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Chiral analysis of methadone and its major metabolites (EDDP and EMDP) by liquid chromatography–mass spectrometry

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Abstract

Racemic methadone (MET) is administered to heroin users undergoing methadone maintenance therapy (MMT) in Australia. The enantiomers of methadone possess different pharmacological effects, and the enantioselective metabolism of methadone to its two major metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) has been demonstrated. Therefore, a stereoselective method capable of quantifying methadone, EDDP and EMDP in biological samples could be of benefit in the monitoring of MMT patients. In particular, the analysis of hair samples would provide a means by which long-term monitoring of MMT patients could be achieved. To date, no HPLC method has been published for the simultaneous separation of the six enantiomers. A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the chiral analysis of methadone, EDDP and EMDP was developed using an α-glycoprotein (AGP) stationary phase. The method development involved the utilisation of factorial analysis experimental designs and the application of artificial neural networks (ANNs) to model the chromatographic response surfaces. The optimal conditions were determined to be 20 mM acetic acid: isopropanol (93:7, pH 7.4), with a flow rate of 0.9 mL/min. The method was validated and subsequently applied to the analysis of 20 hair samples collected from MMT patients.

Keywords: Methadone; EDDP; EMDP; Chiral; α-Glycoprotein; AGP; LC–MS; Artificial neural networks; Hair

1. Introduction

Whilst effective for pain relief, methadone (MET) is an opioid that is primarily used therapeutically in the management of withdrawal symptoms in heroin-dependent users during maintenance therapy. It has μ-opioid receptor agonist activity similar to that observed for morphine [1]. Racemic methadone is administered to heroin users undergoing methadone maintenance therapy (MMT) in Australia. The enantiomers of methadone possess different pharmacological effects, and the (*R*)-enantiomer is almost exclusively responsible for the analgesic and abstinence relieving effects [1]. The main metabolic pathways of methadone (Fig. 1) involve *N*-demethylation followed by spontaneous cyclisation. The two main metabolites of methadone are 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP); both of these compounds are chiral, with the chiral centre denoted by the asterisk (*) (Fig. 1). In MMT patients, 20–60% of the methadone dose is excreted in urine in 24 h, with up to 33% as unchanged drug, up to 43% as EDDP and 5–10% as EMDP [2]. The pharmacokinetics of methadone has been found to be stereoselective, with large inter-individual variability [3]. The (*R*)- and (*S*)-enantiomers of methadone possess different properties with respect to receptor affinity [4], metabolism [5] and protein binding [3]. However, a full appreciation of the effect of inter-individual variability in the metabolism of methadone requires a highly sensitive stereoselective method for the six major enantiomers.

Numerous HPLC methods are available for the chiral separation of methadone (MET) and/or EDDP (but not EMDP) in biological fluids, including urine [6–8], plasma [7,9–14], serum [6,15–18], whole blood [19], hair [20], sweat [21] and

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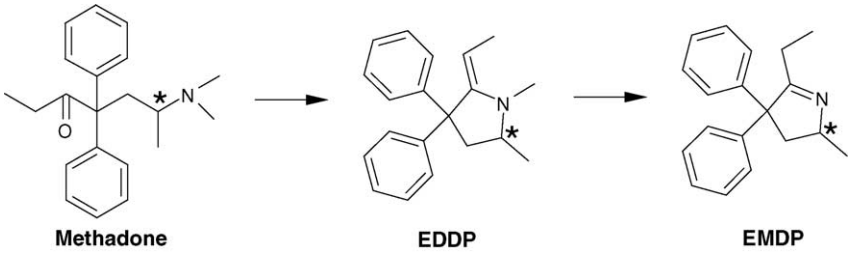

Fig. 1. Main metabolic pathways of methadone (asterisk (*) denotes chiral centre).

saliva [17]. α-Glycoprotein (AGP) and β-cyclodextrin chiral stationary phases (CSPs) have been most commonly used for the chiral resolution of the methadone enantiomers. On the basis of the previous methods utilising CSPs for the stereoselective separation of MET and/or EDDP, the AGP stationary phase was selected for the development of a chiral LC–MS/MS method for the separation of the MET, EDDP and EMDP enantiomers. Rudaz et al. [22] and Foster et al. [3] noted that inability of the Cyclobond I 2000 RSP column (β-cyclodextrin derivatised with *R*,*S*-hydroxylpropyl ether-bonded phase) to resolve the EDDP enantiomers, or to separate the achiral EDDP peak from the (*R*)-methadone peak. Pham-Huy et al. [6] also observed that while the Cyclobond I 2000 RSP stationary phase was able to adequately resolve the methadone enantiomers, it was unsuccessful in the resolution of the EDDP enantiomer pair.

The AGP CSP consists of AGP immobilised onto 5 μm silica particles packed into a HPLC stationary phase. AGP has been observed to stereoselectively bind cationic species, but can be used for the chiral resolution of acidic and basic drugs, as either charged or neutral species. Enantioselectivity is dependent on the sterical structure of the protein. The conformation of the protein is influenced by the chemical composition and pH of the mobile phase, as well as the operating temperature [23,24]. These changes can result in differences in the solute and/or the protein structure. Uncharged organic modifiers that are commonly used in conjunction with the AGP CSP include isopropanol (IPA), acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), and tetrahydrofuran (THF). Other additives, termed non-reversible organic modifiers, can be used to facilitate permanent structural changes in the chiral selector. Some non-reversible organic modifiers that are commonly used with the AGP stationary phase include *N*,*N*-dimethyloctylamine (DMOA), octanoic acid, and quaternary ammonium compounds. To assist the stability of the AGP CSP, recommended working conditions for the stationary phase include a pH range from 3 to 7, up to 25% organic modifier and temperatures of up to 70 °C [25].

Univariate 'trial and error' strategies are commonly applied for the optimisation of HPLC separation methods. However, this approach can prove to be considerably time consuming, and at times unsuccessful due to the large number of factors that may interact and influence the HPLC separation. The application of experimental designs provides a means by which multivariate optimisation of the separation method can be achieved. Identification of factors that influence the separation is achieved by preliminary univariate experiments. Experimental designs are then applied to proficiently and rapidly evaluate the interaction between the identified factors using a condensed number of experiments. Statistical software packages can then be used to interpolate a response-surface model for the separation within the experimental space. In this research, artificial neural networks (ANNs) were utilised for the interpolation of the response-surface models based on the data obtained from the experimental design. ANNs are composed of a large number of highly interconnected processing elements ('neurons') that are linked with weighted connections ('synapses') [26]. A training algorithm is applied to a representative set of input and output data (located within the input and output layers, respectively), whereby the algorithm 'learns' iteratively to adjust the weighted connection between the processing elements (located within the hidden layer(s)) in order to cause the overall 'network' to produce the appropriate result, whilst minimising the error. One example of an ANN architecture type is a radial basis function (RBF) ANN. An RBF is characterised by hidden layers of radial units, whereby the synaptic function is determined by the scaled squared distance of the weight factor from the input vectors [27–29].

Recently, Kelly et al. [30] detailed the first simultaneous chiral separation of methadone, and its two major metabolites, EDDP and EMDP, using capillary zone electrophoresis (CZE). Baseline resolution of each of the three enantiomer pairs was achieved using a 50 μm fused silica capillary with a background electrolyte (BGE) consisting of 1 mM heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (DMβCD) in 100 mM phosphate at pH 2.6. However, to date no HPLC method has been published that details the stereoselective separation of six enantiomers associated with methadone metabolism. Such a method would be of benefit to the monitoring of MMT patients, and it could be used to determine if there is a correlation with MMT failures and inter-individual variation in the levels of each enantiomer. Therefore, the main objective of this study was to develop a fully optimised and validated LC–MS/MS method for the simultaneous determination of all six enantiomers, based on an AGP stationary phase, using experimental designs and ANNs. This method could subsequently be applied to the analysis of various biological fluids, including plasma and hair.

2. Experimental

Unless stated otherwise, all reagents were of analytical grade. (*R*,*S*)-methadone hydrochloride (MET) was kindly donated by Clinical Pharmacology, St. Vincent's Hospital (Darlinghurst, New South Wales, Australia). (*R*,*S*)-methadone-d_3 (MET-d_3, 1 mg/mL MeOH), (*R*,*S*)-EDDP perchlorate (EDDP, 1 mg/mL MeOH) and (*R*,*S*)-EMDP (EMDP, 1 mg/mL ACN) standards were purchased from Diagnostic Consultants (Sydney, NSW, Australia) or Lipomed (Arlesheim, Switzerland). Due to the unavailability of the individual enantiomeric standards, peak allocation for each enantiomer could not be performed during the method development. Glacial acetic acid (CH_3COOH, BDH HiPerSolv for HPLC, Sydney, NSW, Australia), or ammonium acetate (NH_4CH_3COO, BDH, Sydney, NSW, Australia) was used in the preparation of the mobile phases. Ammonia (NH_3, APS Chemicals, Sydney, NSW, Australia) was used for the pH adjustment of the mobile phases. Isopropanol (IPA, Mallinckrodt UltimAR, Selby Biolab, Victoria, Australia) and acetonitrile (ACN, Merck HPLC grade, Crown Scientific, Sydney, NSW, Australia) were used as organic modifiers. Milli-Q water was used throughout the experiments. Prior to reconstitution, extracted samples were evaporated to dryness using a Heto VR Maxi vacuum concentrator (Medos, Sydney, NSW, Australia) at 45 °C.

A 5 μm Chromtech Chiral AGP column (150 mm × 4.0 mm i.d.), with a 5 μm Chromtech AGP guard column (10 mm × 3.0 mm i.d.) was utilised, with a flow rate of 0.9 mL/min, split to 0.35 mL/min into the MS. Both AGP columns were obtained from Adelab Scientific (Adelaide, Australia). Unless stated otherwise, an injection volume of 10 μL and an ambient column temperature (21–25 °C) were used. A reduced column temperature of approximately 15 °C was also investigated. This was achieved by submerging the column in a water bath consisting of ice and cold water; the approximate temperature was monitored over time to ensure that the temperature of the ice bath remained at 15 ± 2 °C.

The method development was performed using a Perkin-Elmer SCIEX API 365 LC–MS/MS system, fitted with an electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) source, a PE Series 200 autosampler, a micro PE Series 200 LC pump and a vacuum degasser (Perkin-Elmer, Sydney, NSW, Australia). The MS parameters were optimised using an aqueous standard solution of each analyte which was infused into the MS via a Harvard 22 syringe infusion pump. A nebulising gas of nitrogen was utilised with a flow rate of 1.8 L/min, while the curtain gas of nitrogen had a flow rate of 1.2 L/min. Multiple reaction monitoring (MRM) of the molecular ions of each analyte and its most predominant fragment in positive ion mode, with an IonSpray voltage of 5 kV, was utilised. The selected MRM transitions for each analyte, with corresponding collision energies given in parentheses, were as follows: MET *m/z* 310 → 265 (25 V), MET-d_3 (internal standard) *m/z* 313 → 268 (24 V), EDDP *m/z* 278 → 234 (46 V), and EMDP *m/z* 264 → 220 (49 V). Quantitation of the analytes was achieved using TurboQuan software, Version 1.0.

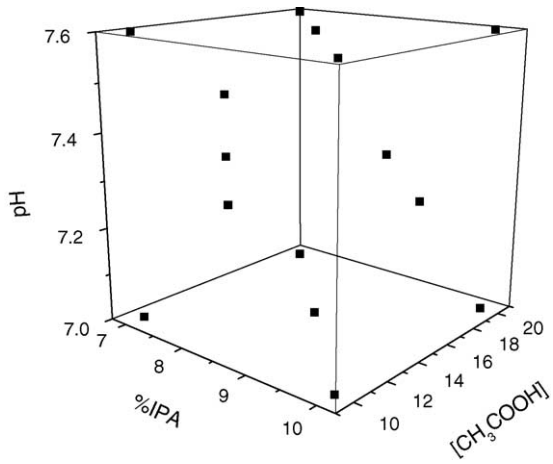

Fig. 2. Schematic of experimental space.

Following preliminary scouting experiments, a central composite experimental design was implemented, based on a three factor design: % organic modifier (IPA), concentration of CH_3COOH, and pH. The experimental space can be summarised as follows: 7–10% IPA; 10–20 mM CH_3COOH; pH 7–7.6; a schematic depicting the experimental space used in the factorial analysis is illustrated in Fig. 2. Duplicate injections of a mixed aqueous standard containing 20 μg/mL of (*R*,*S*)-MET, (*R*,*S*)-MET-d_3, (*R*,*S*)-EDDP and (*R*,*S*)-EMDP were used in each experiment. An analysis time of 90 min was selected. The ANN modelling of the experimental space was performed using Trajan software (Version 6.0), while subsequent three dimensional data evaluation was performed using Origin (Version 6).

The resolution (R_s) of each enantiomer pair was calculated for each chromatogram according to Eq. (1). In cases where partial peak resolution was observed, the baseline peak widths were estimated as twice the peak width from the leading (peak 1) or tailing (peak 2) edge to the apex of the peak. The predicted resolution (R) of each enantiomer pair was calculated for each value within the experimental space according to Eq. (2). The predicted product resolution (PR) value was calculated by Eq. (3).

The method was applied to hair samples obtained from MMT patients ($n = 20$). The study was approved by the St. Vincent's Hospital Human Research Ethics Committee, and the University of Technology, Sydney (UTS) Human Research Ethics Committee. The collection of the hair samples was performed after obtaining written informed consent from each participant. Each hair sample was wrapped in aluminium foil, and stored separately in coded envelopes at room temperature prior to analysis. Each hair sample was prepared based on procedure detailed by Segura et al. [31]. Briefly, 75 mg of finely cut hair was incubated in 1 mL MeOH:TFA (9:1) at 37 °C overnight. Six hundred microliters of the digest

was then transferred to a clean, dry plastic tube, evaporated to dryness using a vacuum concentrator, prior to reconstitution in 200 μL of MilliQ water.

Calibration curves were obtained by analysing drug-free hair fortified with working standard solutions to result in final concentrations of 0.05–2.02 ng/mg MET1/MET2; 0.10–2.02 ng/mg EDDP1/EDDP2; and 1.21–3.23 ng/mg EMDP1/EMDP2. Calibration curves were constructed based on peak area ratios. For the purposes of the calibration, it was assumed that the (*R*,*S*)-standards of each analyte were composed of a 50:50 mixture of the (*R*)- and (*S*)-enantiomers. A quality control (QC) sample was obtained by homogenising hair collected from known MMT patients. Calibration standards and QC samples were analysed daily with each set of samples, with a standard curve constructed using linear regression. Any potential outliers in the calibration curves were identified using regression analysis. Accuracy and precision were evaluated at two concentrations for each enantiomer (0.76 and 2.02 ng/mg for MET and EDDP, and 2.42 and 3.23 ng/mg for EMDP), with three replicates performed at each concentration level. Intra-assay precision and accuracy was assessed by analysing three replicate samples at two concentrations within the same validation batch, while the inter-assay precision and accuracy was evaluated by analysing three replicate samples at those concentrations over three validation batches. Accuracy was expressed as the percentage of the ratio of calculated concentration to nominal concentration, while precision (% coefficient of variation (%CV)) was calculated as the percentage of the average divided by the standard deviation in peak area ratio of the three replicates. Recovery was calculated as the average percentage of the ratio of the peak area of each analyte in the extracted fortified hair samples to the peak area of each analyte of an unextracted aqueous standard. The limit of detection (LOD) was defined as a signal to noise (S/N) ratio of 3:1, while the limit of quantitation (LOQ) was defined as a S/N ratio of 10:1.

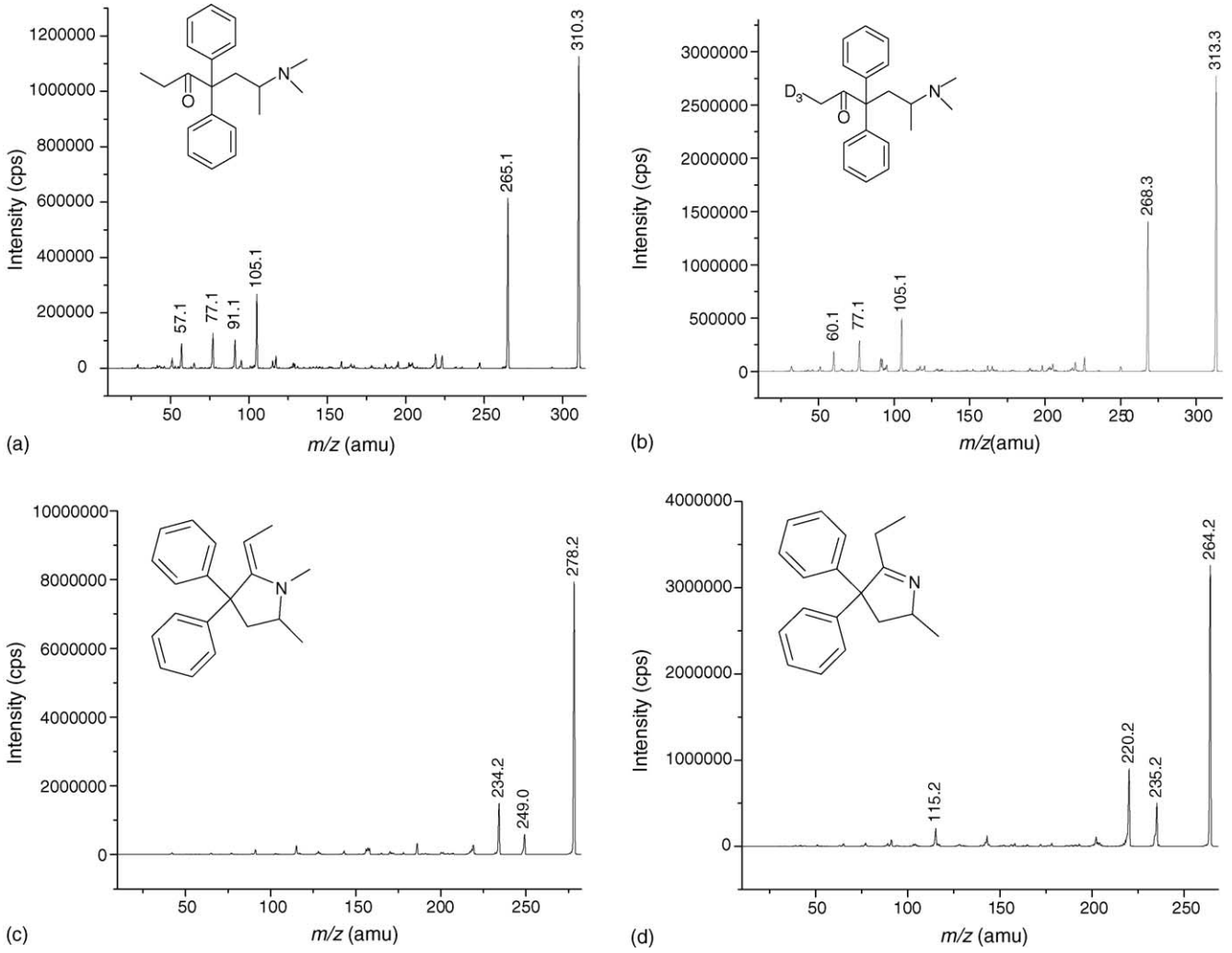

Fig. 3. Product ion scan of: (a) *m/z* 310 (methadone MS/MS spectra); (b) *m/z* 313 (methadone-d_3 MS/MS spectra; (c) *m/z* 278 (EDDP MS/MS spectra); and (d) *m/z* 264 (EMDP MS/MS spectra).

3. Results and discussion

Optimisation of the MS parameters was achieved by continuous infusion of an aqueous 1 μg/mL solution of each analyte (2.5 mL/h) via a syringe infusion pump with 0.2 mL/min flow of methanol. Autotune optimisation software was then used to optimise the MS parameters of each analyte. Following the evaluation of ESI and APCI, both in positive and negative modes, ESI in positive ion mode was selected for the ionisation of all analytes. Example product ion (MS/MS) spectra for methadone, methadone-d_3, EDDP and EMDP are given in Fig. 3(a–d), respectively.

The manufacturer's specifications for the separation of the MET enantiomers were used as the initial experimental conditions, except with the substitution of ammonium acetate as the buffer instead of phosphate buffer (i.e. 10 mM NH_4CH_3COO pH 6: IPA (90:10)). Under these conditions, the enantiomers of MET (and MET-d_3) were baseline resolved (retention times: 11.90 and 15.99 min, R_S 1.06), the EDDP enantiomers were partially resolved (retention times: 9.46 and 10.41 min, R_S 0.41), while the enantiomers of EMDP were barely separated (retention times: 19.96 and 20.85 min, R_S 0.23). The initial LC–MS separation of the MET, EDDP and EMDP enantiomers is illustrated in Fig. 4. The chromatogram for MET-d_3 is not illustrated as it is similar to that observed for MET.

Since pure enantiomeric standards of each analyte were unavailable, peak identification for each enantiomer was not performed during the method development. Therefore, for the purposes of the method development, the enantiomers were

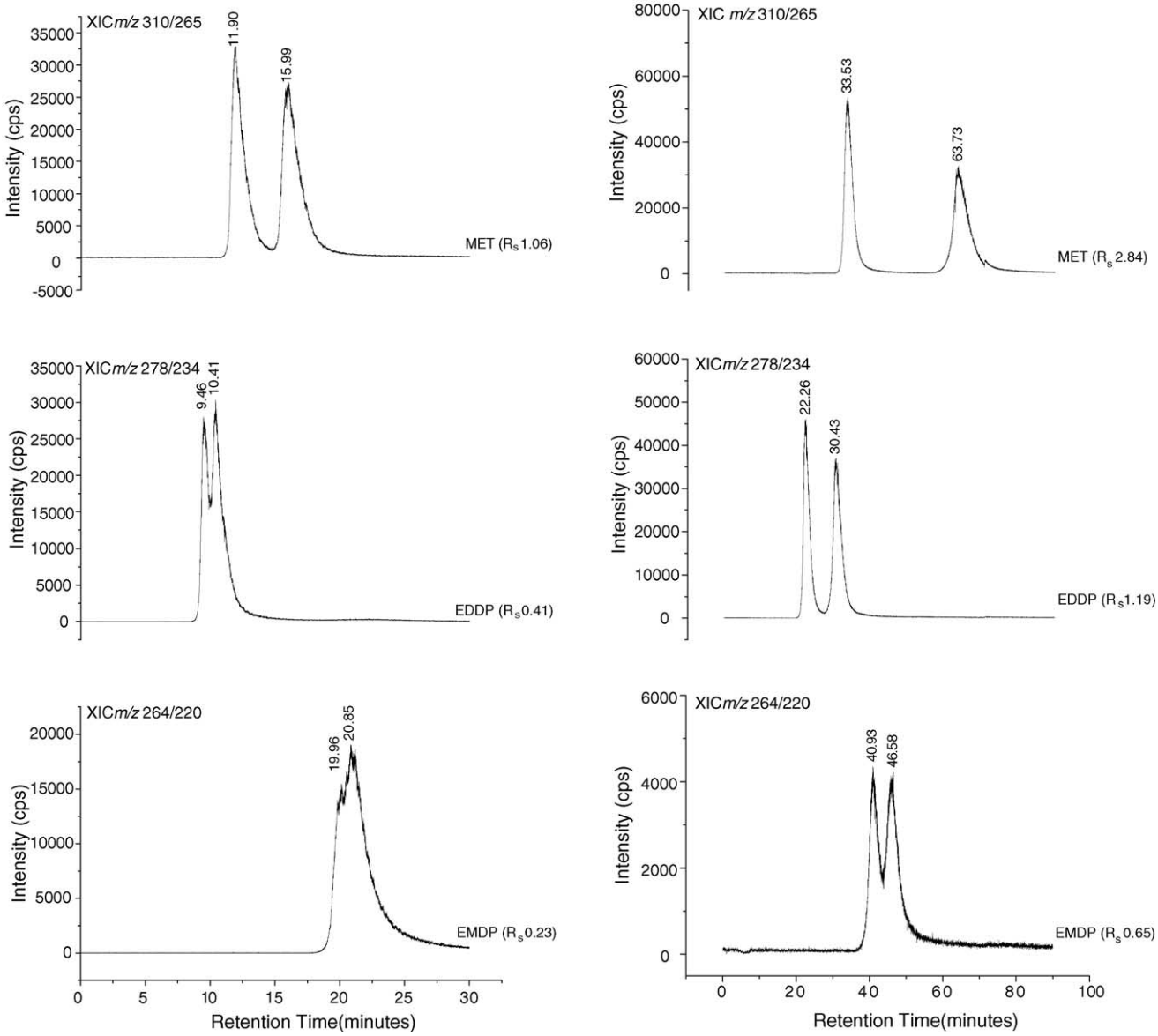

Fig. 4. The initial (left) and optimised (right) LC–MS separation of the MET, EDDP and EMDP enantiomers. Conditions: 10 mM NH_4CH_3COO pH 6:IPA (90:10), 0.9 mL/min (initial); 20 mM CH_3COOH:IPA (93:7) pH 7.4, 0.9 mL/min (optimised).

labelled in elution order, for example, the first and second eluting enantiomers of methadone were MET1 and MET2, respectively. Nevertheless, by considering the elution order observed for previously published methods that have used an AGP column with varying percentage compositions of an organic modifier of either ACN or IPA at a pH ranging from 5.8 to 7, it can be postulated that the (*R*)-enantiomer of both methadone and EDDP elutes prior to the (*S*)-enantiomer [9,10,20,21,32,33].

Following some preliminary scouting experiments (data not shown), a central composite three factor experimental design was implemented: 7–10% IPA; 10–20 mM CH_3COOH; pH 7–7.6. Similar retention times were observed for each enantiomer in the duplicate injections; therefore the average retention times were used for the training of the ANN. In one experiment (7% IPA, 10 mM CH_3COOH, pH 7.6), one of the enantiomers of MET eluted after the selected analysis time of 90 min; therefore this experiment was disregarded for the purposes of training an ANN. The values of the three factors used in each of the remaining experiments ($n = 14$) and the corresponding average retention times of the six enantiomers were then used as input and output, respectively, for the training of an ANN. A thorough search using various ANN topologies and training algorithms was performed, with a randomised selection of 12 training experiments and 2 verification experiments until a suitable ANN was simulated. The suitability of the ANN obtained was based on the minimum training and verification errors which were similar in magnitude. The selected ANN was a radial basis function (RBF) architecture, with three input layers and six hidden layers and six output layers (RBF 3:3-6-6:6); the network illustration is given in Fig. 5. The training and verification errors for the selected ANN were 0.01775 and 0.02186 RMS, respectively.

The predicted retention times generated by this model were found to be consistent with the values obtained experimentally. The observed average retention times obtained for each enantiomer in the 14 experiments were plotted against the predicted values generated by the ANN. An overall strong linear correlation was observed (Fig. 6, $y = 0.9916x + 0.2446$, $r^2 = 0.9844$). The ANN was then used to predict the retention times for each enantiomer within the experimental space grid (CH_3COOH 10–20 mM at increments of 1 mM, %IPA 7–10% at increments of 0.5%, and pH 6.90–7.60 at increments of 0.05). The predicted resolution values, *R*, were then calculated based on the predicted retention times obtained from the ANN. The data generated by the ANN model was sorted in order of descending PR value. The conditions predicted by the ANN model for the optimal PR in the shortest analysis time were 20 mM CH_3COOH:IPA (93:7) at pH 7.40. Example chromatograms of the optimised separation for the MET, EDDP and EMDP enantiomers (20 μg/mL mixed aqueous standard) using 20 mM CH_3COOH:IPA (93:7) at pH 7.4 is illustrated in Fig. 4. The enantiomers of MET and EDDP were baseline resolved (MET R_s 2.84 and EDDP R_s 1.19), while the EMDP enantiomers were partially resolved (EMDP R_s 0.65).

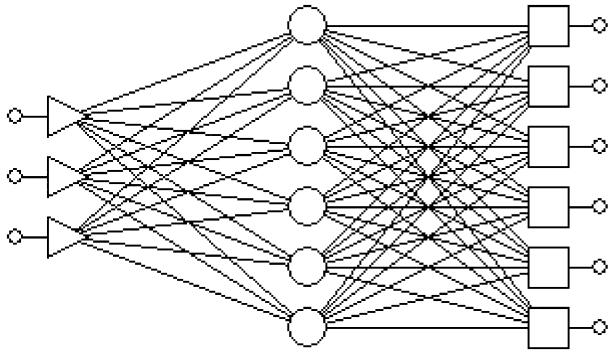

Fig. 5. Network illustration of selected ANN (RBF 3:3-6-6:6).

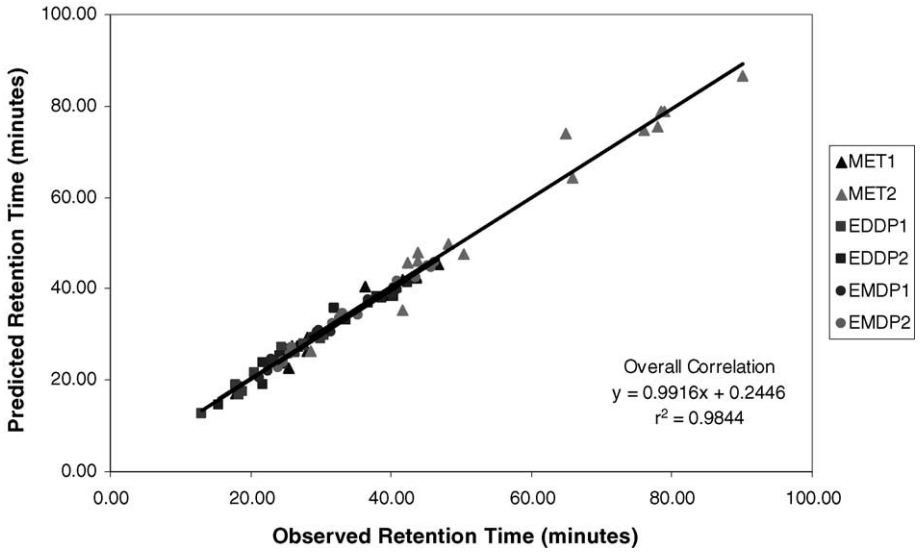

Fig. 6. Predicted retention times vs. observed retention times for each enantiomer for the 14 experiments.

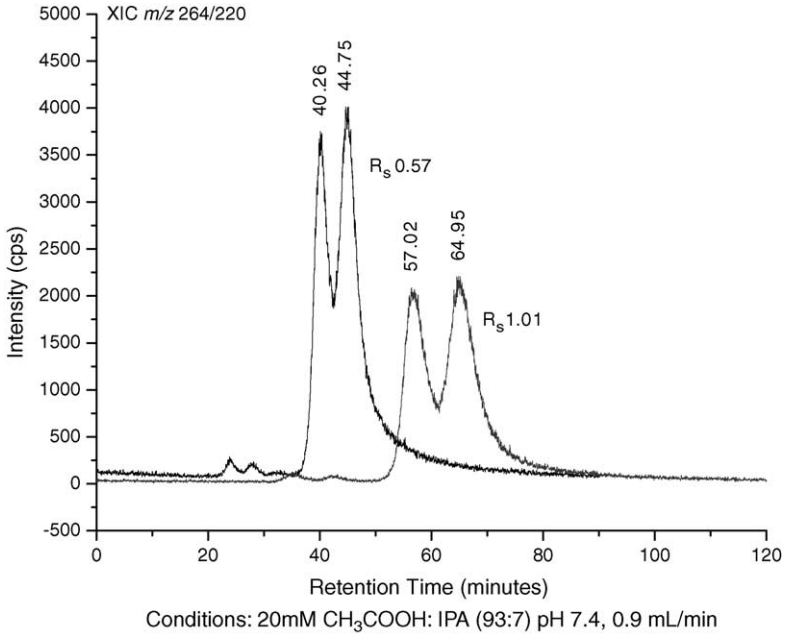

Fig. 7. Separation of EMDP enantiomers at ambient temperature (R_s 0.57) and 15 °C (R_s 1.01). Conditions: 20 mM CH_3COOH:IPA (93:7) pH 7.4, 0.9 mL/min.

The optimal separation conditions of 20 mM CH_3COOH: IPA (93:7) at pH 7.4 were then investigated at a reduced temperature of approximately 15 °C. At 15 °C, the resolution of EMDP was 1.01, which was an improvement compared to that obtained at ambient temperature (EMDP R_s 0.6). A comparison of the separation achieved for the EMDP enantiomers at ambient temperature (21–25 °C) and 15 °C is illustrated in Fig. 7. Under the reduced temperature conditions of approximately 15 °C, MET2 was observed to have a retention time greater than the 90 min cut-off (MET2 RT 113.42 mins). Without the utilisation of a column cooler, the cooling of the column over long sequences may prove to be problematic, leading to unstable column temperatures over time. Therefore, despite the improvement achieved in the EMDP resolution at a temperature of 15 °C, an ambient temperature (21–25 °C) was chosen for this method. However, future studies should consider the utilisation of a column cooler to investigate further the effects of reduced temperatures on the resolution of the EMDP enantiomers.

Following the optimisation using the IPA factorial analysis and ANNs, only partial separation of the EMDP enantiomers (R_s 0.65, Fig. 4) was achieved using the AGP column with an organic modifier of IPA. However, this was a considerable improvement on the separation obtained during the preliminary LC–MS experiments (R_s 0.23, Fig. 4). Furthermore, the resolution achieved for the EMDP enantiomers would be sufficient for quantification.

An additional factorial analysis was performed using an alternate organic modifier of ACN: 10–20% ACN; 10–20 mM CH_3COOH; pH 5–7.6 (data not shown). ACN was chosen as it may influence the conformation of the AGP protein differently to IPA, and therefore differences in the enantioselectivity of the AGP stationary phase toward the analytes may be observed. Following the optimisation using the ACN factorial analysis and ANNs, while complete separation of the methadone and EDDP enantiomers could be achieved, only partial separation of the EMDP enantiomers (R_s 0.52, data not shown) was observed. This resolution value was less than that obtained using an organic modifier of IPA (R_s 0.65). Therefore, the IPA optimal separation conditions of 20 mM CH_3COOH:IPA (93:7) at pH 7.4 provides the best resolution of each of the enantiomer pairs.

The application of experimental designs in conjunction with ANNs proved to be extremely beneficial in the method development, and obtaining the desired result using traditional univariate strategies would have been both difficult and time consuming. It should be noted that the optimised values obtained from the application of the ANN are a local optimum, rather than the global optimum, due to the constraints placed on the variables considered in the factorial analysis. For example, increasing the analysis time constraint may lead to further improvement in the enantioselectivity of EMDP. Nevertheless, using the two selected organic modifiers and with the applied variable constraints, the results of the factorial analyses and subsequent application of ANNs suggests that the AGP column is unable to further separate the enantiomers of EMDP, with conditions that preserve the stability of the stationary phase (pH $\leq$ 7.6) within an analysis time of 90 min. Future work could consider the use of non-reversible organic modifiers, such as DMOA to determine whether an improvement in the resolution of the EMDP enantiomers could be achieved. However, since this would result in a permanent change to the

Table 1
Methadone and EDDP hair concentrations in MMT patients ($n = 20$)

Sample code	Mass hair (mg)	Dosage history (mg/day)	Hair colour	MET1 concentration (ng/mg)	MET2 concentration (ng/mg)	EDDP1 concentration (ng/mg)	EDDP2 concentration (ng/mg)
X1	79	185	Brown	0.67	0.37	0.09	0.05
X2	77	140	Brown-grey	0.88	0.62	0.13	0.12
X3	83	130	Brown	0.89	0.49	0.07	0.03
X4	78	110	Brown	0.49	0.32	0.07	BLOQ
X5	76	130	Brown	1.27	0.87	0.10	0.03
X6	80	120	Brown	0.57	0.34	0.10	0.06
X7	78	90	Brown	0.40	0.23	0.07	BLOQ
X8	75	195	Brown	1.41	0.68	0.17	0.15
X9	83	150	Brown	0.46	0.27	0.06	BLOQ
X10	76	145	Brown (red tint 6 months ago)	1.16	0.57	0.13	0.09
X11	75	100	Brown-grey	0.52	0.39	0.06	0.06
X12	76	130	Black-brown	1.69	0.89	0.18	0.14
X13	75	105	Brown (bleach 1 month ago)	0.18	0.09	0.03	BLOQ
X14	54	110	Brown	0.68	0.38	0.09	0.08
X15	89	125	Brown	1.18	0.52	0.10	0.08
X16	75	140	Brown	1.65	1.06	0.11	0.09
X17	83	150	Brown	1.62	0.94	0.15	0.16
X18	75	115	Brown	0.39	0.26	0.04	BLOQ
X19	78	150	Brown (red tint 2 months ago)	1.36	0.65	0.08	0.09
X20	76	140	Brown	0.37	0.21	0.05	0.04

Abbreviations: below limit of quantitation (BLOQ); LOD: 0.0015 ng/mg MET1/MET2, 0.008 ng/mg EDDP1/EDDP2, and 0.09 ng/mg EMDP1/EMDP2; LOQ: 0.05 ng/mg MET1/MET2, 0.03 ng/mg EDDP1/EDDP2 and 0.30 ng/mg EMDP1/EMDP2.

protein conformation it was not considered in the present study.

Linearity, with correlation coefficients (r^2) ranging from 0.9142 to 0.9982, was established over the concentration range of 0.05–2.0 ng/mg MET1/MET2, 0.1–2.0 ng/mg EDDP1/EDDP2 and 1.2–3.2 ng/mg EMDP1/EMDP2 in hair. The intra- and inter-precision ranged from 0.5 to 16 %CV and from 5.0 to 23 %CV, respectively, while the intra- and inter-accuracy ranged from 94 to 122% and from 94 to 111%, respectively for all three analytes. The recoveries for the methadone analytes in hair ranged from 49 to 96%. The limits of detection in hair were 0.0015 ng/mg MET1/MET2, 0.008 ng/mg EDDP1/EDDP2, and 0.09 ng/mg EMDP1/EMDP2, while the limits of quantitation were 0.05 ng/mg MET1/MET2, 0.03 ng/mg EDDP1/EDDP2 and 0.30 ng/mg EMDP1/EMDP2. The results of the hair validation study showed that both the intra- and inter-assay accuracy and precision values for the method were satisfactory for all three analytes. Furthermore, the recovery data for all of the enantiomers was also satisfactory.

Twenty hair samples were collected from patients undergoing MMT. The MET and EDDP concentrations found in the hair samples are given in Table 1. All of the samples were found to have quantifiable amounts of both methadone enantiomers, with concentrations ranging from 0.18 to 1.69 ng/mg for MET1 and from 0.09 to 1.06 ng/mg for MET2. Most of the hair samples contained EDDP enantiomer concentrations greater than the limit of quantitation (EDDP LOQ 0.03 ng/mg), with concentrations in the range of 0.04–0.18 ng/mg for EDDP1 and below the limit of quantitation—0.16 ng/mg for EDDP2. However, the concentration of the EMDP enantiomers in all the hair samples was determined to be below the limit of detection (EMDP LOD 0.09 ng/mg). Therefore, the method was capable of detecting methadone, EDDP and EMDP in hair. However, the results of the hair samples collected from twenty MMT patients suggest that greater sensitivity is required to detect the EMDP enantiomers in authentic MMT hair samples.

4. Conclusions

An LC–MS/MS method for the simultaneous stereoselective separation of methadone and its major metabolites, EDDP and EMDP was successfully developed and validated, using an AGP stationary phase. This method represents the first HPLC technique for the simultaneous analysis of the six enantiomers related to methadone, which is of significance with respect to the monitoring of patients undergoing methadone maintenance therapy. The optimised conditions for the greatest product resolution of the six enantiomers in the shortest analysis time were determined to be 20 mM acetic acid:isopropanol (93:7), at pH 7.4 with a flow rate of 0.9 mL/min. Using these conditions, the enantiomers of MET and EDDP were baseline resolved ($R_s \geq 1.19$), while the EMDP enantiomers were sufficiently

resolved to allow quantitation (EMDP R_s 0.65). The method was applied to the analysis of the methadone analytes in hair specimens. This paper emphases the potential of experimental designs and ANNs in the optimisation of HPLC separations of drugs, particularly for complex stereoselective applications.

Appendix A

Eq. (1): calculation of resolution (R_s)

$$R_s = \frac{t_2 - t_1}{0.5(w_1 + w_2)} \quad (1)$$

where t_1 and t_2 are the retention times for each peak and w_1 and w_2 are the baseline peak widths for peaks 1 and 2, respectively.

Eq. (2): calculation of predicted resolution (R)

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_B}{1 + k'_B}\right) \quad (2)$$

where k'_B is the capacity factor of the slower-moving species and α is the selectivity factor for the peak pair. For the purposes of the experimental model predictions, N was assumed to remain constant.

Eq. (3): calculation of product resolution (PR)

$$\text{PR} = \text{MET } R * \text{ EDDP } R * \text{ EMDP } R \quad (3)$$

where MET R, EDDP R and EMDP R are the predicted resolution values for the methadone, EDDP and EMDP enantiomer pairs, respectively.

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