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Chiral analysis of methylphenidate and dextromoramide by capillary electrophoresis

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Abstract

Capillary electrophoretic methods have been developed to separate the enantiomers of methylphenidate (MPH) and dextromoramide. For MPH separation was achieved with heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DMCD) as chiral selector in a 100 m*M* phosphoric acid buffer adjusted to pH 3.0 with triethanolamine. Commercial samples of D,L-*erytho*-MPH HCl and D,L-*threo*-MPH HCl were analysed using the method. There was no evidence of the presence of D,L-*threo*-MPH HCl in D,L-*erytho*-MPH HCl and vice versa. The ratio of the enantiomers was determined for each diastereoisomer. Hydroxypropyl-β-cyclodextrin was the chiral selector of choice for the chiral separation of the enantiomers of moramide. The separation which gave a resolution of about 3.5 was achieved in 4 min using only a 6 cm of length of capillary. In a sample of *dextro-R*-moramide tartrate only a small quantity (4.9% w/w) of *levo-S*-moramide was detected with this method.

Keywords: Enantiomer separation; Methylphenidate; Dextromoramide

1. Introduction

Medicinal chemists have long recognised the importance of chirality in drug design since it is known that optical isomers of a drug may have different physiological properties. Until recently it was not economically viable to produce sufficient quantities of a single, pure isomer. In most cases the unwanted enantiomer has been assumed to be harmless. This assumption was seldom verified. In the early 1990s new methods for producing single enantiomers on a commercial scale were developed, thereby overcoming the problem of obtaining sufficient material of a single pharmacologically active enantiomer. However, the other enantiomer may be present in the substance as a degradant or manufacturing impurity and still has to be controlled. The US Food and Drug Administration as well as regulatory authorities in Europe, China and Japan [1], have provided guidelines indicating that only one enantiomer of a new chiral drug should be brought to the market. A test for the enantiomeric impurity in a chirally pure drug plus a simple test of enantiomeric identity, usually optical rotation, has to be included in the quality control dossier [1].

Consequently, separation techniques have taken on an essential role in all stages of drug development because the separation of enantiomers on an ana-

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lytical scale is required for measuring the enantiomeric purity of the chiral drug. Recent papers dealing with application of capillary electrophoresis to the resolution of enantiomers of a chiral drug substances have been published [2–4] over the past few years, as well as comprehensive reviews on chiral CE [5,6]. In most cases, enantioseparations have been achieved with free-solution CE employing cyclodextrins (CDs) or their derivatives as chiral selectors.

A wide range of CD derivatives are currently used in CE for chiral analysis including uncharged methylated-, hydroxyethylated, hydroxypropylated, acetylated CDs and more recently sulfated-, phosphated and carboxymethylated cyclodextrins [5]. The vast choice of chiral selectors and possible sets of conditions makes method development in chiral CE a difficult task. A recent comprehensive review [5] describes method development strategies for the enantioseparation of drugs by CE using cyclodextrins as chiral additives. Fillet's development procedure [7] was used for the development of chiral CE separations of the pharmaceuticals methylphenidate and dextromoramide. These drugs belong to a class of compounds which will be currently reviewed with respect to the biological activities of the individual enantiomers.

Methylphenidate HCl (MPH) is used for the treatment of attention deficit hyperactivity disorder [8] and narcolepsy [9]. It is known that D-*threo*-MPH is pharmacologically more active than L-*erytho*-MPH. There is concern that the biological activity of the final product may vary as a result of changes occurring in the D (active) to L (inactive) enantiomer ratio, resulting in reduced effectiveness of the active enantiomer. To monitor the chiral stability of MPH, a chiral assay method was required which is specific for the four optically active isomers of MPH. Aoyama et al. [10] reported a stereoselective gas chromatographic–mass spectrometric method for the quantitative analysis of MPH in plasma and urine, based on derivatisation of MPH and an internal standard with heptafluoro butyryl-L-propyl chloride. Also the enantiomeric separation of D,L-*threo*-MPH by HPLC using the macrocyclic antibiotic vancomycin as chiral selector was reported [11]. Lin et al. [12] examined the chiral stability of MPH by HPLC using a Chirobiotiv V™ stationary phase.

Dextro-R-moramide tartrate is a narcotic analgesic drug derived from the unripe seeds of the opium poppy, which has powerful pain relieving properties [13]. *Dextro-R*-moramide (DM) is used for the relief of severe pain following injury or surgery, or in long-term illness such as cancer. It is often given with a tranquillising drug, which enhances its analgesic effect [14]. Compared with similar drugs, DM has a rapid onset of action, but this is followed with a short duration. DM is marketed, as indicated by the name, as the pure *R*-isomer of moramide. The *levo-S*-enantiomer has no analgesic properties. Several methods have been published describing either the determination of *dextro-R*-moramide in the presence of other different narcotic drugs [14,15] or in biological matrices [16–18]. However, there are no reports to date that quantify the *levo-S*-enantiomer of moramide in the final bulk drug substance.

This paper reports the development of validated, fast and selective CE methods for the quantification of the two pairs of optical isomers of MPH as well as the two enantiomers of moramide in bulk drug material using CE.

2. Experimental

2.1. Chemicals

Ortho-phosphoric acid (85% w/w in water) and triethanolamine (TEA) were obtained from BDH Laboratory Supplies (Poole, UK). Heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DMCD) and hydroxypropyl-β-cyclodextrin (HPCD) were purchased from Sigma–Aldrich Chemicals (Poole, Dorset, UK). Samples of D,L-*threo*-methylphenidate HCl and D,L-*erytho*-methylphenidate HCl were obtained from Macfarlan Smith Ltd. (Edinburgh, UK). Samples of racemic moramide and *dextro-R*-moramide tartrate were also supplied by Macfarlan Smith Ltd. Diphenylpyraline HCl (DPP, Fig. 1) was a gift from the Medicines Testing Laboratory Ltd. (Edinburgh, UK). Water was glass distilled and filtered through 0.45-μm nylon filters (Whatman, Kent, UK).

2.2. Apparatus

CE separations were obtained with a SpectraPHORESIS® ULTRA CE system equipped with PC 1000 software and a SpectraPHORESIS®

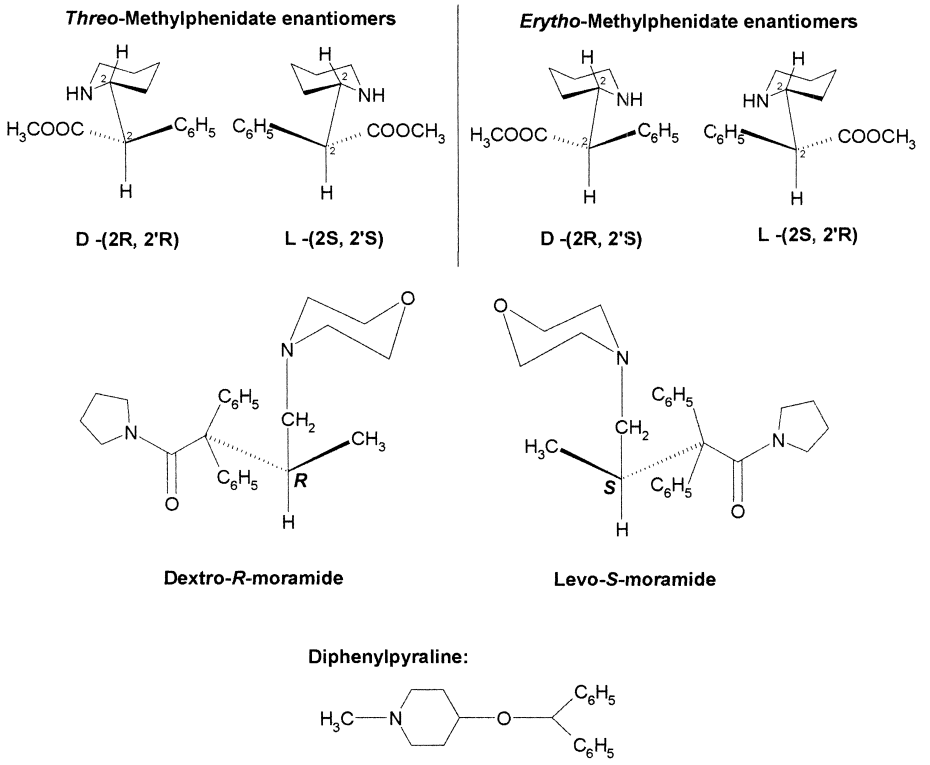

Fig. 1. Structural formulae of methylphenidate, moramide and the internal standard diphenylpyraline.

UV 3000 scanning detector (Thermo Separation Products, San Jose, USA). The capillary (375 μm O.D., and 50 μm I.D.) from Composite Metal Services Ltd (Hallow, UK) was cut to a total length of 40 cm. The polyimide coating on the capillary was burnt off 6 cm from the cathodic end to provide a window at 34 cm (l_{eff}) of the capillary length. Detection was by on-capillary UV absorbance measurement at 210 nm with a rise time of 0.5 s. The capillary was maintained at 25°C. Initially, the capillary was conditioned by flushing it for 5 min with 0.1 *M* NaOH at 50°C prior to sequential washing with water and the running buffer at the temperature of analysis. The first two injections, in order to equilibrate the capillary, were running buffer using normal CE conditions. Between injections the capillary was washed with the running buffer for 1 min. On completion of the analyses the capillary was flushed with water and dried by passing air through it before storage.

Injections of solutions of methylphenidate were made by applying a pressure (0.5 p.s.i.) for 2 s to the anodic end of the capillary. Separations were performed at a voltage of 25 kV for 15 min resulting in a field strength of 625 V/cm and a current of 53 μA.

Solutions of moramide were injected by applying a pressure (0.5 p.s.i.) for 2 s to the cathodic end of the capillary. Separations were performed using only 6 cm of effective capillary length at a voltage of −25 kV for 15 min resulting in a field strength of −625 V/cm and a current of −55 μA.

2.3. Running buffer

A volume (5.6 ml) of ortho-phosphoric acid was dissolved in 1 L of distilled water to give a 100 m*M* phosphoric acid solution adjusted to pH 3.0 with TEA. A quantity (0.499 g DMCD, 0.548 g HPCD) of each chiral selector was separately dissolved in a volume (25 ml) of 100 m*M* phosphoric acid–TEA buffer solution to give a final concentration of 15 m*M* for each chiral selector. The final buffer solutions were filtered through a 0.45-μm membrane

filter and degassed for 15 min in an ultrasonic bath before use.

2.4. Method validation

2.4.1. Methylphenidate

Optimum separation of the enantiomers of methylphenidate was achieved using a running buffer of 15 m*M* DMCD in 100 m*M* phosphoric acid–TEA buffer (pH 3.0). A 10 mg/ml stock solution of DPP HCl was prepared for use as an internal standard (I.S.). Appropriate volumes of this I.S. solution were added to the sample and standard solutions in order to obtain a final concentration of 1.0 mg/ml of DPP in each solution. The method was validated with respect to the following parameters.

Linearity of detector response was determined with four concentrations of each epimer (D,L-*threo*-MPH HCl and D,L-*erytho*-MPH HCl) of 0.2 to 0.8 mg/ml dissolved in 10 m*M* phosphoric acid–TEA buffer (pH 3.0). The 10 m*M* phosphoric acid–TEA buffer (pH 3.0) was used as a blank. Peak areas of the analytes in the electropherograms were normalised by dividing them with their corresponding migration times [19] and the peak area ratios of analyte to internal standard DPP were then calculated. Each standard solution was injected twice and regression equations determined for the calibration plots for each compound. Relative standard deviations (RSD) of the slopes and the intercepts were calculated. The within-day precision of the method was determined from twelve replicate injections of a mixture containing D,L-*threo*-MPH HCl (1.0 mg/ml) and D,L-*erytho*-MPH HCl (1.0 mg/ml) in 10 m*M* phosphoric acid–TEA buffer pH 3.0. The peak area of each analyte was divided by the corresponding migration time, and the peak area ratio of analyte-to-I.S. and the RSD values determined. The inter-day precision was determined in the same way over a period of 5 days taking the average of four replicate injections per day. Detection limits and quantification limits were estimated for each enantiomer using an approach used for chromatographic methods [20].

2.4.2. Dextromoramide

The optimum separation of the optical isomers of moramide was achieved in 4 min using a running buffer of 15 m*M* HPCD in 100 m*M* phosphoric acid–TEA pH 3.0 with short-end injection mode. The CE method was validated with respect to the following parameters.

Linearity of response was determined with five concentrations of racemic moramide dissolved in 10 m*M* phosphoric acid–TEA buffer (pH 3.0) in the range 0.1 to 0.5 mg/ml. A 10 m*M* phosphoric acid–TEA buffer (pH 3.0) was used as a blank. The analyte peak areas in the electropherograms were normalised by dividing them by their corresponding migration times [13]. The peak area ratio of analyte to internal standard (DPP) was calculated. Two injections were made per standard solution and regression equations determined for each calibration solution. Relative standard deviations (RSD) of the slopes and the intercepts were calculated. The within-day precision of the method was determined from twelve replicate injections of a standard mixture solution, prepared from racemic moramide, containing *dextro-R*-moramide (0.5 mg/ml) and *levo-S*-moramide (0.5 mg/ml) in 10 m*M* phosphoric acid–TEA buffer (pH 3.0). The peak area of each analyte was divided by the migration time, and the peak area ratio of analyte-to-internal standard (DPP) and the RSD values determined. The intra-day precision was determined in the same way over a period of 5 days averaging four replicate injections per day. Detection limits and quantification limits were estimated for each enantiomer using an approach used for chromatographic methods [20].

2.5. Sample preparation

Stock solutions of MPH HCl were prepared by dissolving ca. 50 mg of each sample in 10 ml of 10 m*M* phosphoric acid–TEA buffer solution (pH 3.0). A stock solution of *dextro-R*-moramide was prepared by dissolving 102.1 mg of the sample in 20 ml of 10 m*M* phosphoric acid–TEA buffer (pH 3.0). Duplicate dilutions of these solutions were prepared with the running buffer to give a final concentration of 1.0 mg/ml for MPH and 0.5 mg/ml in case of DM. Diphenylpyraline HCl (1 mg/ml) was added as internal standard to the diluted sample solutions before making up to volume, after which the diluted samples were analysed ($n=2$). Electropherograms were recorded and the areas of the peaks due to the analytes normalised by dividing them by their re-

spective migration times. The analyte to I.S. peak area ratio was calculated for each compound.

3. Results and discussion

With the advent of reliable commercial CE instruments and improved methodology it is possible to obtain RSD values of 1–2% for the analysis of compounds [21]. However, the major source of imprecision is due to variabilities in injection volume. Reproducible injection of nanolitre sample volumes presents a significant engineering challenge. However the use of an internal standard will help to overcome this problem [22]. Diphenylpyraline HCl (Fig. 1) was chosen as internal standard because of its structural similarity to MPH and its lack of a chiral centre.

A good separation of the enantiomers of *erythro*- and *threo*-MPH was obtained with DMCD as chiral selector (Fig. 2).

A CE method should be well controlled in terms of consistent migration times as the peak area obtained for an analyte is related to both sample concentration and migration time [23]. The RSD values (D,L-*erytho*-MPH HCl: 2.0%; D,L-*threo*-MPH HCl: 1.0%) for the relative migration times (relative to the internal standard) indicate a relatively large drift in migration times. Peak normalisation [24] was undertaken to improve peak area precision. However, still comparatively poor RSD values were obtained for twelve replicate injections of a standard mixture (D,L-*erytho*-MPH HCl: 3.5%; D,L-*threo*-MPH HCl: 2.5%; Table 1).

In order to obtain high precision in CE, concentrated samples with long injection times should be used [25]. However, to obtain good separation of enantiomers with a chiral selector a very short injection plug length is required to produce a narrow analyte zone. In this study since the injection volume was only approximately 3 nl, the peak area precision obtained can be considered as acceptable. Also the small difference between the inter- and intra-day values of precision confirms that injection imprecision is the most likely cause for the peak area variations (Table 1).

The high efficiency of CE (*erytho*-MPH: 90 000 plates; *threo*-MPH: 60 000 plates; Table 1) gives

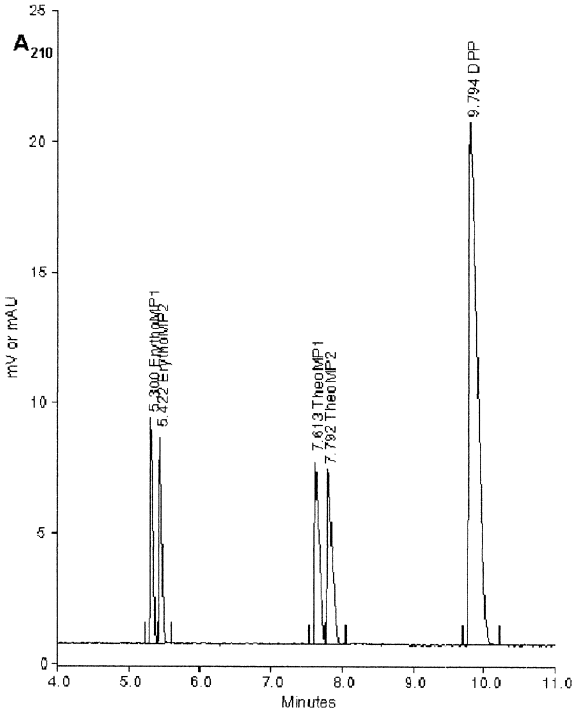

Fig. 2. Electropherogram of a standard mixture of D,L-*erytho*-MPH HCl, D,L-*threo*-MPH HCl and DPP HCl (internal standard). Conditions: Running buffer — 15 m*M* DMCD in 100 m*M* phosphoric acid–TEA buffer pH 3.0; applied voltage 25 kV (25°C), hydrodynamic injection for 2 s at 0.5 p.s.i. and detection at 210 nm.

sufficient resolution between the pairs of enantiomers (Table 1) to allow an accurate and precise determination of each enantiomer.

Dimethyl-β-cyclodextrin (DMCD), the chiral selector, which successfully separated the optical isomers of methylphenidate, showed no selectivity for the enantiomers of racemic moramide. However, excellent resolution (4.5) was obtained with the hydroxypropyl-β-cyclodextrin (HPCD). Since the resolution was good it was decided to use the short-end of the capillary for the injection to reduce the analysis time from 10 min to less than 4 min with the internal standard DPP eluting after the isomers (Fig. 3). Using this approach the value for the resolution of the enantiomers was about 3.5. The method was validated with respect to migration time precision, normalised peak area precision (inter- and intra-day), linearity and LOD/LOQ levels (Table 1).

Table 1
Validation data for the separate assays used for the determination of methylphenidate and racemic moramide

Sample	D,L-*erytho*-MPH HCl		D,L-*threo*-MPH HCl		Moramide	
	Erytho 1	*Erytho* 2	*Threo* 1	*Threo* 2	*R* isomer	*S* isomer
Migration time [min]	5.34	5.47	7.69	7.87	2.53	2.94
Relative migration time [min]	0.54	0.55	0.78	0.80	0.67	0.78
($n=10$) (RSD, %)	(2.03)	(1.99)	(0.95)	(0.88)	(0.32)	(0.22)
Resolution		1.75		1.42		3.59
Efficiency of peak N ($\times 10^3$)	94.2	80.9	57.6	52.4	11.0	8.9
Linearity:						
Slope	0.228	0.253	0.258	0.259	1.0587	1.064
(RSD, %) ($n=2$)	(0.65)	(0.80)	(0.63)	(1.05)	(0.35)	(0.42)
Intercept ($\times 10^3$)	−0.0013	−0.0011	−0.0025	−0.0025	−0.0051	−0.0064
Correlation coefficient (r^2)	0.9998	0.9999	0.9996	0.9996	0.9987	0.9980
Precision:						
Within day ($n=12$) RSD [%][a]	3.62	3.06	2.47	2.46	2.18	2.04
Day-to-day ($n=5$) RSD [%][a]	3.81	4.08	3.10	3.57	1.44	1.07
LOD [μg/ml]	2.50	2.74	3.01	3.08	1.06	1.18
LOQ [μg/ml]	8.34	9.12	10.03	10.28	3.53	3.92

Key: *$\lambda=210$ nm; LOD=limit of detection; LOQ=limit of quantification (based on Ref. [20]).
[a] Conc. [μg/ml] for MPH: D,L-*erytho*-MPH=1.0; D,L-*threo*-MPH=1.0; Conc. [μg/ml] for DM: *dextro*-*R*-moramide=0.5; *levo*-*S*-moramide=0.5.

In CE it is important to maintain a constant temperature throughout a series of injections, since temperature affects both viscosity and electrophoretic mobility [26]. The need for temperature control applies to the capillary, sample vials and electrolyte reservoirs. Viscosity decreases with increased temperature. Therefore, when the temperature is increased, more sample solution enters the capillary for the same injection time. Also electrophoretic mobility increases with temperature, which reduces migration times [27]. A disadvantage of using the short-end of a capillary for the separation is that this part of the capillary is outside the capillary temperature control oven of the CE instrument. This could be the source of error, which would explain the observed RSD data (ca. 2.0%; Table 1) for the inter-day precision. Interestingly the RSD values for the intra-day precision were lower than the data obtained for the inter-day precision (Table 1) and this indicates that the source of error is consistent from day to day. Nevertheless, excellent resolution (R_S 3.59; Table 1) was obtained between the peaks for *dextro*-*R*-moramide and *levo*-*S*-moramide in a very short analysis time of 4 min (Fig. 3).

Samples of D,L-*erytho*-MPH HCl (MPH 1) and of D,L-*threo*-MPH HCl (MPH 2) were analysed by CE using DMCD as the chiral selector and DPP HCl as internal standard. Two solutions of each sample were prepared and all solutions were analysed in duplicate. There was no evidence of the presence of D,L-*threo*-MPH in D,L-*erytho*-MPH and vice versa. The ratio of the enantiomers was determined for each analyte (Table 2).

A sample of dextro-*R*-moramide tartrate was also analysed by 'short-end' CE using HPCD as chiral selector and DPP HCl as internal standard. A small quantity (4.9% w/w) of levo-*S*-moramide was detected in the sample (Table 2).

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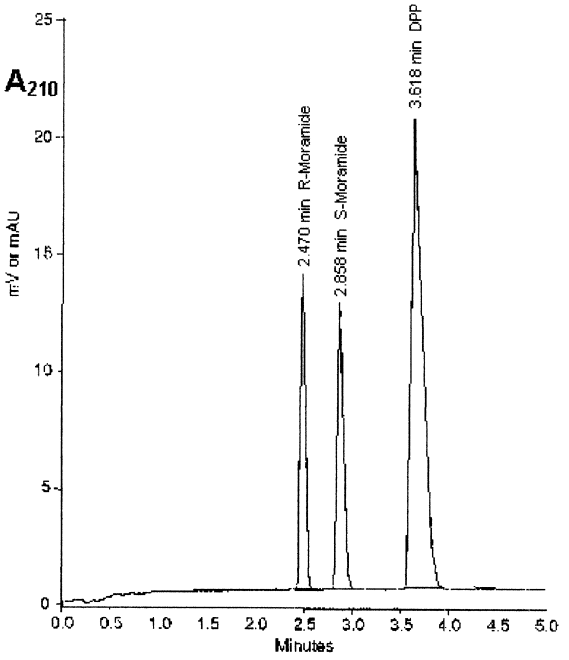

Fig. 3. Electropherogram of a mixture of racemic moramide and DPP HCl (internal standard). Conditions: Running buffer — 15 m*M* HPCD in 100 m*M* phosphoric acid–TEA pH 3.0 with detection at 210 nm. Hydrodynamic, short-end injection for 2 s at 0.5 p.s.i.; applied voltage −25 kV (25°C).

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Table 2
Analysis of samples of methylphenidate and dextromoramide

Sample	Analyte	Mean rel. t_M ($n=4$) [min]	Mean NPA I.S. ratio ($n=4$)	D/L ratio [%]	RSD [%] ($n=4$)
MPH 1	*Erytho*-MPH1	0.528	0.212	50.16	3.73
	Erytho-MPH2	0.537	0.210	49.84	4.33
	Threo-MPH1	n/d	n/d	–	–
Threo-MPH2n/dn/d––					
MPH 2	*Erytho*-MPH1	n/d	n/d	–	–
	Erytho-MPH2	n/d	n/d	–	–
	Threo-MPH1	0.760	0.233	49.86	1.14
Threo-MPH20.7820.23450.141.51					
dextro-moramide	*R*-moramide	0.668	1.256	–	0.37
	S-moramide	0.816	0.0053	4.89%[a] (w/w)	0.21

Key: n/d = not detected; rel. t_M = relative migration time; RSD = relative standard deviation of mean; NPA I.S. = ratio of analyte to internal standard using normalised peak area.

[a] Amount quantified in relation to calibration curve for this isomer.

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