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Stereoselective determination of the enantiomers of methadone in plasma using high-performance liquid chromatography

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ABSTRACT

An enantioselective high-performance liquid chromatographic assay for the quantification of methadone in human and beagle plasma is described. The procedure involves extraction of methadone from alkalized plasma into hexane–isoamyl alcohol (99:1, v/v). Stereoselective separation was achieved with a silica column with covalently bound α_1-acid glycoprotein (Chiral-AGP) without any derivatization procedure. The detection wavelength was set at 215 nm. Using an internal standard provided reliable control of the extraction procedure as well as quantification of the enantiomers of methadone. The limit of quantification was found to be 2.5 ng/ml. The method was demonstrated to be sufficiently sensitive for stereoselective pharmacokinetic studies of methadone.

INTRODUCTION

Methadone (MET) is a central-acting analgesic with high affinity for μ-opioid receptors; its chemical structure is related to that of morphine. Within the last decades MET has acquired an important role in the treatment of severe pain, *e.g.* cancer pain, and in maintenance treatment of opioid addicts [1–3].

For the quantification of MET in biological fluids or tissues (*e.g.* plasma, serum, urine, bile and brain) various non-stereospecific techniques have been published, including gas chromatography [4–9], mass fragmentography [10,11] and radioimmunoassay [12]. Some high-performance liquid chromatographic (HPLC) assays have been reported recently employing post-column ion-pair extraction with on-line fluorescence detection [13] or UV detection at a wavelength of 215 or 210 nm, respectively [14,15]. Recently, a chiral HPLC assay has been published [16]. The method, however, did not employ an internal standard (I.S.) and the limit of detection was only 10 ng/ml.

MET exists as two enantiomers, and in humans *R*-MET is about 25-fold more potent as an analgesic than *S*-MET following oral or intramuscular administration [1,17–19]. However, it is not known whether these differences in potency are only the result of pharmacodynamic differences or whether to stereoselective pharmacokinetic disposition also plays a part [20–22] since no highly sensitive stereoselective analytical assay is available. Consequently, the inter-individual variability in stereoselective disposition of MET has not been investigated.

This paper describes a simple and rapid meth-

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od for the stereoselective determination of both enantiomers of MET in plasma (human, beagle), using a commercially available α_1-acid glycoprotein (Chiral-AGP) HPLC column [23] and UV detection at 215 nm. The employed I.S. provides a reliable determination of MET as well as good precision and accuracy data. No derivatization has to be performed. In order to compare the racemic concentrations of the two enantiomers and the amount of racemic MET, a conventional gas chromatographic procedure was used [8].

EXPERIMENTAL

Chemicals

The MET enantiomers (*R,S*-6-dimethylamino-4,4-diphenyl-3-heptanone) as the pure compounds (hydrochloride salts) were donated by Hoechst (Frankfurt/Main, Germany). The optical purity of both enantiomers was >98%. All other organic solvents and chemicals were of HPLC or reagent grade. The mobile phase was freshly prepared, filtered (0.45 μm) and degassed in a vacuum prior to use. Dextropropoxyphene, (1*S*,2*R*)-(1-benzyl-3-dimethylamino-2-methyl-1-phenylpropyl)propionate, used as the I.S., was obtained as a gift from the Department of Forensic Medicine (University of Erlangen, Erlangen, Germany). The stock standard solutions of MET enantiomers (hydrochloride salts) were prepared by dissolving an appropriate amount in distilled water. Working standards were prepared in drug-free plasma from the stock standard to yield concentrations of 2.5–400.0 ng/ml (free base) of plasma and were kept at 5°C in darkness. No degradation was detectable within twelve weeks.

Chromatographic system

The HPLC system consisted of a Model 8810 SP pump (Spectra Physics, Darmstadt, Germany), a Model SP 100 UV monitor with a 10-mm analytical cell (Spectra Physics) fitted with a Model 232 diluter–autosampler (Gilson/Abimed, Langenfeld, Germany) and a CR 3A Shimadzu integrator (Egling, Germany). Stereoselective separation was achieved with a Chiral-AGP HPLC column (ChromTech, Norsborg, Sweden), 100 mm × 4.0 mm I.D., 5 μm particle size (Grom, Herrenberg, Germany). Owing to the temperature dependence of the chiral separation a column thermostat (Chemdata, Sinsheim, Germany) set to 20°C was used [24]. The mobile phase consisted of 2-propanol–0.01 *M* phosphate buffer, pH 6 (9.5:90.5, v/v). The flow-rate was set at 0.6 ml/min and UV detection was at 215 nm. The system was used in an air-conditioned room (20°C).

Analytical procedure

For the quantification of the enantiomers of MET, 2.0 ml of plasma (standard, quality control or sample from a dosed animal or patient) and 50 μl of the prepared solution (10 μg/ml in mobile phase) of the I.S. were transferred to a conical glass tube, with a PTFE-lined screw cap, then alkalized with 400 μl of 1 *M* potassium carbonate solution. It was extracted into 5.75 ml of *n*-hexane–isoamyl alcohol (99:1, v/v) by agitating for 20 min at room temperature. The organic layer (5 ml) was evaporated to dryness under a gentle stream of dry nitrogen, followed by rinsing the sides of the tube with 0.5 ml of *n*-hexane and evaporation. The dry residue was redissolved in mobile phase (100 μl). An 80-μl aliquot of the sample was injected onto the HPLC column. Standard curves were prepared by injecting plasma extracts spiked with various amounts of the *R*- and *S*-enantiomers of MET, simulating the concentration range following intravenous (i.v.) administration of common clinical dosages. For the quantification of the plasma samples peak-area ratios for MET relative to the I.S. were used.

Precision of the assay

Four samples each of eight plasma standards in the range 2.5–400.0 ng/ml (MET, base) and four quality control samples were determined in a day in order to achieve the intra-day precision values. The inter-day precision variabilities were obtained by performing single measures of spiked plasma samples over six days in the range 2.5–400 ng/ml. Each day, two quality control samples were analysed as well. Concentrations were back-calculated after plotting peak-area ra-

tios of MET to I.S. *versus* concentrations of the standards.

Recovery values

Recovery values were calculated by comparing extracted spiked samples of each MET enantiomer with the unextracted standard solutions in mobile phase in the range 2.5–400.0 ng/ml.

Application

The utility of the method was demonstrated after single i.v. administration to four, 4-year-old, male beagles (0.5 mg/kg *R*/*S*-MET) weighing between 17 and 20 kg or to five female patients, 39–60 years of age, suffering from severe cancer pain (0.07 mg/kg *R*-MET) (cancer of rectum, carcinoma of the pancreas, breast cancer). Blood samples were collected up to 100 h from an indwelling catheter in the right cubital vein. The blood samples were centrifuged and the separated plasma was then stored at −30°C with plasma quality control samples pending analysis.

RESULTS AND DISCUSSION

The assay described allows for the quantification of both enantiomers of MET in human and beagle plasma with a lower limit of quantification and better precision and accuracy than published recently by Beck *et al.* [16]. The analytical procedure consists of a single extraction with organic solvents employing an I.S., followed by the separation of the enantiomers with a Chiral-AGP HPLC column and detection at 215 nm. Typical chromatograms of blank human plasma and spiked human plasma are shown in Fig. 1. The retention times were 7.8, 11.2 and 17.4 min for the I.S., *R*-MET and *S*-MET, respectively. The limit of quantification (the lowest concentration that could be determined during the inter-day validation with either precision or accuracy of less than or equal to 15% [25]) for each enantiomer of MET in plasma was 2.5 ng/ml. The recovery values of the I.S. and the enantiomers of MET are 90.6% and within 86–99% (mean 93%), respectively (Table I). The

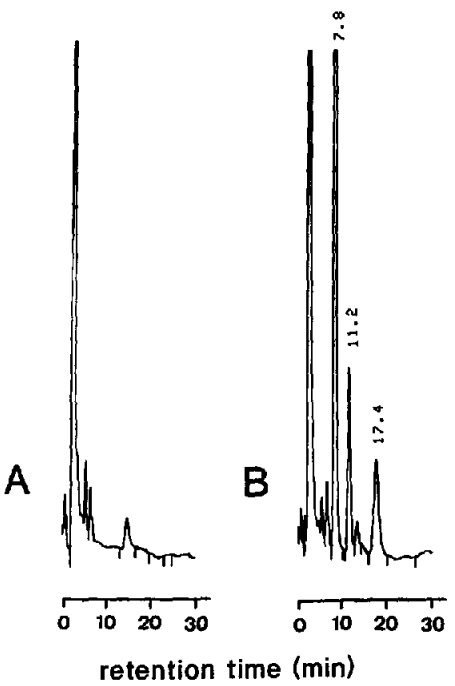

Fig. 1. Chromatograms of plasma extracts. (A) Blank human plasma; (B) human plasma spiked with racemic methadone hydrochloride (55.8 ng/ml; equivalent to 25 ng/ml *R*-methadone and 25 ng/ml *S*-methadone) and dextropropoxyphene hydrochloride (500 ng/ml; internal standard). The retention times were 7.8, 11.2 and 17.4 min for dextropropoxyphene, *R*-methadone and *S*-methadone, respectively.

peak-area ratios of the compounds were linearly related ($r > 0.999$) to the amount of MET enantiomers added (as hydrochloride salts) to human or beagle plasma in the ranges 2.5–25 and 25–400 ng/ml using two calibration curves. The inter-day and intra-day precision of the assay in plasma over seven days are given in Table II. The precision data using beagle plasma (data not shown) showed no difference as compared with human plasma. Additionally, the accuracy of the stereoselective HPLC determination was controlled comparing the evaluated concentrations of the enantiomers of MET to their sum using a conventional gas chromatographic assay [8]. It was within the range 96.6–99.9%.

The application of the assay was demonstrated after administration of MET to healthy beagles and patients suffering from severe cancer pain. Racemic MET and *S*-MET were administered to

TABLE I

ANALYTICAL RECOVERY OF METHADONE ENANTIOMERS AND OF THE I.S. IN HUMAN AND BEAGLE PLASMA

The recovery values of *R*/*S*-MET from beagle plasma are given in parentheses.

Amount added (ng/ml)	Recovery (mean ± S.D., n = 4) (%)		
	R-Methadone	*S*-Methadone	I.S.
2.5	96.9 ± 2.3	93.7 ± 4.8	
	(95.6 ± 3.1)	(94.2 ± 4.2)	
5.0	99.1 ± 5.1	96.0 ± 8.5	
10.0	91.9 ± 6.3	99.2 ± 6.5	
	(96.5 ± 7.1)	(93.1 ± 5.8)	
25.0	86.2 ± 7.3	89.8 ± 3.1	
50.0	94.8 ± 1.4	95.9 ± 6.1	
	(96.8 ± 2.8)	(93.6 ± 4.9)	
100.0	86.7 ± 4.7	89.0 ± 3.2	
	(89.4 ± 4.1)	(92.9 ± 6.5)	
200.0	93.5 ± 6.3	92.6 ± 4.1	
	(90.8 ± 7.0)	(89.7 ± 5.9)	
400.0	92.2 ± 3.6	94.1 ± 5.8	
	(94.8 ± 5.1)	(90.3 ± 3.9)	
500.0			90.6 ± 5.5

beagles only, because in Germany only the *R*-enantiomer of MET has been approved for human use. No inversion of *R*-MET to *S*-MET or *vice versa* was found in beagle plasma within the concentration range tested. Characteristic plasma concentration *versus* time profiles of MET following a single i.v. administration of 0.5 mg/kg racemic MET to a beagle and 0.07 mg/kg *R*-MET to a patient suffering from severe cancer pain of the rectum are shown in Fig. 2. In humans too, *R*-MET was not inverted to *S*-MET as determined by plasma analysis.

The inter-individual variability in analgesic potency of MET in patients treated with *R*-MET cannot be explained by an inter-individual variability in inversion. However, other processes of stereoselective disposition cannot be excluded. They deserve further investigation, *e.g.* employing the method described above.

As the AGP column is based on immobilized protein, the separation power, which decreases with an increasing number of analysed samples (*ca.* 300–500 plasma samples can be separated

TABLE II

INTRA-DAY (DAY 1) AND INTER-DAY PRECISION OF METHADONE ENANTIOMERS OVER SIX DAYS IN HUMAN PLASMA

C.V., coefficient of variation.

Concentration added (ng/ml)	Concentration found (ng/ml)									
	Day 1 (mean ± S.D., n = 4)	C.V. (%)	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Days 2–7 (mean ± S.D.)	C.V. (%)
R-MET										
2.5	2.5 ± 0.16	6.3	2.7	2.4	2.3	2.6	2.8	2.4	2.5 ± 0.2	7.9
5.0	4.9 ± 0.35	7.1	5.5	5.4	4.8	5.2	4.7	5.3	5.2 ± 0.3	5.8
10.0	9.8 ± 0.72	7.3	11.1	9.4	9.6	11.1	9.9	10.3	10.2 ± 0.7	6.8
25.0	24.9 ± 1.13	4.5	25.2	24.9	24.5	23.5	25.2	25.9	24.9 ± 0.8	3.2
50.0	50.8 ± 1.5	2.9	52.3	51.7	54.1	51.6	48.9	50.2	51.5 ± 1.8	3.4
100.0	99.9 ± 4.5	4.5	102.1	100.6	96.1	104.9	95.1	106.1	100.8 ± 4.5	4.5
200.0	197.0 ± 7.6	3.9	208.3	192.9	191.6	195.0	210.3	199.6	199.6 ± 8.0	4.0
400.0	399.6 ± 10.3	2.6	386.1	408.2	407.3	396.9	393.8	414.5	401.1 ± 10.6	2.6
S-Met										
2.5	2.4 ± 0.20	8.1	2.2	2.6	2.4	2.5	2.8	2.3	2.5 ± 0.2	8.0
5.0	5.4 ± 0.49	9.8	5.0	5.1	4.7	5.4	5.1	4.8	5.0 ± 0.4	8.1
10.0	10.5 ± 0.2	1.9	9.9	9.3	10.1	10.8	9.7	11.0	10.1 ± 0.7	6.9
25.0	24.9 ± 1.6	6.5	23.9	25.5	23.8	24.6	25.0	24.8	24.6 ± 0.7	2.8
50.0	50.2 ± 1.5	3.0	51.8	52.5	53.1	49.5	49.1	51.3	51.2 ± 1.6	3.1
100.0	98.0 ± 5.4	5.5	95.0	98.0	102.7	92.4	100.4	96.3	97.5 ± 3.7	3.8
200.0	198.8 ± 5.9	2.9	212.2	197.4	195.7	191.9	201.2	207.3	201.0 ± 7.6	3.8
400.0	397.9 ± 7.9	2.0	390.9	404.1	427.0	382.3	401.6	395.8	400.3 ± 15.2	3.9

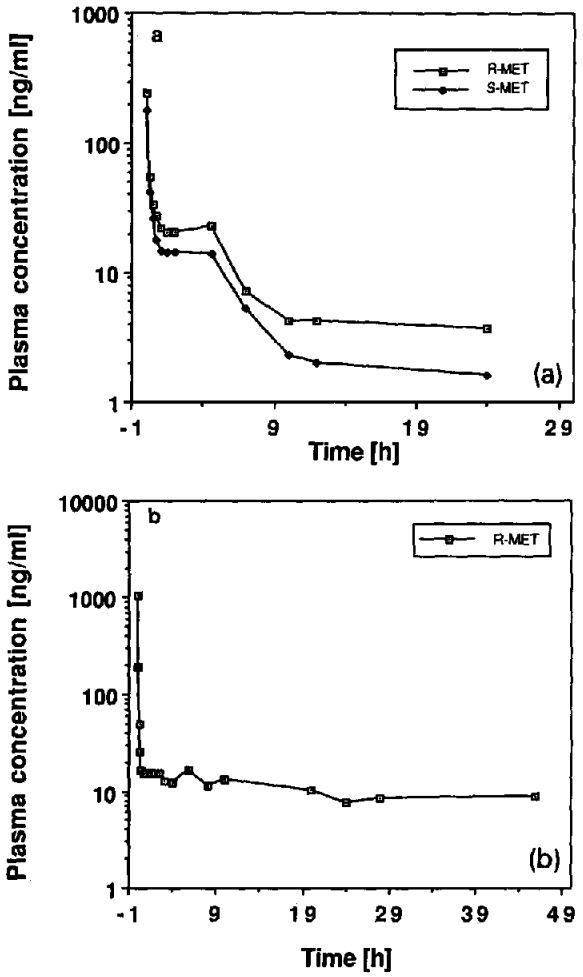

Fig. 2. Plasma concentration *versus* time profile of methadone enantiomers following a single i.v. injection of 0.5 mg/kg body weight racemic methadone to a beagle (a) and 0.07 mg/kg body weight *R*-methadone to a patient suffering from cancer pain (b).

per column), may differ from one column to another. Furthermore, the chiral separation using AGP columns is very sensitive to changes in chromatographic conditions (ion strength, pH, column temperature) [26,27]. Consequently, this assay may require minor methodological changes when switching from one column to another.

Many investigators have determined MET in biological fluids employing non-stereospecific methods [4,5,8,12–15]. Recently, a chiral assay for MET has been published [16]. The chiral assay we have developed is more sensitive (2.5 *versus* 10 ng/ml) than other HPLC methods (5 ng/ml [14], 10 ng/ml [15]) and mass fragmentography (5 ng/ml [10]) and almost as sensitive as radioimmunoassay (3 ng/ml [12]) and gas chromatography (1 ng/ml [8], 2.5 ng/ml [9]). In addition, our method employs an I.S., resulting in good accuracy and precision within the whole concentration range (Table II).

In conclusion, the HPLC assay presented above allows for the stereoselective determination of the enantiomers of MET in plasma. The method is more sensitive than the chiral assay published recently and provides good accuracy and precision. The analytical procedure is not inexpensive but easy to handle, and no derivatization of the enantiomers has to be performed. We have demonstrated its applicability for the stereoselective disposition of MET.

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REFERENCES

1 K. M. Foley, *N. Engl. J. Med.*, 313 (1985) 84.
2 J. Hansen, C. Ginman, P. Hartvig, P. A. Jakobson, M. I. Nilsson, A. Rane, J. Säwe and E. Änggard, *Acta Anaesthesiol. Scand., Suppl.*, 74 (1982) 124.
3 W. H. Horn, M. Rado and A. Goldstein, *Clin. Pharmacol. Ther.*, 17 (1975) 636.
4 G. K. Gourlay, P. R. Wilson and C. J. Glynn, *Anesthesiology*, 57 (1982) 458.
5 M. J. Kreek, *N.Y. State J. Med.*, 23 (1973) 2773.
6 M. J. Kreek, C. L. Gutjahr, J. W. Garfield, D. V. Bowen and F. H. Field, *Ann. N.Y. Acad. Sci.*, 281 (1976) 350.
7 H. R. Sullivan and D. A. Blake, *Res. Commun. Chem. Pathol. Pharmacol.*, 3 (1972) 467.
8 F. Magora, J. Chrubasik, D. Damm, J. Schulte-Mönting and Y. Shir, *Anesth. Analg.*, 66 (1987) 1308.
9 P. Jacob, J. F. Rigod, S. M. Poud and N. L. Benowitz, *J. Anal. Toxicol.*, 5 (1981) 292.
10 H. R. Sullivan, F. J. Marshall, R. E. McMahon, E. Änggard, L.-M. Gunne and J. H. Holmstrand, *Biomed. Mass Spectrom.*, 2 (1975) 197.
11 G. J. Kang and F. S. Abbott, *J. Chromatogr.*, 231 (1982) 311.
12 G. S. F. Ling, J. G. Umans and C. E. Inturrisi, *J. Pharmacol. Exp. Ther.*, 217 (1981) 147.

13 H. Derendorf and E. R. Garret, *J. Pharm. Sci.*, 72 (1983) 630.
14 K. Wolff, M. Sanderson, A. W. M. Hay and D. Raistrick, *Clin. Chem.*, 37 (1991) 205.
15 E. R. Garret, H. Derendorf and A. G. Mattha, *J. Pharm. Sci.*, 74 (1985) 1203.
16 O. Beck, L. O. Boreus, P. Lafolie and G. Jacobsson, *J. Chromatogr.*, 570 (1991) 198.
17 A. F. Casy and R. T. Parfitt, *Opioid Analgesics, Chemistry and Receptors*, Plenum Press, New York, 1986.
18 N. B. Eddy, H. Halbach and O. J. Braenden, *Bull. WHO*, 14 (1957) 353.
19 H. F. Fraser and H. Isbell, *Bull. Narc.*, 14 (1962) 25.
20 A. L. Misra and S. J. Mule, *Nature*, 241 (1973) 281.
21 B. A. Judson, W. H. Horns and A. Goldstein, *Clin. Pharmacol. Ther.*, 20 (1976) 445.
22 M. J. Kreek, D. L. Hachey and P. D. Klein, *Life Sci.*, 24 (1979) 925.
23 J. Hermansson, *Trends Anal. Chem.*, 8 (1989) 251.
24 S. Björkman, *J. Chromatogr.*, 414 (1987) 465.
25 V. P. Shah, K. K. Midha, S. Dighe, I. J. McGilveray, J. P. Skelly, A. Yacobi, T. Layloff, C. T. Vinswanathan, C. E. Cook, R. D. McDowall, K. A. Pittman and S. Spector, *J. Pharm. Sci.*, 81 (1992) 309.
26 G. Blaschke, in W. Fresenius, H. Guinzler, W. Huber, H. Keiker, S. J. Lüderwald, G. Tölg and H. Wisser (Editors), *Analytiker Taschenbuch*, Vol. 7, Springer, Berlin, 1988, p. 123.
27 G. Schill, I. W. Wainer and S. A. Barkan, *J. Liq. Chromatogr.*, 9 (1986) 641.