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# Stereoselective determination of methadone in serum by HPLC following solid-phase extraction on disk[1]

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**Abstract**

A solid-phase extraction (SPE) technique for methadone has been developed using a mixed-mode solid-phase extraction disk which contains both hydrophobic and cation-exchange functional groups. The SPE technique was used to isolate the drug from the biological matrix and to prepare a cleaner sample prior to stereoselective analysis by HPLC on a silica column with covalently bound $\alpha_1$-acid glycoprotein (Chiral-AGP) followed by ultraviolet detection at 205 nm.

The within-run precision was less than 5% for the complete method over the therapeutic range. The quantification limit was 25 ng $ml^{-1}$. The between-run precision was less than 15% at the quantification limit. The between-run precision at other concentrations was less than 8.5% with an accuracy of more than 95%. The mean recovery for *R*-methadone was 78.5% and the mean recovery for *S*-methadone was 73.4%. The complete procedure has been validated. This method was successfully used for the analysis of 15 clinical cases.

*Keywords:* Methadone; $\alpha_1$-Acid glycoprotein column; Chiral analysis; Solid-phase extraction; Extraction disk; Mixed-mode silica bonded phase

## 1. Introduction

Methadone is a synthetic opiate largely used as an analgesic drug, with pharmaceutical properties similar to those of morphine [1–4]. It has also been used, for many years, to treate heroin addicts undergoing methadone maintenance programs [5–8]. In Switzerland, a great number of people are following a methadone treatment [9].

The structure of the compound is shown in Fig. 1. Methadone possesses an asymmetric carbon and exists in *R* and *S* forms, therefore it is commercially available as a racemic mixture or as a pure enantiomer. Nowadays, methadone is administered as the racemate in many countries. However, it is well known that the *R*-enantiomer is 50 times more potent than the *S*-enantiomer in humans [10,11] which means that one has to have

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analytical methods capable of determining the concentrations of the enantiomers in biological matrices such as serum or whole blood.

Chromatographic separations have recently been developed for the enantioselective determination of methadone, such as GC [12] or HPLC [13–18]. Nevertheless, a direct separation is more suitable for this kind of determination and HPLC, with bonded $\alpha_1$-acid glycoprotein (Chiral-AGP) and $\beta$-cyclodextrin columns, is the method of choice.

Due to the complexity of serum or whole blood, the sample must be purified before chromatography in order to eliminate interfering compounds. Over the last few decades, this step has not undergone the same development as the chromatographic techniques and liquid–liquid extraction (LLE) [19–22] and solid-phase extraction (SPE) [19,23–26] are still the most commonly used methods. Recently, disks have been developed in order to enhance the performance of the SPE and reduce the solvent consumption. Some of these new supports are made of rigid glass fiber material embedded with bonded silica [27,28]. All of the bonded-phase chemistries typically found in conventional packed SPE devices, such as a mixed-mode silica, are available. This kind of support contains hydrophobic and sulfonic acid groups and permits the retention of basic compounds such as methadone selectively, as already demonstrated with conventional cartridges [23,25,29–31].

This paper describes the extraction of methadone, in serum or whole blood, on SPE disks containing a mixed-mode silica bonded phase. The purification step is coupled off-line with a chromatographic separation on a Chiral-AGP column. This method has been validated and used in real clinical cases in order to determine the enantiomeric ratio.

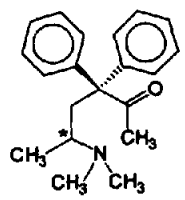


Fig. 1. Structure of methadone showing the position of the chiral carbon atom (asterisk).

## 2. Experimental

### 2.1. Material

Racemic methadone (*R/S*-6-dimethylamino-4,4-diphenyl-3-heptanone) hydrochloride was obtained from Hänseler AG (Herisau, Switzerland) and *R*-methadone hydrochloride was a gift from Hoechst (Frankfurt-am-Main, Germany). Deionised water was obtained from a Milli-Q RG ultrapure water system (Millipore, Molsheim, France). Drug standards used to test interferences were obtained from Siegfried AG (Zofingen, Switzerland). Human sera standards were obtained from Boehringer (Basel, Switzerland). Blood samples were obtained from patients participating in the methadone maintenance programs of the Fondation Phenix (Geneva, Switzerland). The dose range of racemic methadone hydrochloride given to the patients was 40–135 mg per day.

All compounds used to prepare buffer and extraction solutions were purchased from Fluka (Buchs, Switzerland). Ammonium Hydroxide (25% in water) was of puriss quality. Other chemicals were of HPLC or analytical grade and were used without any further purification.

For the SPE procedure, Toxi-Lab SPEC® plus ™MP3 columns (MP3-plus) (15 mg disk mass, 3 ml column size) were supplied by Ansys (Irvine, CA).

### 2.2. Solutions for extraction

Phosphate buffer (0.1 M) was prepared by dissolving 6.81 g of potassium dihydrogen phosphate in 450 ml of deionised water, adjusting the pH to 6.0 ($\pm$ 0.1) with 1.0 M potassium hydroxide, and making the total volume up to 500.0 ml with deionised water.

Acetic acid solution (10 mM) was prepared by adding 5.75 $\mu$l of glacial acetic acid to a 100.0 ml volumetric flask and making up to volume with deionised water. The eluting solution, isopro-

panol–$NH_4OH$–dichloromethane (20:2:78 v/v/v), was prepared daily by adding 1.0 ml of ammonium hydroxide to a 50.0 ml volumetric flask containing 10.0 ml of isopropanol. After 2 min of sonication, dichloromethane was slowly added and made up to volume.

### 2.3. Preparation of sample

#### 2.3.1. Whole blood preparation

Blood (5 ml) was sonicated in an ultrasonic bath for 15 min at room temperature. The sample was vortexed for 30 s. The blood was centrifuged at 3000 rev $min^{-1}$ for 10 min and the pellets were discarded. The serum obtained was treated according to the following procedure.

#### 2.3.2. Serum preparation

Serum (1.0 ml) was pipetted into an appropriate labelled test tube. To each specimen tube was added 2.0 ml of 0.1 M phosphate buffer pH 6.0. The sample was vortex mixed for 1 min.

### 2.4. SPE

The extraction was performed with MP3-plus disks installed on a 12-port Model vacuum Visiprep manifold (Supelco, Bellafonte, PA). The total procedure is outlined below.

(1) The column was conditioned with 1 ml of methanol and subsequently with 1 ml of phosphate buffer (0.1 M, pH 6.0) at a flow rate of 2 ml $min^{-1}$ (the flow rate was controlled by adjusting the vacuum of the extraction system).

(2) The pretreated blood or serum sample (3 ml) was transferred to the column and pulled through completely at a flow rate of 0.8 ml $min^{-1}$.

(3) The column was washed with 1 ml $H_2O$ and the pH of the extracting system was adjusted by passing 1 ml of acetic acid (0.01 M, pH 3.3) at a flow rate of 1.5 ml $min^{-1}$.

(4) The column was dried under vacuum for 5 min and 3 ml of methanol was added. The column was then dried again under vacuum for 2 min.

(5) A labelled evaporation tube was placed into the manifold basin and 1 ml of the eluting solution was added twice to the column and eluted completely at a flow rate of 0.5 ml $min^{-1}$.

(6) The solution was evaporated to dryness under a gentle stream of nitrogen. The dry residue was redissolved in 100 $\mu$l of methanol. A 60 $\mu$l aliquot of the sample was injected into the HPLC column.

### 2.5. Chromatography

The HPLC system consisted of a Model L-6200A pump, a Model L-4250 UV–Vis detector, a Model D-2500 chromato-integrator from Merck–Hitachi (Darmstadt, Germany) fitted with a Model 232 diluter–autosampler (Gilson, France). Stereoselective separation was achieved with a Chiral-AGP precolumn, 10 mm × 3 mm i.d., and a Chiral-AGP HPLC column (Chrom-Tech, Norsborg, Sweden), 100 mm × 4.0 mm i.d., 5 $\mu$m. Owing to the temperature dependence of chiral separation, an oven Model 7955 column chiller (Jones, COL) set to 20°C was used. The mobile phase consisted of isopropanol–phosphate buffer (10 mM) –*N*,*N*–dimethyloctylamine (DMOA), (900:100:1 v/v/v) (pH 6.6). The mobile phase was delivered at 0.6 ml $min^{-1}$ and UV detection was set at 205 nm.

## 3. Results and discussion

### 3.1. SPE

Often the preparation step is the bottleneck of an analytical procedure. The conventional techniques are tedious and time-consuming, can induce losses and contaminations and are not easily automated. Therefore, the development of SPE on disks is a very promising approach to avoid the above-mentioned disadvantages. In order to extract methadone from serum or whole blood selectively, we used a MP3-plus disk which contains a mixed-mode silica bonded phase. This disk exhibits hydrophobic and cation-exchange extraction mechanisms and can be used without any pretreatment of the sample, except for a dilution step [32].

A preliminary study (data not yet published) showed that this disk gave satisfactory results for the extraction of methadone from serum in com-

parison with conventional cartridges (C18 and Bond Elut Certify (BEC)(Analytichem Int., Harbor City, CA) silica sorbents) and with other disks such as a C18, a cationic exchanger SCX and a mixed-mode MP3 (Ansys, Irvine, CA) support.

Actually, the C18 silica bonded phase used either in a disk or in a cartridge retains methadone only by hydrophobic interactions. This mechanism is not selective enough and therefore, in a matrix such as serum, the extract contains too many interfering compounds for further chiral separation. In the same way, with a cationic exchanger disk (SCX), the selectivity was not sufficient. Only the BEC cartridges, the MP3 and the MP3-plus disks, which contain mixed-mode silicas, were able to give clean extracts for the enantiomeric separation. However, with serum samples, the MP3-plus disk was more appropriate due to its advanced filter designed to prevent clogging. Thus, the repeatability was better and the recoveries were greater than with the other supports. The total SPE procedure is described under experimental. The disk was first activated with methanol and buffer at pH 6.0. Then, the serum sample, buffered at pH 6.0, was percolated through the disk at a flow rate of 0.8 ml $min^{-1}$. At this pH, methadone was in its cationic form ($pK_a = 8.3$) and was then retained by the two mechanisms of interaction. Neutral and acid compounds were eliminated in the washing step, and the support was dried under nitrogen. The elution of methadone was achieved with a basic solvent which was then evaporated under nitrogen. The sample was reconstituted in 100 $\mu$l methanol allowing an enrichment factor of 10. This solution was then directly injected into the chromatograph.

### 3.2. Stereoselective determination

The enantiomeric separation of methadone was performed on a Chiral-AGP column. This chiral stationary phase has already been used by several authors for the analysis of methadone [15–18]. Actually, the plasma $\alpha_1$-acid glycoprotein, used as the chiral selector, bonds methadone with a high stereoselectivity in the human body [33].

Under the optimal chromatographic conditions (results to be published) reported in Fig. 2, the

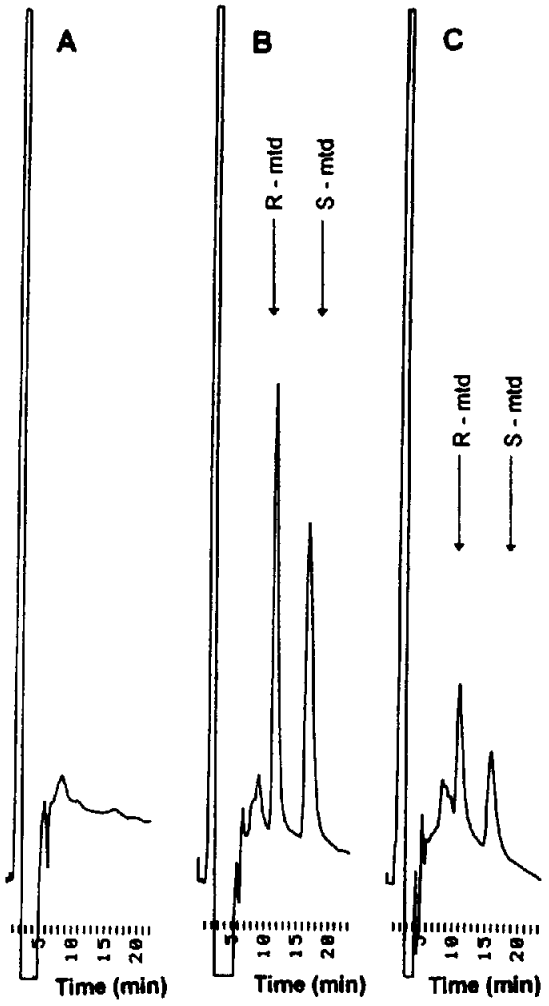


Fig. 2. Chromatograms of human serum extracts analysed as described using an AGP column with a mobile phase composed of isopropanol–phosphate buffer (10 mM)–DMOA(900:100:1 v/v/v) (pH 6.6). (A) Human blank serum. (B) Human serum spiked with 250 ng $ml^{-1}$ of each enantiomer. (C) Case No. 2, patient serum 24 h after a dose of 125 mg administered orally. R-mtd: *R*-(−) methadone; S-mtd: *S*-(+) methadone. Flow rate: 0.6 ml $min^{-1}$. UV detection: 205 nm. Temperature: 20°C (60 $\mu$l injected).

analysis is achieved in less than 20 min. It was found that the retention and stereoselectivity on Chiral-AGP can be varied by changing the pH of the aqueous mobile phase and the content of charged and uncharged organic compounds [34].

The pH has a strong influence on chromatographic separation as it modifies the complexing

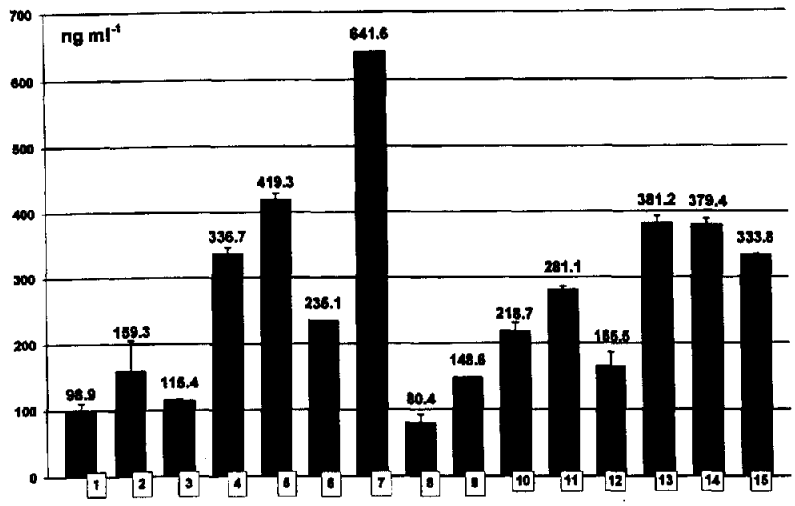


Fig. 3. Concentration of total methadone as the sum of l-and d-methadone in 15 previously heroin-addicted patients following a treatment.

properties of the stationary phase vis-a-vis methadone. In order to obtain the best resolution and satisfactory capacity factors, pH 6.6 was found to be optimal to separate the methadone from the matrix.

The major effect of adding isopropanol is to decrease the capacity factors without any effect on the enantioselectivity. A modification of the proportion of isopropanol in the mobile phase allowed us to regulate the analysis time and the addition of DMOA enhanced the efficiency of the chromatographic column.

The use of a precolumn, which contains the same phase as the column, preserved the analytical column. In order to maintain the efficiency of the system, this precolumn had to be replaced after every 100 injections of the sample extracts.

A regeneration of the analytical column is necessary after every 50 injections in order to recover the initial performance. For this purpose we percolated, through the column, 60 ml of a solution of phosphate buffer (pH 3.5; 10 mM) –isopropanol (85:15, v/v) at a flow rate of 0.5 ml $min^{-1}$ and then 150 ml of a solution of water–isopropanol (75:25, v/v) at a flow rate of 0.2 ml $min^{-1}$. This procedure was applied more than 30 times without significant loss in the performance of the analytical column.

The chromatographic parameters, under the optimal conditions, are shown in Table 1.

### 3.3. Quantitative analysis

Calibration curves were prepared with spiked serum over the range 25–1000 ng $ml^{-1}$ of methadone enantiomers. Spiked serum samples were processed by the SPE and chromatographic stereoselective determination. The correlation coefficients ($r$) for calibration curves were 0.9947 for $R$-methadone and 0.9987 for $S$-methadone. The regression equation for $R$-methadone gave a slope of $1914.0 \pm 81.7$ (RSD = 2.1%) and an intercept of $29\,667.9 \pm 39\,480.7$. A slope of $1817.8 \pm 38.6$ (RSD = 1.3%) and intercept of $-6420.9 \pm 18\,615.0$ were determined for $S$-methadone. The peak-area ratios of the compounds were linearly

Table 1
Chromatographic parameters of stereoselective determination of methadone (for conditions see text)

| Parameter | $R$-methadone | $S$-methadone |
|---|---|---|
| $k'$ | 6.8 | 10.1 |
| Asymmetry | 1.18 | 1.41 |
| $N$ | 1451 | 1222 |
| Enantioselectivity | 1.48 | |
| Resolution | 2.58 | |

Table 2
Precision and relative accuracy data[a] of methadone enantiomers in human plasma over 3 days

| Concentration added (ng ml$^{-1}$) | Concentration found (ng ml$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Within-run | | | Between-run | | |
| | Day 1 (Mean ± RSD; %) | Day 2 | Day 3 | Mean | Precision % | Accuracy % |
| *R*-Methadone | | | | | | |
| 25 | 20.8 ± 3.6 | 29.4 | 30.4 | 26.9 | 14.8 | 107.5 |
| 100 | 94.0 ± 2.0 | 94.5 | 98.9 | 95.8 | 3.4 | 95.8 |
| 250 | 267.5 ± 1.5 | 245.6 | 246.2 | 253.1 | 6.1 | 101.2 |
| 500 | 492.7 ± 0.7 | 503.4 | 501.6 | 499.2 | 2.1 | 99.8 |
| *S*-Methadone | | | | | | |
| 25 | 21.8 ± 4.9 | 33.5 | 36.4 | 30.6 | 13.5 | 122.3 |
| 100 | 91.0 ± 3.6 | 97.2 | 104.5 | 97.6 | 8.4 | 97.6 |
| 250 | 270.5 ± 2.2 | 248.2 | 252.7 | 257.1 | 5.9 | 102.9 |
| 500 | 491.7 ± 0.7 | 493.5 | 513.2 | 499.5 | 3.7 | 99.9 |

[a] Precision is expressed as the RSD (%) and accuracy as the assayed concentration, relative to the amount added (%) ($n = 3$).

related to the amount of methadone enantiomers added. This was confirmed by statistical analysis (Student's *t*-test). For each calibration curve, the intercept was not statistically different from zero.

In human serum, within-run precision was assessed by performing three replicate determinations of four serum standards, for concentrations ranging from 25 to 500 ng ml$^{-1}$ per enantiomer. Between-run precision of the method was assessed by analysing three replicates of four concentrations of serum standards on three separate days. Precision was expressed as the RSD (%) and accuracy as the mean of the assays relative to the amount added (%). Results are shown in Table 2. The within-run precision was less than 5% for the complete method and over the therapeutic range. The detection limit for each enantiomer, given by a signal-to-noise ratio of 3:1, was determined to be 10 ng ml$^{-1}$ and the quantification limit (signal-to-noise ratio of 10:1) was determined to be 25 ng ml$^{-1}$.

The between-run precision was less than 15% at the quantification limit. The between-run precision on the other concentrations was less than 8.5% with an accuracy of more than 95%.

Fig. 2 shows the chromatograms of an extract from a human blank serum, a human serum spiked with 250 ng ml$^{-1}$ of each enantiomer and an extract of a clinical case.

To simplify the chromatogram and the extraction process, no internal standards were used. Potential chromatographic interference was assessed for cocaine, morphine, codeine and dextropropoxyphene. For these experiments, and for clinical trials, no interfering peaks were obtained.

### 3.4. Extraction recoveries

Calibration curves were prepared with standard solutions in methanol, ranging in concentration from 10 to 1000 ng ml$^{-1}$ of racemic methadone. Peak-areas of methadone were measured and the calibration curves were generated by least-squares linear regression. The regression lines were used to calculate the extraction yields including the enrichment factor of 10 in the spiked samples. Results are shown in Table 3. Human serum standards, spiked with 50, 200, 500 and 1000 ng ml$^{-1}$ of racemic methadone, were used to obtain recovery values in the expected range of therapeutic concentration. The mean recovery for *R*-methadone was 78.5% and the mean recovery for *S*-methadone was 73.4%. The RSDs ($n = 5$) were lower than 4% except for the lowest concentrations where RSDs were approximately equal to 14%. In biological samples the recoveries are sim-

Table 3
Analytical recovery[a] of methadone enantiomers in human serum using SPE disks

| Concentration added (ng ml$^{-1}$) | Recovery (mean ± RSD; %) | |
|---|---|---|
| | *R*-Methadone | *S*-Methadone |
| 25 | 84.1 ± 13.8 | 69.1 ± 13.4 |
| 100 | 80.1 ± 1.8 | 78.1 ± 3.1 |
| 250 | 78.2 ± 1.5 | 75.6 ± 2.6 |
| 500 | 71.6 ± 2.2 | 70.7 ± 2.2 |
| Mean | 78.5 ± 4.8 | 73.4 ± 5.3 |

[a] Recoveries (%) are expressed as the actual assayed peak area relative to the expected area from direct injection of pure standards in methanol ($n = 5$).

ilar to those obtained by other authors who followed an SPE by an achiral analysis of methadone [29,35].

The quantitation of methadone was further validated by comparing the sum of *R*-methadone and *S*-methadone with the results obtained with a conventional LLE procedure coupled to a GC–NPD (Nitrogen-Phosphorous-Detector) analysis. This last method is used routinely in forensic laboratories in order to determine the total methadone concentration. The results obtained are reported in Table 4. The results from the two methods were found to correlate well, according to the equation LLE–GC–NPD = SPE–HPLC × 1.000 – 0.364, with a correlation coefficient of 0.9994.

### 3.5. Clinical applications

This method was successfully used in the analysis of 15 samples from previously heroin-addicted patients following a treatment with methadone. Each sample was analysed twice; the obtained means are given in Fig. 3.

All the measured concentrations were in the expected range and the repeatability of the analysis was satisfactory. The variations of the measured concentrations can be attributed to the different dosages given to the patients and to individual metabolisation.

Fig. 4 shows that the enantiomeric *R*/*S* ratio of methadone is extremely variable, ranging from 0.57 to 1.54. Our results are in accordance with previous studies which have indicated inter-individual differences between the enantiomeric *R*/*S* ratio of methadone. The differences in the ratios could be important in determining the dose to be administered.

## 4. Conclusion

We have developed a simple extraction procedure for methadone contained in serum. This was achieved by using an extraction disk made of rigid glass fiber meterial with a mixed-mode silica bonded phase. The simultaneous hydrophobic and ion-exchanging interactions selectively retain methadone from a complex matrix such as human serum.

Using extraction disks presents the advantages, over the conventional cartridges, of reducing the volume of solvents and decreasing the time needed to dry the phases, thus ensuring a good repeatability of the analyte extraction. We have also shown that the extraction recoveries were good and reproducible in the explored concentra-

Table 4
Comparative data[a] for LLE–GC–NPD and SPE–HPLC–UV for methadone determination

| Concentration added (ng ml$^{-1}$) | LLE GC analysis | | SPE HPLC analysis | |
|---|---|---|---|---|
| | Accuracy (%) | Precision (%) | Accuracy (%) | Precision (%) |
| 50 | 84.0 | 6.0 | 85.2 | 4.1 |
| 200 | 99.1 | 5.5 | 92.5 | 2.6 |
| 500 | 103.6 | 5.1 | 107.6 | 1.8 |
| 1000 | 99.2 | 5.2 | 98.4 | 0.7 |

[a] Precision is expressed as the RSD (%) and accuracy as the assayed concentration, relative to the amount added (%) ($n = 3$).

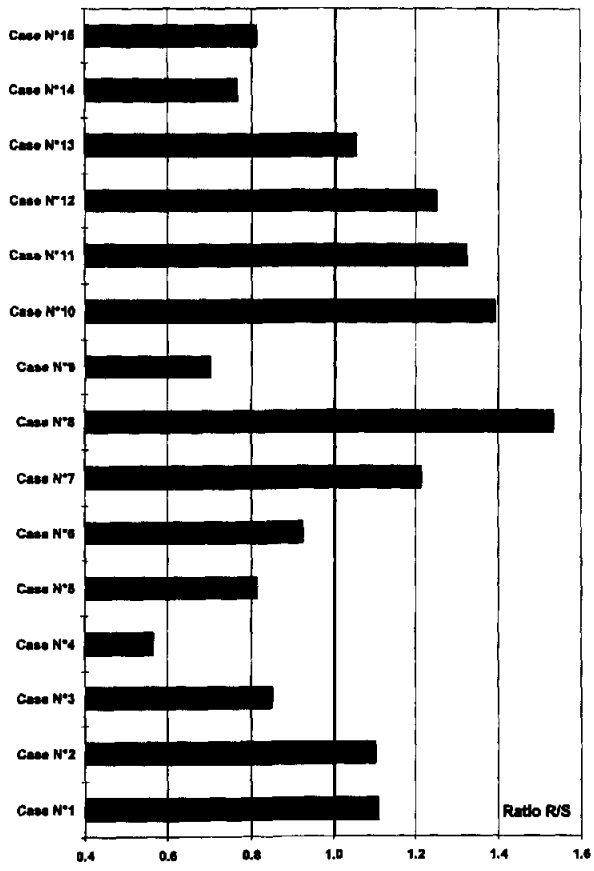


Fig. 4. *R/S* ratios of samples from patients treated with racemic methadone.

tion range. This procedure, coupled with a chiral stationary phase (a Chiral-AGP column) has enabled us to determine the enantiomeric ratio of methadone. The complete procedure has been validated. We have shown that this method is precise and can be applied to clinical cases. Preliminary trials on 15 subjects have proved the applicability of our method at these dosage levels, and further trials are underway. The next step will be to introduce this method in our laboratory as an automatic procedure.

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