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Chiral high-performance liquid chromatography methodology for quality control monitoring of dexfenfluramine

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Abstract

The objective of this study was to measure the concentration of *d*-fenfluramine · HCl (the desired product) and *l*-fenfluramine · HCl (enantiomeric impurity), in the final pharmaceutical product, in the possible presence of its isomeric variants. Sensitivity, stability and specificity were enhanced by derivatizing the analytes with 3,5-dinitrophenylisocyanate utilizing a Pirkle chiral recognition approach. Analysis of the calibration curve data and quality assurance samples showed an overall inter-assay precision of 1.78 and 2.52%, for *d*-fenfluramine · HCl and *l*-fenfluramine, with an overall intra-assay precision of 4.75 and 3.67%, respectively. The minimum quantitation limit was 50 ng/ml, having a minimum signal-to-noise ratio of 10, with relative standard deviations of 2.39 and 3.62% for *d*-fenfluramine and *l*-fenfluramine. The method is capable of accurately and consistently determining as low as 0.01% *l*-fenfluramine in the *d*-fenfluramine final pharmaceutical product.

1. Introduction

After successfully developing a chiral HPLC method for the analysis of dexfenfluramine for the clinical trial involving human plasma and urine [1], we investigated adapting this methodology for application in the monitoring the final pharmaceutical product in the quality control phases.

For purposes of clarity (Fig. 1), *d,l*-fenfluramine · HCl is N-ethyl-α-methyl-*meta*-(trifluoromethyl)phenethylamine, and is referred to throughout this paper as m-I,II. *ortho-d,l*-Fenfluramine · HCl has the trifluoromethylphenethylamine in the *ortho* position, and is referred to as o-I,II; *para-d,l*-fenfluramine · HCl has the trifluoromethylphenethylamine in the *para* position, and is referred to as p-I,II. The roman numeral I refers to the *d*-enantiomer and the II refers to the *l*-enantiomer.

The literature appeared lacking any HPLC method for the analysis of *d*-fenfluramine in the bulk raw drug or final pharmaceutical product. Therefore, a chiral method was developed for the quantitative enantiomeric determination of m-I and m-II in the possible presence of its isomeric variants o-I,II and p-I,II. This was accomplished by derivatizing the analytes into their 3,5-dinitrophenyl urea derivatives, separat-

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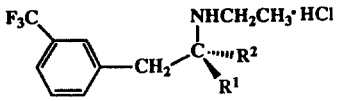
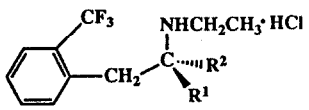
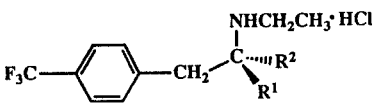
d,l-Fenfluramine·HCl (m-I,II)



Ortho-d,l-Fenfluramine·HCl (o-I,II)



Para-d,l-Fenfluramine·HCl (p-I,II)



N-Methylphenethylamine (N-MPEA)

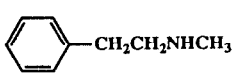


Fig. 1. Basic chemical structures. $R^1 = H$ and $R^2 = CH_3$ for the *d*-configuration; $R^1 = CH_3$ and $R^2 = H$ for the *l*-configuration.

ing them on different chiral stationary phases consisting of (*R*)- and (*S*)-1-(1-naphthyl)-ethylurea covalently bound to silica through a propyl linkage. The procedure is based on a chiral stationary phase originally described by Oi et al. [2], utilizing a convenient method for urea formation introduced by Pirkle et al. [3].

2. Experimental

2.1. Reagents

Glacial acetic acid (HPLC grade) and hydrochloric acid (reagent grade) were purchased from Fisher Scientific, Fairlawn, NJ, USA. Acetonitrile, hexane, methanol, methylene chloride and toluene (all HPLC grade) were purchased from Burdick & Jackson, Baxter Scientific, Edison, NJ, USA. 3,5-Dinitrobenzoylchloride (>98%) and sodium azide (>99.5%) were purchased from Fluka, Ronkonkoma, NY, USA. N-Methylphenethylamine (N-MPEA, 99%) and sodium hydroxide pellets (99.99%) were purchased from Aldrich, Milwaukee, WI, USA. m-I, m-I,II, o-I, o-I,II, p-I and p-I,II (all hydrochloride salt reference standards) were acquired through Wisconsin Analytical Research Services and Servier Technologie, Paris, France. Water, Milli-Q/HPLC grade; was produced in our laboratory.

2.2. Equipment

Similar equipment was used as previously described [1], except for the analytical columns consisting of (*R*)-naphthylurea Chiral, 250 × 4.6 mm, 5 μm particle size for system A, and the equivalent in the (*S*) configuration for system B, respectively (Supelco, Bellefonte, PA, USA).

2.3. Computerization

Similar computerization was used as previously described [1]. All concentrations were reported in the salt (HCl) form.

2.4. Preparation of reagent stock solutions

The preparation of 3,5-dinitrobenzoyl azide (DNB-N_3) and stock 3,5-dinitrophenylisocyanate (DNP-NCO) is described elsewhere [1]. A portion of this DNP-NCO stock solution was diluted with methylene chloride, 1:100 (v/v) for the m-I assay (HPLC system A) and 1:10 (v/v) for the m-II assay (HPLC system B), to give a final working solution concentration of approximately $2 \cdot 10^{-5}$ *M* for m-I and $2 \cdot 10^{-4}$ *M* for m-II. The higher concentration of DNP-NCO for the m-II assay was due to the much higher relative concentration of m-I as compared to m-II for this

assay and the reagent needed to be added in excess of all reacting species.

2.5. Placebo/blank preparation

Placebo/blank solutions were prepared by emptying one capsule (150 mg) into a 1-l class A volumetric flask. The solution was stirred, centrifuged (1500 rpm = 1250 *g*), and the supernatant was saved for later use.

2.6. Standard solution preparation

All sample preparations and analyses were conducted under yellow lighting in order to avoid possible sample degradation.

Analytical standard/stock and intermediate analytical standard/stock solutions were prepared in placebo solution. Concentrations were 60.0 and 6.00 μg/ml for m-I, m-II, o-I, o-II, p-I and p-II.

Internal standard stock solutions were prepared in 1 m*M* HCl using HPLC-grade water. Final concentration was 6.25 μg/ml for N-MPEA.

Working solutions were prepared in placebo solution by appropriate serial dilution of standard/stock solutions. Final concentrations were 2000, 1000, 500, 200, 100 and 50 ng/ml.

2.7. Preparation of solutions for the determination of m-II in the m-I analytical standard

Solutions of 60.00 μg/ml of the m-I analytical standard were prepared in HPLC-grade water. This yielded relative m-II concentrations that were on the calibration curve if m-II is approximately 0.1% of m-I.

2.8. Standard curve

Each calibration curve was generated with 1.00 ml of drug-free placebo solution fortified with 50.0, 100, 200, 500, 1000 and 2000 ng/ml of m-I and m-II, in duplicate. Internal standard concentration was maintained at 1000 ng/ml.

2.9. Sample solution preparation

Determination of m-I

The contents of a 150-mg capsule were emptied into a 1000-ml class A volumetric flask, filled with HPLC-grade water, and stirred for 1 h. After the solution sat for 30 min, the colorless solution was decanted into centrifuge tubes and spun at 1500 rpm (1250 *g*) for 5 min. The supernatant was removed and 1.00 ml was placed in a 50-ml class A volumetric flask and filled to the mark with HPLC-grade water.

Determination of m-II

Since m-II is expected to be much lower in relative concentration to that of m-I, the following dilution scheme was used.

The contents of the 150-mg capsules containing 5, 15 or 30 mg of m-I were emptied into a 100-, 250- or 500-ml class A volumetric flask, respectively, and filled to the mark with HPLC-grade water. The solutions were stirred, allowed to settle, the colorless solutions were removed, placed in centrifuge tubes, and spun at 1500 rpm (1250 *g*) for 5 min. The supernatants were collected for later use. This resulted in m-I concentrations of 50, 60 and 60 μg/ml. With relative m-II concentrations expected to be in the range 0.1–3% to that of m-I, the final dilution concentrations should produce data that will fall in the linear calibration curve range of 50–2000 ng/ml.

2.10. Extraction and derivatization

Control blanks were prepared by combining 1.00 ml of placebo solution, 100 μl of HPLC-grade water and 100 μl of 0.1 *M* NaOH.

Standards and samples were prepared by adding 100 μl of internal standard solution (1.00 μg/ml of N-MPEA) and 100 μl of 0.1 *M* NaOH to 1.00 ml of the respective solution.

A 2.00-ml volume of the appropriate concentration DNP-NCO solution was added to all and vortexed. The reaction was allowed to go to completion (15 min), the aqueous (top) layer

was removed and placed in HPLC autosampler vials ready for injection.

2.11. HPLC conditions

Our previous study [1] involved using a mobile phase composition of hexane–isopropanol–acetonitrile. However, we found better resolution, peak symmetry, and fewer interfering peaks when we substituted methylene chloride and methanol for isopropanol (IPA), especially since methylene chloride was already being used as the DNP-NCO diluent. Since alcohols also react with DNP-NCO, and not just primary and secondary amines, a previously interfering peak was removed that may now be attributed to the IPA–DNP-NCO product.

Individual solvent components were degassed under conditions of high vacuum and bumping, and then filtered through a 0.2-μm nylon (or equivalent) membrane.

HPLC system A (quantitation of m-I)

Combine hexane, methylene chloride, methanol and acetonitrile, in the ratio 81.0:17.5:1.0:0.5 (v/v).

Chromatographic run time and flow-rates were 30 min at 1.50 ml/min. Detection was at 235 nm using a sensitivity of 0.005 AUFS. A 30-μl volume of the final solution was injected. Retention times were approximately 8.3 min for o-I, 9.0 min for o-II, 10.3 min for m-I, 14.6 min for p-II and 17.6 min for N-MPEA.

HPLC system B (quantitation of m-II)

Combine hexane, methylene chloride, methanol and acetonitrile, in the ratio 85.0:13.5:1.0:0.5 (v/v).

Chromatographic run time, flow-rate and detection parameters were identical to that of HPLC system A. A 20-μl volume of the final solution was injected. Retention times were approximately 7.8 min for o-II, 8.4 min for o-I (inter-system check by comparison to HPLC system A), 10.1 min for m-II, 14.2 min for p-I and 18.3 min for N-MPEA.

3. Results and discussion

The chiral *S* column yielded a favorable separation of m-II from m-I over the *R* column for this application over our previous choice [1], since m-II eluted prior to m-I and the reagent. This is preferable when analyzing a clean sample (i.e. not biological in origin) and the objective is to determine the % m-II in m-I. Two slightly different chromatographic conditions were required in order to quantitate within acceptable error limits.

Isomeric elution order was established by derivatization and chromatographing a sample of m-I, m-I,II, o-I, o-I,II, p-I and p-I,II. No peak correspondence to the antipode, in excess of the stated purity (Certificate of Analysis), was observed. This demonstrated that no racemization occurs under the extraction and/or derivatization conditions [4].

Given the present state-of-the-art in analytical chiral HPLC columns, only relatively moderate resolution and efficiency were experienced throughout these studies. Attempts at manipulating many of the customary HPLC variables (i.e. column particle size, length and temperature, mobile phase composition and linear velocity, etc.) in order to achieve adequate separation of all species in one run, were futile.

In order to determine if experimental conditions, i.e. selectivity (α), capacity factor (k'), theoretical plates (N), and subsequently resolution (R_s), were appropriate prior to the onset of any tray, test mixes (usually the high standard) were run in order to determine if the various separation factors were adequate. Typical overall physical characteristics of the separation were in the order of that expected. A range is specified to indicate the values for the first through last analytes of interest. Individual analytes should fall within the ranges specified herein. The *S* column's (HPLC system A, Fig. 2) α values ranged from 0.9 to 1.5 (analyte peak to closest neighbor), k' from 3.1 to 7.8 (using t_0 equal to the system dead volume in units of minutes), and $N_{1/2\ h}$ from 9600 to 12 500 plates/column. The *R* column's (HPLC system B, Fig. 3) α values ranged from 0.9 to 1.3, k' from 3.5 to 9.8, and

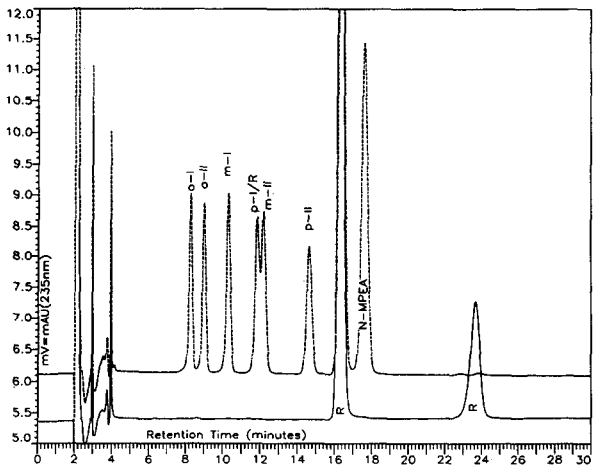


Fig. 2. Chromatographic overlays of HPLC system A showing a control blank (bottom) and 500 ng/ml m-I,II, o-I,II and p-I,II with 625 ng/ml N-MPEA (top).

$N_{1/2\,h}$ from 5700 to 7100 plates/column, and coincided with our previous study [1].

The calibration curves and resultant statistical data were consistent and accurate. Table 1 shows the overall inter-assay precision for standards and Table 2 shows the overall intra-assay precision. The overall relative standard deviation (R.S.D.) was less than 5%. Calibration curve data produced a minimum mean coefficient of determination, R^2 (correlation coefficient squared), of >0.9992 with a slope R.S.D. of $<3\%$.

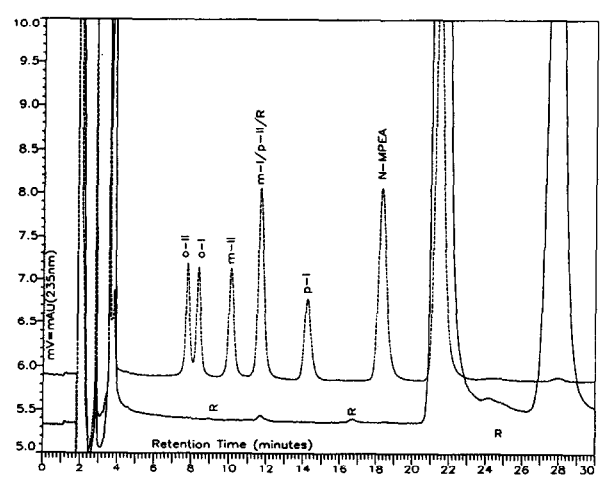


Fig. 3. Chromatographic overlays of HPLC system B showing a control blank (bottom) and 500 ng/ml m-I,II, o-I,II and p-I,II with 625 ng/ml N-MPEA (top).

Both HPLC systems utilized high signal-to-noise (S/N) ratios when the minimum quantitation limit (MQL) of 50 ng/ml was selected. HPLC system A had typical baseline noise (maximum − minimum) equal to 10 μV or approximately $10 \cdot 10^{-6}$ AU, while the 50 ng/ml m-I standard had a peak height of approximately 250 μV with $N_{1/2\,h}$ of 10 800 plates/column. This resulted in a S/N of 25. Similar data were observed with HPLC system B, having as typical baseline noise of $8 \cdot 10^{-6}$ AU, yet the peak height was approximately 100 μV for the 50 ng/ml m-II standard with $N_{1/2\,h}$ of 6400 plates/column. This resulted in a S/N of 12.5. Since efficiency was reduced for system B, the resultant decreased S/N was as expected.

Since the analytical standards employed throughout this work had stated enantiomeric purity specifications of $>99\%$ m-I and $\leq 0.12\%$ m-II (Certificate of Analysis and determined by capillary GC), we needed to determine and compare enantiopurity using this method prior to analyzing any product. High concentrations (≥ 60.00 μg/ml) of the m-I standard were prepared so that the endogenous relative concentration of m-II in the m-I standard could be accurately evaluated. The mean m-II concentration was found to be 52.3 ng/ml, or 0.083% m-II in the m-I analytical standard, with a 1.20% R.S.D. This correction was then incorporated for all subsequent analyses on the product. Table 3 shows all data for the 5-, 15- and 30-mg capsules.

Using the sample preparation scheme herein, we were able to consistently determine that the m-I analytical standard contained 0.08% m-II, which is within the specifications listed in the Certificate of Analysis ($\leq 0.12\%$ m-II) provided by the manufacturer and determined by capillary GC. If necessary, lower, valid determinations could be made, as low as 0.01%, by altering the sample preparation scheme, e.g. increasing the initial m-I analytical standard solution concentration from 60 μg/ml to 500 μg/ml if m-II were as low as 0.01%.

Adequate separation of the enantiomers of the individual corresponding isomers was evidenced.

Table 1
Summary of inter-assay precision from the analysis of the calibration curve data

	STD = 50	STD = 100	STD = 200	STD = 500	STD = 1000	STD = 2000
d-Fenfluramine (m-I) (n = 7)						
Mean	50.94	99.39	200.58	494.25	991.11	2016.41
S.D.	1.22	0.71	3.68	3.61	8.00	14.19
R.S.D. (%)	2.39	0.71	1.83	0.73	0.81	0.70
Overall R.S.D. (%)	1.78					
l-Fenfluramine (m-II) (n = 8)						
Mean	51.67	98.64	198.35	493.97	1006.35	2002.18
S.D.	1.87	2.3	3.89	8.59	10.39	16.74
R.S.D. (%)	3.62	2.33	1.96	1.74	1.03	0.84
Overall R.S.D. (%)	2.52					

All concentration units are in ng/ml. STD = Analytical standard concentration.

However, when all the isomers were combined, the chromatographic requirements become more complicated, and necessitated the need for dual conditions. Fig. 2 shows the separation on HPLC system A. Note that this system can only resolve o-I, o-II, m-I and p-II (4 of the 6 analytes needed); p-I co-elutes with a reagent peak and m-II. Since it was important to be able to quantitate m-I *and* m-II, a second set of chromatographic conditions was required. We chose using the (*R*)-naphthylurea column for several reasons, however, mainly for reversed enantiomer elution order over that of the corresponding (*S*) column (Fig. 3). With this column in place, and by using slightly different mobile phase composition, m-II and p-I were resolved from interfering peaks.

In addition, the percent m-II as compared to m-I in random clinical specimens 2 and 3 from our previous study [1], were approximately 1.8%. This result coincides with that determined here. This proves that physiological interconversion was not apparent in the clinical trial, since any m-II was coming from the product and not from metabolic origin.

This method could have been used to quantitate the isomeric variants mentioned herein, but this was beyond our need. Any evidence of any

Table 2
Summary of intra-assay precision from the analysis of the calibration curve data

	STD = 50	STD = 100	STD = 200	STD = 500	STD = 1000	STD = 2000
d-Fenfluramine (m-I) (n = 7)						
Mean ratio	1.014	1.022	1.003	1.007	1.007	1.009
S.D.	0.055	0.059	0.068	0.054	0.048	0.040
R.S.D. (%)	5.42	5.77	6.78	5.36	4.77	3.96
Overall R.S.D. (%)	4.75					
l-Fenfluramine (m-II) (n = 8)						
Mean ratio	0.992	0.990	1.000	1.078	1.043	1.031
S.D.	0.058	0.020	0.013	0.069	0.042	0.032
R.S.D. (%)	5.85	2.02	1.30	6.40	4.03	3.10
Overall R.S.D. (%)	3.67					

All concentration units are in ng/ml. STD = Analytical standard concentration.

Table 3
Verification of analytical standard purity and final product content

	Prepared m-I concentration (mg/l)	Determined m-II concentration (ng/ml)	Calculated % m-II	m-I[a] (ng/ml)	m-I[b] (mg/capsule)	m-II[a] (ng/ml)	m-II[b] (μg/capsule)	% m-II[c]
m-I standard (n = 20)								
Mean	63.11	52.32	0.0829					
S.D.	3.88	2.35	0.0014					
R.S.D. (%)	6.15	4.49	1.69					
5-mg capsule (n = 20)								
Mean				466.6	4.666	856.7	85.67	1.84
S.D.				28.9	0.289	15.8	1.58	
R.S.D (%)				6.19	6.19	1.84	1.84	
15-mg capsule (n = 20)								
Mean				475.8	14.28	1037.6	260.0	1.82
S.D.				22.9	0.69	25.7	6.02	
R.S.D. (%)				4.81	4.83	2.48	2.32	
30-mg capsule (n = 20)								
Mean				592.7	29.64	1065.3	533.0	1.80
S.D.				25.9	1.3	22.0	11.0	
R.S.D. (%)				4.37	4.39	2.07	2.06	

[a]Concentration determined by HPLC.
[b]Calculated mass per capsule, calculated by multiplying HPLC concentration by appropriate dilution.
[c]Calculated by dividing mass m-II by mass m-I and multiplying by 100.

of the isomeric variants of fenfluramine would show production problems.

4. Conclusions

The literature has only a few references on dexfenfluramine and all are related to its analysis from a biological matrix. The prime focus of this study was to develop and validate, an analytical stereospecific HPLC method intended to quantitate the concentrations of *d*-fenfluramine (major product) and *l*-fenfluramine (impurity) in the final pharmaceutical product. Only qualitative data were required for the possible isomeric variants and their enantiomers. This new methodology has been designed for quality assurance purposes for monitoring commercial production of the drug.

Efforts addressed at producing a single HPLC condition for resolving all the analytes of interest was futile given the resources allocated to this project and its intended use. When looking at Van der Waals models of the three isomers, and each isomer's two enantiomers, similar molecular surface characteristics were evident. Other separation approaches were considered, such as using a reversed-phase HPLC column coupled in-line to a chiral HPLC column, to separate the isomers and the enantiomers, respectively. However, solvent–stationary phase compatibility issues would be very time consuming and quite difficult to determine. Reversed-phase HPLC coupled orthogonally to chiral HPLC was another option, but we believe that such a system would have been more susceptible to error than we have demonstrated here with this system.

With the resultant statistical data shown herein, it is clear that this experimental approach is capable of providing accurate and reliable data

that can be used for monitoring the production of dexfenfluramine.

Acknowledgements

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