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Enantioselective Separation of Methadone and Its Main Metabolite in Human Hair by Liquid Chromatography/Ion Spray–Mass Spectrometry*

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ABSTRACT: Optical isomers exhibit significant differences in their affinities for receptor sites, biotransformation and binding to serum and tissue proteins. Methadone has been used for the substitution of heroin addicts since 1964. The racemic form is used, i.e., a mixture of the biologically active R-form and the practically inactive S-form. To investigate methadone distribution, a chiral separation of the isomers was developed in human hair samples. The method involves decontamination of hair with water and acetone, pulverization in a ball mill, enzymatic hydrolysis in presence of deuterated internal standards, solid-phase extraction, and liquid chromatography/ion spray-mass spectrometry. Enantioselective separation of methadone and its main metabolite, EDDP, was obtained using an alpha1-acid glycoprotein column (100 by 4 mm ID). In all nine specimens obtained from subjects under racemic methadone treatment in a detoxification center, R- and S-enantiomers of methadone and EDDP were identified with the following concentrations: 2.58–10.22, 1.89–9.53, 0.42–1.73, and 0.40–2.10 ng/mg for R-methadone, S-methadone, R-EDDP, and S-EDDP, respectively. Results are suggestive of a predominance of the R-enantiomer of methadone in human hair.

KEYWORDS: forensic science, forensic toxicology, chiral separation, methadone, hair, gas chromatography-mass spectrometry

Methadone therapy is important for the effective treatment of opiate dependance in heroin addiction. Methadone is used in detoxification and maintenance programs for the management of physical dependance of narcotics. The drug provides pharmacological stability and cross-tolerance to opiate drugs and, hopefully, eliminates the necessity for illicit opiate drugs. Extra methadone is frequently ingested by the self-user on maintenance, irrespective of the presence of withdrawal symptoms or reduction of symptoms after dose ingestion.

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The pharmacokinetic parameters of methadone differ from that of other opioids. Its almost complete oral bioavailability favors its use by the oral route (1). Studies in maintenance patients indicate that a potential of accumulation exists due to a wide interindividual range of the terminal half-life of the drug (2). This variability may require dose adjustment on the basis of individual pharmacokinetic parameters.

Methadone contains a chiral carbon atom and therefore exists in two stereoisomeric forms. (R)-(−) methadone, also named levomethadone, has been estimated to be 25 to 50 times more potent than (S)-(+) methadone in clinical studies (3,4). Although the enantiomers show different pharmacodynamic and pharmacokinetic parameters, it is usually the racemate that is administered. In Germany, methadone substitution started with 1-Polamidon, the levo-isomer in 1992. Meanwhile, levo- and racemic forms are used currently.

With the purpose of examining the possible stereoselective absorption and disposition of methadone, a sensitive method for the measurement of the enantiomers is needed. Recently, hair testing has been demonstrated to have some advantages over urine testing for monitoring and managing patients on methadone maintenance (5). A major limitation of blood and urine analyses is the relatively short retrospective time period for drug exposure. Most drugs of abuse are detected in urine during 2 to 4 days after a single exposure. To be effective, two urine tests have to be performed each week. Therefore, hair analysis greatly expands the time window for the detection of exposure to illicit drugs because it can reveal exposures from weeks to months prior to a test (see review Ref 6).

Few papers have presented the determination of methadone and/or its major metabolite, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP) in human hair (7–10). In all cases, only racemic methadone was measured.

This paper describes a method for the stereoselective determination of both enantiomers of methadone and EDDP in human hair using an alpha1-acid glycoprotein (Chiral-AGP) HPLC column and mass spectrometric detection.

Material and Methods

Chemicals and Equipment

Methanol and isopropanol were HPLC grade (Merck, Darmstadt, Germany). All other chemicals were analytical grade and supplied by Merck. Methadone, EDDP, methadone-d_3 and EDDP-d_3 were purchased from Radian (Austin, TX, USA). β-glucuronidase (12

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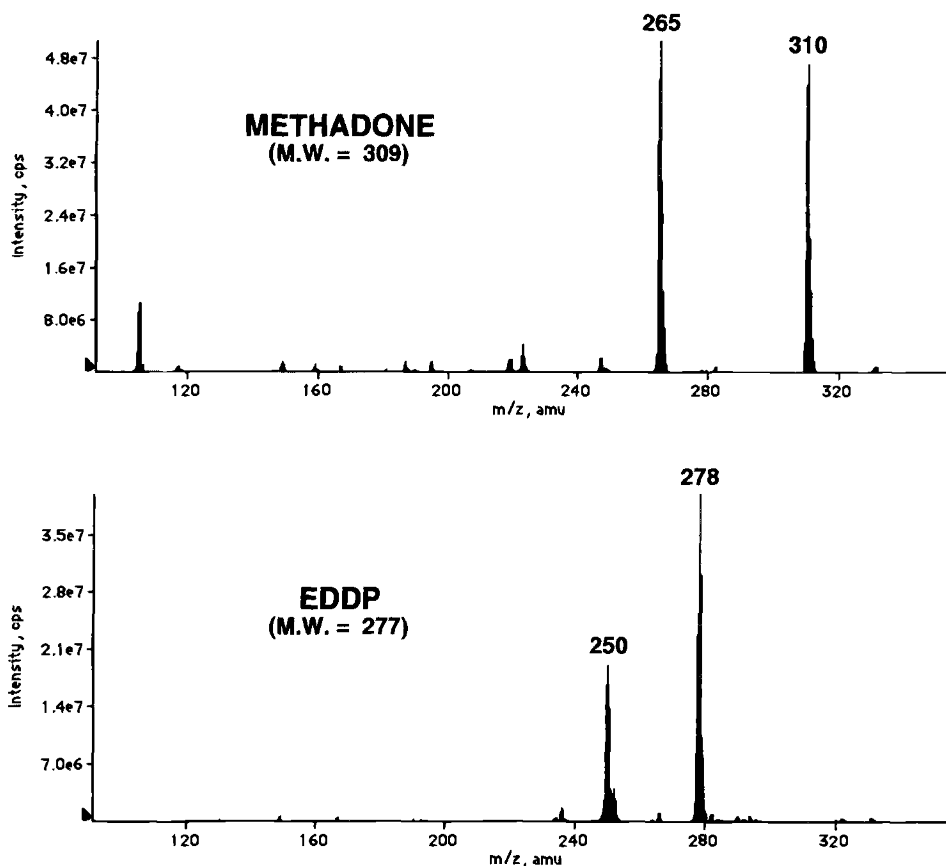

FIG. 1—*Background-substracted ISP-MS spectrum of methadone (upper graph) and EDDP (lower graph). Conditions: Infusion (5 μL/min) of 10-μg/mL standards in isopropanol/2 mM NH_4COOH buffer, pH 5.8 (20:80, v/v).*

units/mL)/arylsulfatase (60 units/mL) was obtained from Boehringer (Mannheim, Germany). The solid phase extraction was carried out with Chromabond® C18 ec (200 mg, 3 mL) extraction columns (Macherey-Nagel, Dülmen, Germany) and Vac-Elut® SPS 24 system (Analytichem International; Frankfurt, Germany). The ball mill (type MM2) was purchased from Retsch (Haan, Germany). The chiral column 5-μm CHIRAL-AGP (100 by 4.0 mm, ID) was obtained from Chromtech (Norsbory, Sweden).

Specimens—Hair samples were obtained from nine subjects, aged between 20 to 30 years, who had been admitted several months before to a detoxification center. All reported an history of intravenous heroin abuse.

Extraction—Hair samples, weighing at least 100 mg, were cut as close as possible to the skin from the posterior vertex. To eliminate external contaminants, the hair was washed with warm water (5 min) and acetone (1 min) and then dried in a stream of warm air.

To 60 mg of pulverized hair in a ball mill, were added 6 mL acetate buffer (pH 4.0) and 300 ng of the deuterated standards (methadone-d_3 and EDDP-d_3). The sample was hydrolyzed with 180 μL β-glucuronidase/arylsulfatase for 1.5 h at 40°C.

The extract was then neutralized with $NaHCO_3$ and deposited on a Chromabond C18 extraction column that has been previously conditioned with 2 times 3 mL methanol and 3 mL distilled water. The column was washed consecutively with 3 mL of distilled water, 3 mL of $NaHCO_3$ (5%) and 3 mL of distilled water, then dried by passing air through for 10 min and centrifuging at 4000 units/min for 15 min. The adsorbed drugs were eluted with 2 mL acetone/dichloromethane (3:1, v/v). The eluent was evaporated to dryness under a stream of nitrogen at 60°C, and then reconstituted in 50μL of methanol.

Liquid Chromatography/Mass Spectrometry

A 2-μL portion of the sample was injected into the LC/MS system. A 20 mL dual-syringe HPLC pump (Applied Biosystems mod. 140B) was used to deliver the pulse-free, low flow rates required by the Ionspray (ISP) interface. Samples were manually injected using a 100-μL gastight syringe (Hamilton mod. 1710) and a Rheodyne mod. 8125 low-dispersion valve equipped with a homemade 2.0-μL PEEK loop (0.005" ID).

The HPLC separations were performed on a 5-μm CHIRAL-AGP Chromtech column (100 by 4.0 mm ID), operated at ambient temperature and protected by a 5-μm alpha1-AGP (ChromTech) guard cartridge (10 by 3.0 mm, ID). Each 16.5 min chromatographic run was carried out with a binary mobile phase of isopropanol (ISO)/2 mM NH_4COOH, pH 5.8 buffer, using the following gradient: ISO at 8% hold for 8 min, up to 20% at 9 min then isocratic to 16 min; down to 8% at 16.5 min. The flow rate was 500 μL/min (operating pressure in the range 4.7 to 8.1 MPa) with a post-column split (a zero-dead volume tee with 2 outlets of unequal length) of 1:9, to reduce at 50μL/min the flow rate infused into the ISP. An equilibration time of 5 min at 8% ISO was allowed between 2 successive runs. Before use, the components of the mobile phase were degassed and filtered through 0.45-μm filters

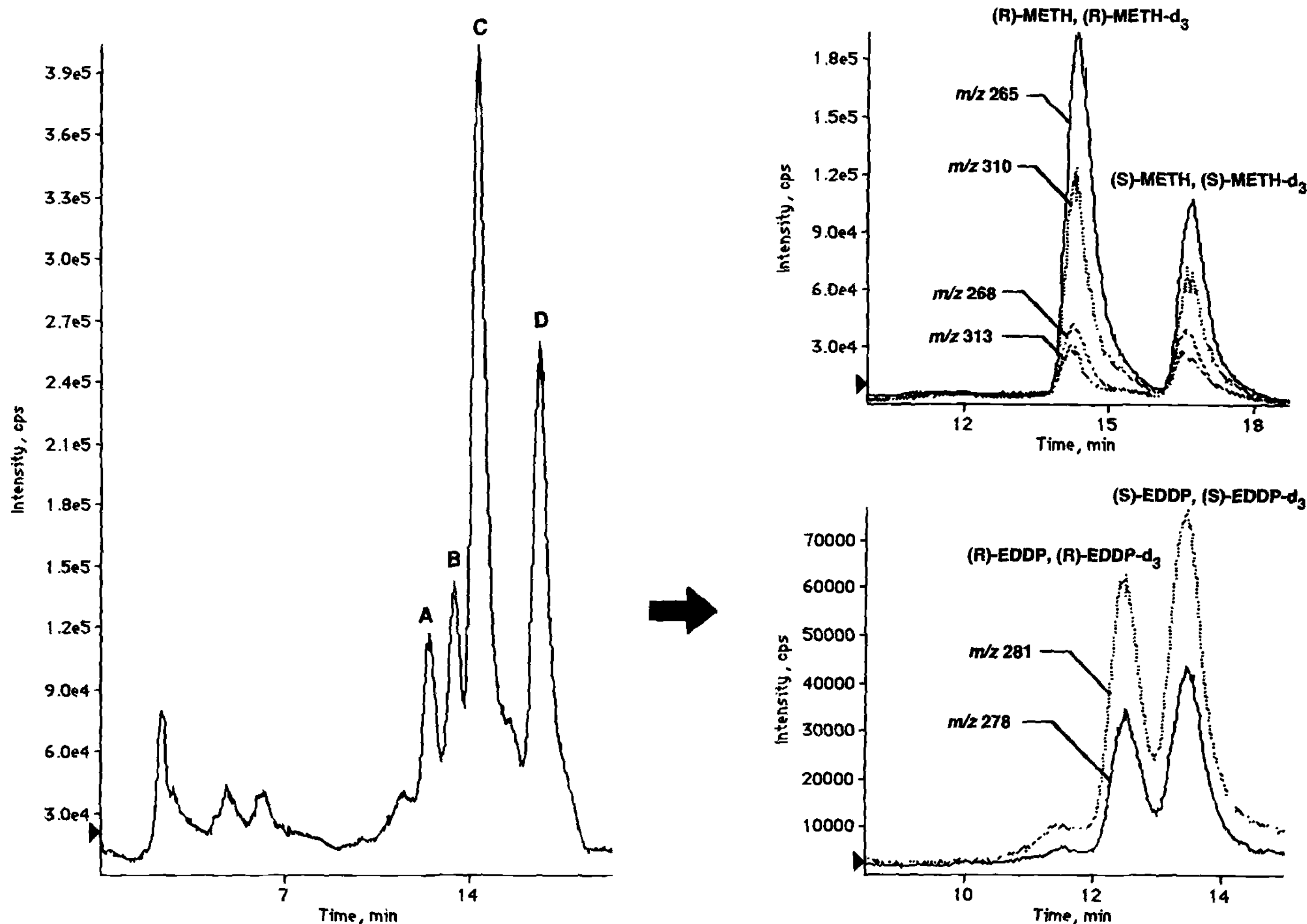

FIG. 2—*HPLC/ISP-MS chromatogram from the hair extract Nb 9.* Left: *MIM chromatogram (m/z 265 + 268 + 278 + 281 + 310 + 313); Peak A (12.47 min): (R)-EDDP; Peak B (13.41 min): (S)-EDDP; Peak C (14.25 min): (R)-methadone; Peak D (16.61 min): (S)-methadone.* Right: *Extracted chromatograms at: 1) Upper graph: m/z 265 and 310 (methadone), 268 and 313 (methadone-d_3); 2) Lower graph: m/z 278 (EDDP) and 281 (EDDP-d_3). Calculated concentrations: (R)-methadone, 10.22 ng/mg; (S)-methadone, 5.93 ng/mg; (R)-EDDP, 1.38 ng/mg; (S)-EDDP, 1.38 ng/mg.*

(Durapore GVWP 047, Millipore) with a Pyrex filter holder (Millipore); at the end of each chromatographic session, the column was unplugged from the detector, then thoroughly rinsed with ISO/deionized water (25:75 for 3 h) at a flow rate of 500 μL/min.

MS detection was carried out using a Perkin-Elmer Sciex API-100 double-quadrupole instrument. Nitrogen (purity grade *U*, i.e., 99.95%, 40 psi) was used as the nebulizing gas (flow rate 1.16 L/min). The instrument was operated in the positive ionization mode with a voltage of +4.5 KV applied to the sprayer during all experiments. To prevent solvent vapors and contaminents from entering the vacuum chamber, the area in front of the orifice was continuously flushed with a 'curtain gas' (N_2 purity U, 40 psi) at a flow rate of 1.08 L/min during all experiments, and 0.14 L/min when the instrument was set in overnight standby.

The system was weekly tuned by using a continuous infusion at 5 μL/min of the standard mixture of high molecular weight propylene glycols, and monitoring the ions at m/z 59, 175, 616, 907, 1255, 1545, 1836, and 2010 for mass calibration, lens optimization, and peak width adjustments. For routine determination, the main instrument setting were OR, +30 V; Q0, −10 V; IQ1 (lens), −12 V; ST (lens), −15 V .Q1, −16 V; EM, +2400 V. MS data were collected as either total ion chromatograms (TIC) by monitoring the signal over a large, continuous mass range or in the single/multiple ion monitoring (SIM/MIM) mode by focusing the detector on a sole mass, or on a discrete list of masses, respectively.

The following ions were selected for identification and quantification (Fig. 1): m/z 265 and 310, 268 and 313, 278 and 281 for methadone, methadone-d_3, EDDP, and EDDP-d_3, respectively.

Results and Discussion

Under the chromatographic conditions used, there was no interference with the drugs or the internal standards by any extractable endogenous materials present in hair. The chromatograms obtained showed a good separation between the optical isomers. Figure 2 presents the chromatogram obtained after extraction of a hair sample from a subject under methadone treatment.

Responses for all compounds were linear in the range 0.5–20, and 0.2–10.0 ng/mg for methadone and EDDP, respectively, irrespective of the isomers. The recoveries after extraction with solid-phase were about 80 ± 8%, and 70 ± 11% for methadone and EDDP, respectively (10).

Accuracy was monitored in nine samples obtained for patients receiving racemic methadone. The determination of R-isomers and S-isomers concentration by this method was summarized and

compared with the result of a total methadone and EDDP determination by a validated GC/MS procedure (10) (Fig. 3). The equations were LC = GC by 0.63 + 1.45, and LC = GC by 0.72 + 0.37 for methadone and EDDP, respectively, with a correlation coefficient of 0.968 and 0.978.

The day-to-day precisions, estimated by daily analysis of an aliquot of hair spiked with racemic methadone and EDDP to obtain a final value for each enantiomers of 5.0 and 1.0 ng/mg for R- and S-methadone, and R- and S-EDDP, respectively, over a period of 10 days, ranged from 13.6% (EDDP) to 17.1% (methadone). No differences were noticed between the enantiomers.

By this method, concentrations of 0.2 and 0.1 ng/mg for methadone and EDDP could be detected, irrespective of the isomer. These detection limits (LOD) were evaluated by decreasing concentration of drugs, until a response equivalent to three times the background noise was observed. LOD of 0.22 ng/mg were reported using GC/MS (10), thus our LC/MS procedure appears to be as sensitive as capillary gas chromatography.

The application of the assay was demonstrated after administration of racemic methadone to drug addicts in a detoxification program. R- and S-enantiomers of both methadone and EDDP were identified and quantified in all 9 subjects that were tested. Concentrations, reported Table 1, were in the range 2.58–10.22, 1.89–9.53, 0.42–1.73, and 0.40–2.10 ng/mg for R-methadone, S-methadone, R-EDDP, and S-EDDP, respectively.

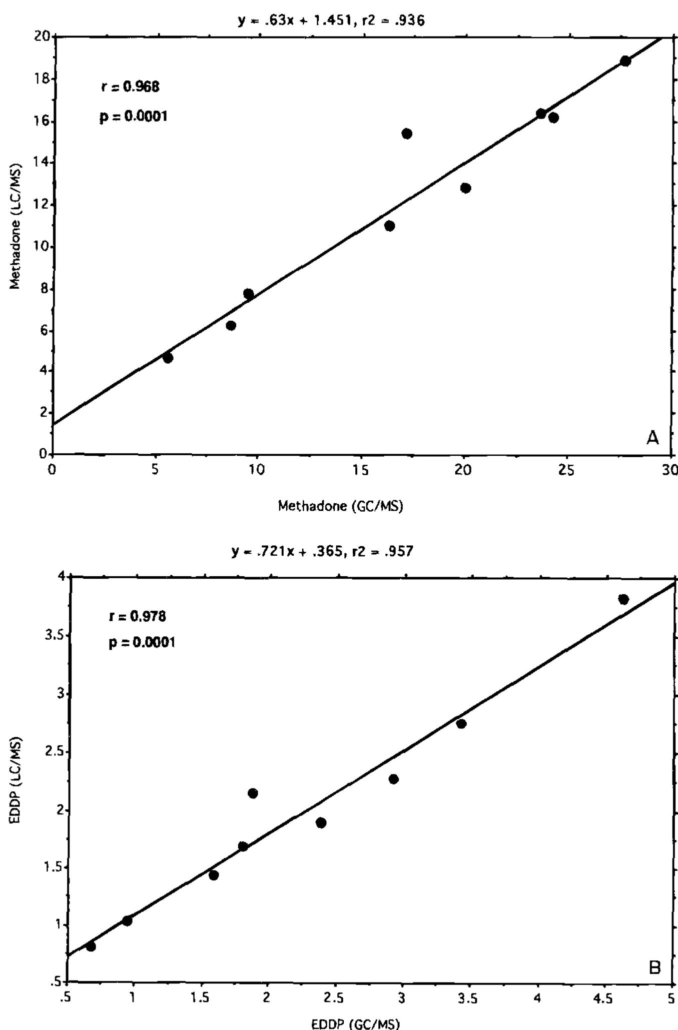

FIG. 3—*Correlation between the hair concentrations (ng/mg) of: (A) methadone, and (B) EDDP measured by LC/MS and a reference GC/MS method.*

TABLE 1—*Concentrations (in ng/mg) of R- and S- enantiomers of methadone and EDDP in nine subjects incorporated in a detoxification program.*

Subject	R-methadone	S-methadone	R-EDDP	S-EDDP
1	5.07	2.72	0.82	0.87
2	6.57	6.25	1.73	2.10
3	2.58	2.09	0.42	0.40
4	9.35	9.53	1.01	1.26
5	4.34	1.89	0.70	0.34
6	6.52	4.49	1.14	0.76
7	8.99	7.38	0.77	1.38
8	7.94	7.45	0.47	0.96
9	10.22	5.93	1.38	1.38

As it is generally the case for other drugs, the concentrations of the metabolite were lower than those of the parent drug. Methadone was present at concentrations approximately 4 to 10 times higher than EDDP. When the concentrations of the enantiomers were summarized, they were in the range of previously published data (10,11).

The data provide strong evidence that both enantiomers of methadone and EDDP can be detected in hair following oral administration of a racemic mixture. Although the limited number of subjects in this study precludes generalization, results are suggestive of a predominance of the R-enantiomer of methadone in human hair. The radio between R- and S-methadone was in the range 0.98–2.30 and was higher than one in eight samples (Table 2). The paired t-Test (StatView II soft, version 1.02, Macintosh) applied to the results showed that the difference [R-met − S-met], with a mean value of 1.54, is significantly different from 0 at a probability $p < 2\%$. Although data suggest R-enantiomer predominance the statistically difference should be taken with caution in light of the low number of subjects tested (9) and the apparent uncertainty in the exact composition of the racemic methadone. In the case of EDDP, the differences between the 2 isomers were lower, with a ratio R/S range of 0.49–2.06, and a predominance of the S-enantiomer in 5 cases. In urine, the concentration of R-methadone is generally higher than the concentration of S-methadone, and the ratio R/S could be up to 5 (12). At the opposite in serum, measurements of the ratio between R- and S-methadone showed inter individual differences, but generally, the S-enantiomer was found in higher amount (13–15). This predominance was explained by the shorter elimination half-life and the lower bioavailability of

TABLE 2—*Distribution of methadone enantiomers reported in the literature and comparison with our data.**

Specimen	Number of subjects	R-methadone	S-methadone	ratio R/S	Number of cases R > S
Urine (12)	40	6–16783	4–6291	1.45–4.89	40 (100%)
Hair	9	2.58–10.22	1.89–9.53	0.98–2.30	8 (89%)
Plasma (13)	6	not given	not given	0.71–2	3 (50%)
Serum (14)	5	42–254	54–228	0.5–1.1	1 (20%)
Serum (15)	2	not given	not given	<1.0	0 (0%)

*Concentrations are expressed in ng/mL, ng/mg and ng/mL for urine, hair and blood, respectively.

the R-enantiomer (13). The unique findings of higher levels of S-methadone in serum and R-methadone in hair appear contradictory as incorporation of drugs from the blood stream was proposed as one of the mechanism by which drugs are deposited into hair (16). Water-soluble drugs are excreted into sweat/sebum, which bathe the hair, and are incorporated after the hair emerges from the skin. Although methadone has been identified in sweat (17), no enantiomeric determination of the drug was achieved. In order to understand drug incorporation in hair better, this has to be done in the near future.

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