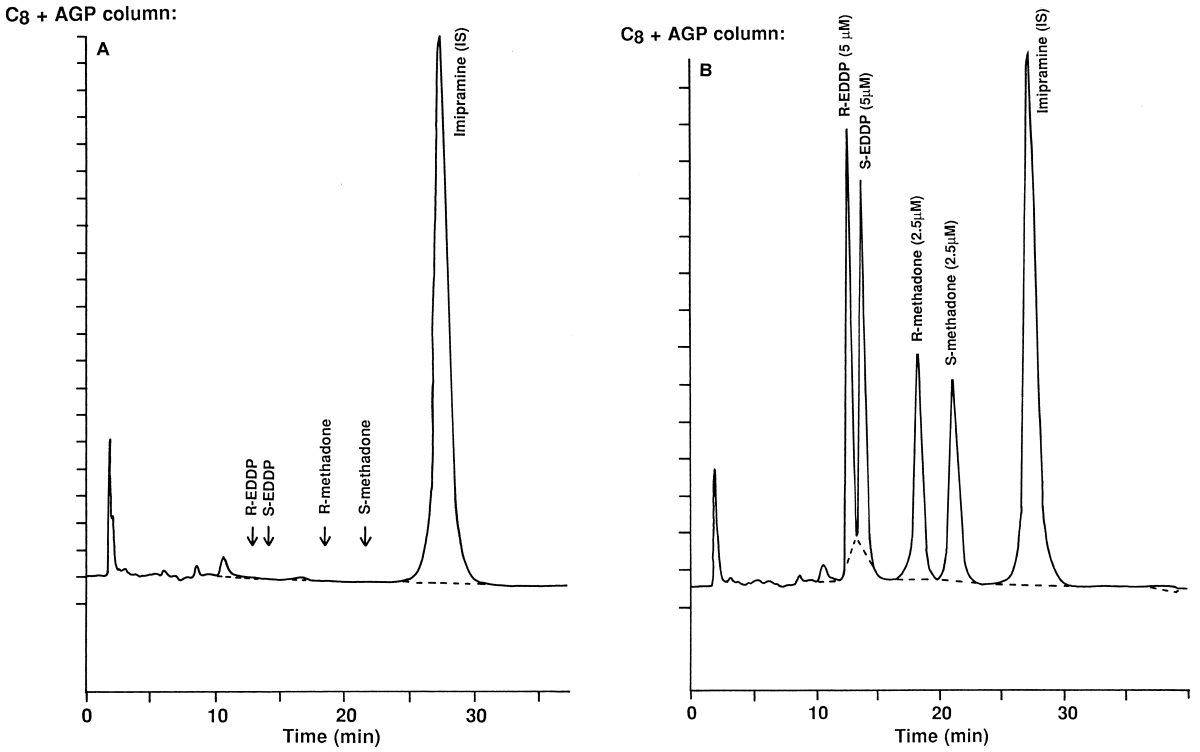

Fig. 2. Chromatograms of human urine extracts, analysed as described in Section 2. (A) Blank urine. (B) Urine spiked with methadone and the main metabolite EDDP. I.S.=Internal standard.

combination of a CN analytical column with a chiral APG column [7]. The latter chromatographic principle could not be used for the simultaneous quantitative determination of methadone and EDDP enantiomers in most urine and serum samples from addicts due to interfering peaks from their use of illicit drugs.

The comparison of the result of the sum of the enantiomers of EDDP and methadone determined by this new method with the result of total EDDP and methadone determined by a conventional GC method [14] showed a reasonable agreement. Only urine samples found positive for ketobemidone in a TLC test had to be omitted due to interfering peaks, whereas urines samples determined positive for the content of morphine and benzodiazepine did not show any interfering peaks in the described chromatographic system.

In the extraction procedure addition of DMF during the evaporation and redissolvating step seemed to prevent disappearance of the EDDP metabolite. Without DMF a lower and more variable recovery was obtained especially in cases where the samples were not redissolved immediately. With DMF the evaporated samples were stable for more than 1 h. Addition of other compounds such as diethylamine or HCl did not have the same effect. The recoveries of methadone and the metabolite after extraction from urine were about 90% and 80%, respectively. Unlike other authors we did not find an increased recovery by using extraction solvents with higher polarity than hexane (butylchloride, 2% butanol) [4,9].

The S/R enantiomeric ratio and the EDDP/methadone ratio ranges determined from analysis of urine samples of 21 methadone maintenance patients are wider than those found by Lanz and Thormann [12] and Phang Huy et al. [15], but in both of these studies only a few patients were included. The S/R enantiomeric ranges confirm the stereoselectivity in methadone excretion.

In summary, this study describes a simple enan-

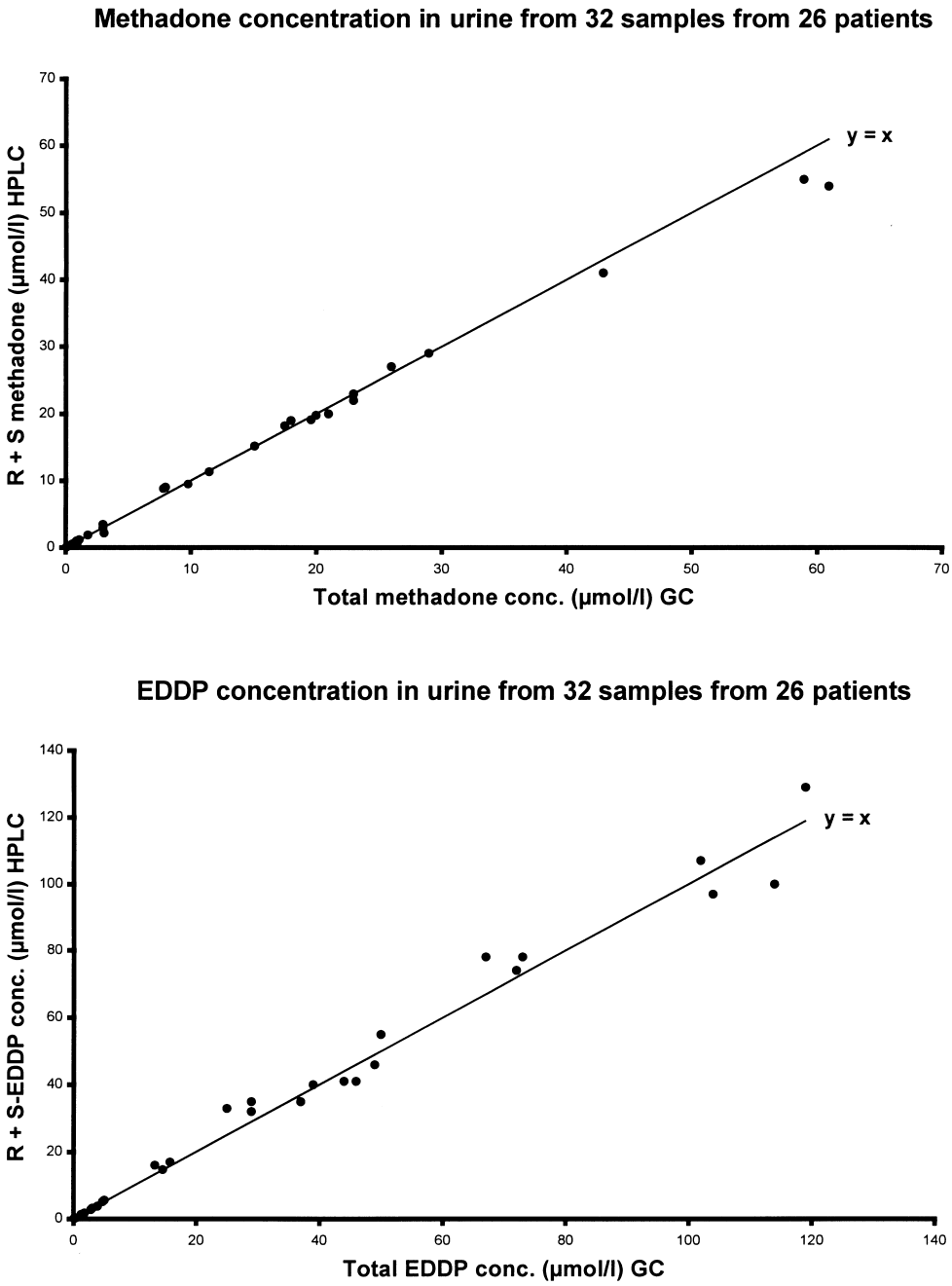

Fig. 3. Correlation of results from urine samples from 21 addicts and five pain patients receiving methadone analysed by the described method and by a conventional racemic GC method [14].

tioselective method for determination of enantiomers of both methadone and EDDP in urines from most methadone maintenance patients and with a limit of quantitation (0.03 μmol/l) sufficient for measuring methadone and EDDP in urines from pain patients. The method has been proven suitable for determi-

Table 2
Enantiomeric and EDDP/methadone ratios in 21 urine samples from 21 methadone maintenance patients

	Mean	Range
S/*R*-methadone ratio	0.5	0.2–0.9
S/*R*-EDDP ratio	1.4	0.4–2.1
(*R*+*S*)-EDDP/(*R*+*S*) methadone ratio	3.2	1.3–12.7

nation of (+)-(*R*)-EDDP, (−)-(*S*)-EDDP, (−)-(*R*)-methadone and (+)-(*S*)-methadone in urine samples, therefore it could be included in future studies investigating the stereoselective kinetics and metabolism/excretion of methadone and its major metabolite EDDP in pain patients.

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