Development of an Enantiomeric Separation of D & L Valine as Their Corresponding Isoindole Adducts By RP-HPLC for Utilization of the L-Valine toward Pharmaceutically Relevant Materials

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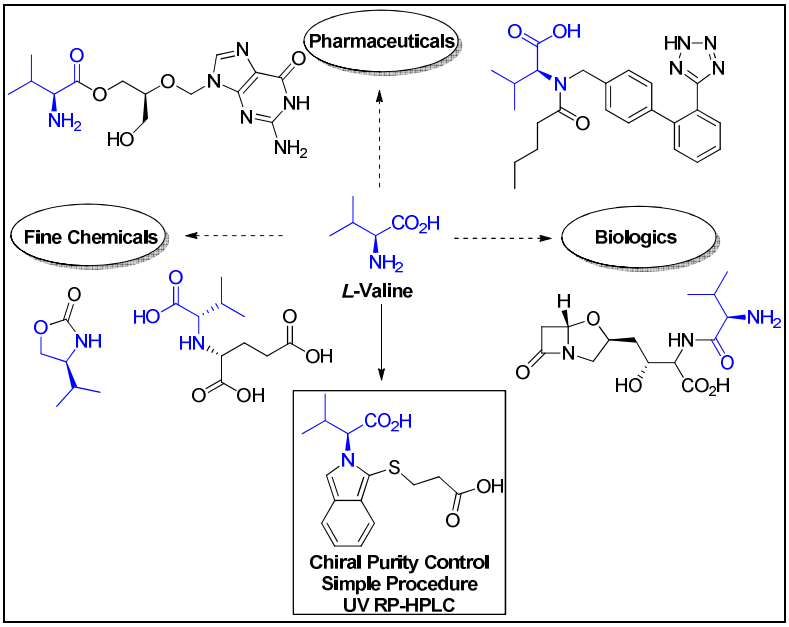
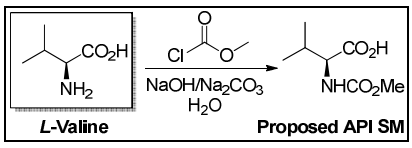


The utilization of L-Valine for its chiral centre is increasing in the fine chemical, biologic, and pharmaceutical sectors. Therefore, a simple and reliable chirality procedure is needed to assure that trace levels of D-Valine are not present. The present work is aimed at the development of a RP-HPLC method which can quantify D-Valine to the 0.05% chiral impurity level within L-Valine. Several method development and regulatory aspects are discussed, as well as method applicability to other related amino acids (Alanine, Norvaline, Isoleucine, Leucine, and tert-Leucine).

Introduction

L-Valine is an essential amino acid which has been increasingly incorporated into compounds found in the fine chemical, biologic, and pharmaceutical sectors[1]. Examples of pharmaceuticals which incorporate *L*-Valine or its isopropyl chiral centre include Valacyclovir[2a], Valganciclovir[2b], Valsartan[2c], and Elvitegravir[2d]. Due to growing scrutiny from regulatory entities, increasing amounts of information regarding the synthesis of an active pharmaceutical ingredient (API) are asked to be disclosed (Figure 1). Potential push back from regulatory agencies could force a proposed GMP starting material derived from *L*-Valine back to *L*-Valine itself. Therefore, analytical methods and specifications which meet the ICH guidelines[3] must be in place for *L*-Valine's impurity profile and

Figure 1. Example of a Proposed API Starting Material from *L*-Valine



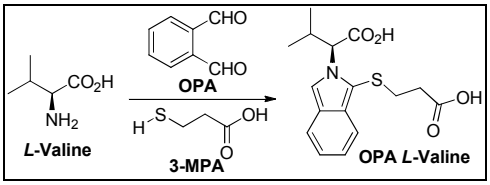
chirality. This paper focuses on the latter subject, the development of a chiral purity method for *L*-Valine.

The USP-NF, European, and JP pharmacopoeias[4] test the chirality of *L*-Valine *via* optical rotation. Given that the optimal sensitivity desired for a chiral method would be detection of the unwanted enantiomer *D*-Valine to the 0.05% level, optical rotation would not be suitable and an alternate detection method was sought out.

The literature was surveyed next regarding the separation of the *D*- and *L*-enantiomers of Valine by HPLC. Separation was indeed possible for the two enantiomers utilizing a variety of strategies (Cu^{2+} mediated ligand exchange[5], crown ether stationary phases[6], macrocyclic antibiotic stationary phases[7], polysaccharid-based stationary phases[8], etc.). But typically the resulting peaks are either broad or the inherent chromophore of Valine would lead to difficulties in quantitation of the undesired D-enantiomer to the desired chiral impurity level of 0.05%.

To circumvent the poor chromophore property of valine and related amino acids, derivatization of amino acids containing a strong chromophore has become a popular strategy for their identification and purity determination. The resulting derivatized amino acids typically produce good peak shape and increase the chromophore of the resulting derivatized adduct. One particular strategy is the *o*-phthalaldehyde (OPA)/thiol derivatization of primary amino acids which results in the corresponding isoindole adducts[9]. Several reviews have been written regarding the use of OPA/thiol derivatization[10] as well as the use of chiral thiols[11] to determine the chiral purity of some amino acids. This led us to believe that the OPA/thiol derivatization strategy to make the corresponding isoindole adduct of Valine (OPA *L*-Valine, Figure 2) could provide a suitable chiral method for *L*-Valine.

Figure 2. Derivatization of *L*-Valine to the OPA *L*-Valine Adduct



In order to make a simple reliable chiral method we set the following criteria to guide our method development efforts:

(1) Simple reagents needed to be employed (no chiral reagents)
(2) Basic instrumentation needed to be employed (HPLC-UV rather than fluorescence detection)
(3) The *D/L*-enantiomers should display baseline resolution
(4) The *D*-enantiomer should be able to be quantitated down to the 0.05% chiral impurity level
(5) The run time for the separation of the *D/L*-enantiomers should be no more than 25 minutes

If the above criteria were met, we were confident that the developed method could be transferred to laboratories around the world.

Results and discussion

A 1.0 mg/mL solution of OPA *D/L*-Valine was prepared and screened with various chiral columns. It was found that the OPA *D/L*-Valine adducts could be separated utilizing a Chiralcel OD-3R column in less than 20 minutes with excellent peak shape for the two major chiral adducts, see Figure 3. Upon inspection of the data, it was clear by the peak ratio that there were some other impurities related to the derivatization reaction which were co-eluting with the later eluting peak corresponding to the OPA *L*-Valine adduct. Since solution stability had not been established, fresh samples were prepared and method optimization was performed. The interfering impurities observed in the original data were separated by increasing the column temperature to 40°C, which provided a peak ratio of 49.9 to 50.1 for the OPA Valine enantiomers, see Figures 3-5. Further method optimization provided baseline resolution of OPA *D*-Valine at 14.8 minutes and OPA *L*-Valine at 16.0 minutes with a total run time of 25 minutes which included column re-equilibration.

Figure 3. Initial separation chromatogram of 1 mg/mL OPA *D/L*-Valine solution

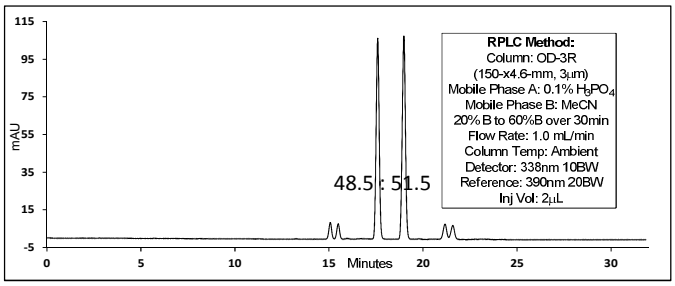


Figure 4. Selective separation of OPA *D/L*-Valine from other impurities produced by the derivatization

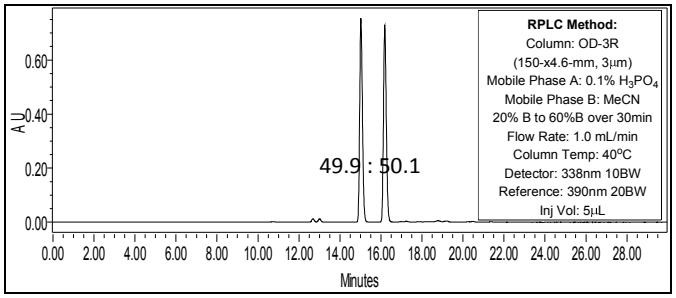
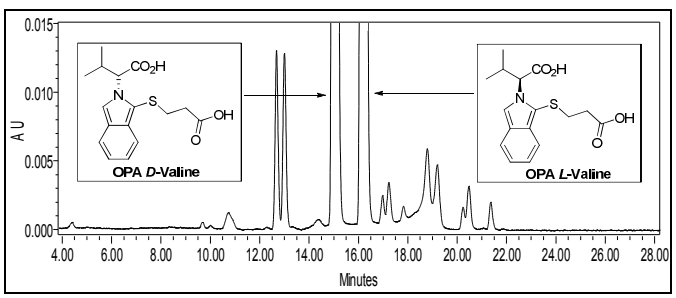


Figure 5. Expanded chromatogram of 1 mg/mL OPA *D/L*-Valine injection



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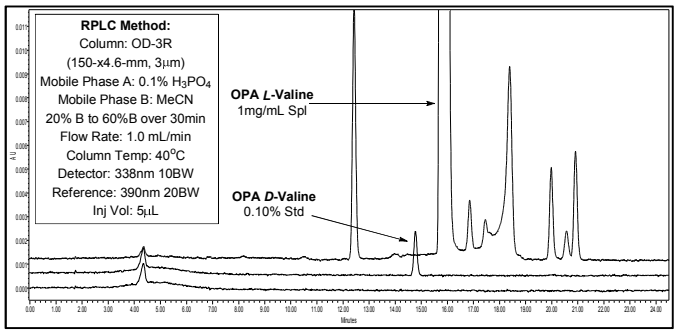
Inspection of the overlay of the derivatization blank, the 0.10% OPA *D*-Valine Standard, and 1.0 mg/mL OPA *L*-Valine sample utilizing the optimized conditions provided further evidence that proper resolution was obtained, see Figure 6. Likewise, the derivatization OPA/thiol strategy should be able to adequately quantitate OPA *D*-Valine within a sample of 1.0 mg/mL OPA *L*-Valine to the desired 0.05% chiral impurity level.

Figure 6. Overlay of a derivatization blank chromatogram, a 0.10% standard chromatogram, and a expanded 1 mg/mL sample chromatogram utilizing optimized conditions



Adequate precision (less than 5.0 % RSD) was achieved for the 0.10% OPA *D*-Valine Standard Injections, see Table 1. Injections of 0.02% and 0.05% OPA *D*-Valine solutions both showed signal to noise values of >10:1, see Tables 2 and 3. Typically, the limit of detection (LOD) and limit of quantitation (LOQ) are defined by signal to noise ratios of 3:1 and 10:1, respectively. However, after taking into account the somewhat elevated baseline seen in the 1 mg/mL OPA L-Valine sample, the 0.02% and 0.05% OPA *D*-Valine solutions were set as LOD and LOQ to allow for a more robust method that is reproducible between different sites and vendors. If needed, a lower level of quantitation of the *D*-Valine within *L*-Valine should be possible utilizing this method.

Table 1. 0.10% OPA *D*-Valine Standard Injection Data

0.10% OPA D-Valine Standard Injection Data			
Name	Area	Ave Area	% RSD
OPA D-Valine	16837.5		
OPA D-Valine	16578.8		
OPA D-Valine	16770.7		
OPA D-Valine	16362.1	16637.3	1.3
OPA D-Valine	16651.3		
OPA D-Valine	16754.4		
OPA D-Valine	15967.1		
OPA D-Valine	16788.3	16588.8	1.8

Table 2. Limit of Detection Data of 0.02% OPA *D*-Valine

0.02% OPA D-Valine Injection Data		
Name	Area	USP_SN
OPA D-Valine	3356.6	14.8
OPA D-Valine	3298.6	11.7
OPA D-Valine	3295.7	15.2
Ave (n=3)	3316.9	13.9
% RSD	1.0	

Table 3. Limit of Quantitation Data of 0.05% OPA D-Valine

0.05% OPA D-Valine Injection Data		
Name	Area	USP_SN
OPA D-Valine	7637.0	55.8
OPA D-Valine	8194.6	67.1
OPA D-Valine	8076.4	30.9
Ave (n=3)	7969.3	51.3
% RSD	3.7	

Linearity assessment of the OPA *D*-Valine adduct was conducted over the concentration range of 0.0005 to 0.005 mg/mL which is equivalent to 0.05% to 0.5% chiral impurity assuming an *L*-Valine sample concentration of 1 mg/mL. The correlation coefficient for OPA *D*-Valine was greater than 0.99 (Found 0.9996), see Table 4. OPA *D*-Valine was indeed linear over the specified range.

Table 4. Linearity of OPA *D*-Valine Data

OPA D-Valine Linearity Data		
Name	Conc mg/mL	Area
0.05% D-Valine	0.00052	8213.3
0.10% D-Valine	0.00105	17062.5
0.20% D-Valine	0.00210	34209.1
0.40% D-Valine	0.00420	64468.6
0.50% D-Valine	0.00525	82226.4
Correlation		0.9996

Spiking experiments were performed to demonstrate accuracy by introducing 0.05%, 0.10%, and 0.20% *D*-Valine to the *L*-Valine sample, followed by derivatization of the mixutre. The recoveries ranged from 96% to 106% over the three impurity levels tested with a % RSD of less than 2.0% for triplicate injections at each level, see Table 5. The accuracy results also indicate that an appropriate amount of derivatization reagent is utilized for the method. If not, the recovery of the spiked *D*-Valine would fail the expected recovery range for an impurity method of 80 to 120%.

Table 5. Recovery of OPA *D*-Valine Spiked into 1.0 mg/mL *L*-Valine Data

Accuracy Data From Spike Recovery Experiments			
Name	% Recovery	Ave % Recovery	% RSD
0.05%-1	95.82		
0.05%-2	97.09	96.9	1.0
0.05%-3	97.79		
0.10%-1	99.52		
0.10%-2	97.40	98.5	1.1
0.10%-3	98.46		
0.20%-1	104.35		
0.20%-2	106.03	104.8	1.1
0.20%-3	103.92		

Solution stability of the OPA *D*-Valine adduct was investigated by comparing the response factor of the 0.10% OPA *D*-Valine injections over approximately 11 hours, with sample storage within an auto-sampler set to 4°C. The absolute difference from T = 0 and T = 11 hours was only 2.0% which indicates stability of the OPA *D*-Valine adduct under these conditions. The 0.10% OPA *D*-Valine

solution was then stored under ambient conditions (normal light and room temperature) for 24 hours and was compared to a freshly prepared 0.10% OPA *D*-Valine solution. The 0.10% OPA *D*-Valine solution was not stable at ambient conditions since the resulting absolute difference of the freshly prepared and aged solutions was greater than 10.0%. Upon storage of the 0.10% OPA *D*-Valine solution in the refrigerator for 24 hours, the absolute % difference was 8.8%. No further solution stability experiments were performed and we recommend that all solutions should be prepared on the day of the analysis.

Table 6. Solution Stability of the 0.10% OPA *D*-Valine Adduct Over 11 Hours.

Solution Stability 0.10% OPA D-Valine Data Over 11 hours			
Name	Rfactor	Ave (n=2)	% Abs Diff
OPA D-Valine T= 0	16042157.8		
OPA D-Valine T= 0	15795654.5	15918906.1	
OPA D-Valine T = 4h	15978532.4		
OPA D-Valine T = 4h	15589144.2	15783838.3	0.8
OPA D-Valine T = 8h	15864733.5		
OPA D-Valine T = 8h	15962989.0	15913861.3	0.0
OPA D-Valine T= 11h	15212845.1		
OPA D-Valine T = 11h	15995256.2	15604050.7	2.0

Table 7. Solution Stability of the 0.10% OPA D-Valine Adduct at different storage conditions after 24 hours.

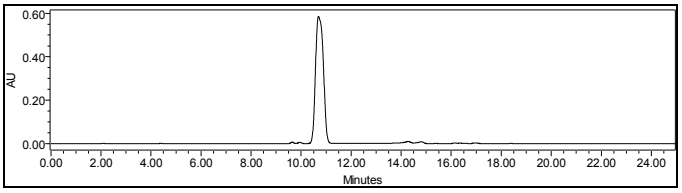
Solution Stability of 0.10% OPA D-Valine Data		
Name	Ave Rfactor	% Abs Diff
24h Ambient Storage	13356435.9	17.0
Fresh OPA D-Valine	16087223.3	
24h Refrigerator Storage	14092944.2	8.8
Fresh OPA D-Valine	15457440.2	

OPA/thiol derivatization of primary amines involves five chemical steps which are dehydration, thio-addition, cyclization followed by dehydration, and finally aromatization to obtain the isoindole adduct. Therefore, it is not suprising that other peaks are observed in the chromatograms seen in Figures 3-6. These other peaks observed could be due to degradation of the isoindole adduct (supported by the solution stability data Table 7) and/or side reactions taking place during the derivatization process (one example being dimerization instead of cyclization). No attempt was made to determine the identity of these other peaks. As illustrated from the recovery experiments (Table 5) incomplete derivatization is not likely otherwise unacceptable recoveries would have resulted (Recoveries outside of the typical 80-120% range).

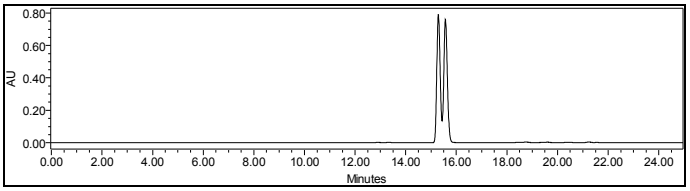
The applicability of the method toward related amino acids (Alanine, Norvaline, Isoleucine, Leucine, and *tert*-Leucine) was explored, see Figure 7. Without any further optimization, the following was observed for the OPA *D/L*-Adducts: no resolution for Alanine; partial resolution for Norvaline, Leucine, and *tert*-Leucine; and baseline resolution for Isoleucine. Increasing the run time and slowing the gradient should provide better separation of the partially resolved OPA *D/L*-Adducts. Another strategy would be to increase the length of the chiral column to obtain better resolution of the partial resolved OPA *D/L*-Adducts. Note that none of the OPA *D/L*-Adducts investigated interfered at the retention time of OPA *D*-Valine (approximately 14.8 minutes) which indicates the method has high specificity for the OPA *D*-Valine adduct.

Figure 7. Chromatograms of related *D/L*-amino acids as their OPA adducts

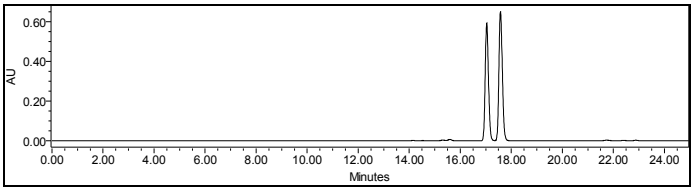
OPA *D/L*-Alanine (1.0mg/mL) no separation of OPA adducts observed.



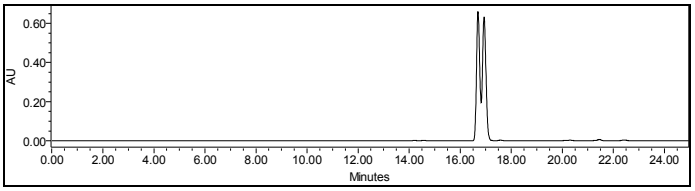
OPA *D/L*-Norvaline partial separation of OPA adducts observed.



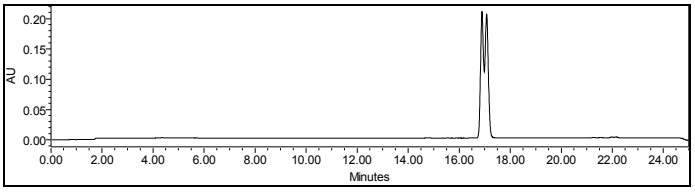
OPA *D/L*-Isoleucine complete separation of OPA adducts observed.



OPA *D/L*-Leucine partial separation of OPA adducts observed.



OPA *D/L-tert*-Leucine partical separation of OPA adducts observed.



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Experimental

Chemicals and reagents

The following chemicals and reagents were utilized to perform the analytical method:
Acetonitrile (**MeCN**) HPLC grade
Phosphoric acid, 85% wt
Water HPLC grade or equivalent
D-Valine, Sigma-Aldrich
L-Valine, Sigma
O-Phthalaldehyde (**OPA**), Acros
3-Mercaptoproprionic acid (**3-MPA**) HPLC grade, Sigma
NaH_2PO_4 monobasic dihydrate, Fischer Scientific
NaOH pellets semiconductor grade (10M NaOH = 40g into 100 mL of water)

Solution preparation

Diluent: 40 mM Na_2HPO_4 pH 7.8
12.5 g of $NaH_2PO_4*2H_20$ in 2L followed by pH adjustment with 10 M NaOH solution
Note: The Na_2HPO_4 solution was made utilizing NaH_2PO_4 at a pH of 7.8 the phosphate buffer is the disodium salt.

Mobile Phases:
Mobile Phase A: 0.1% H_3PO_4 in water (Transferred 2.0 mL of H_3PO_4 to 2-L of water)
Mobile Phase B: MeCN

*Derivatization Reagent (***OPA-R***):* Transfer ~250 mg of O-Phthalaldehyde to a 25-mL vol. flask and dissolve in ~20 mL of MeCN. Add 200 µL of 3-mercaptoproprionic acid to the flask and dilute to volume with MeCN. This solution is a clear/pale yellow homogeneous solution.

Stock D-Valine (1 mg/mL): Transfer ~10 mg of *D*-Valine to a 10-mL vol. flask and add ~5mL of diluent. Sonicate the flask for 5 minutes, then dilute to volume with diluent.

Intermediate Solution (0.01 mg/mL): Transfer 1.0 mL from the stock solution to a 100-mL vol. flask and dilute to volume with diluent.

Derivatization Blank: Transfer 2 mL of OPA-R to a 10-mL vol. flask and add ~5mL of diluent to the flask. Sonicate the flask 5 minutes with intermediate shaking, then dilute to volume with diluent.

0.10% D-Valine (0.001mg/mL): Transfer 1.0 mL of Intermediate Solution to a 10-mL vol. flask, add 2mL of OPA-R and 4 mL of diluent to the flask. Sonicate the flask for 5 minutes with intermediate shaking, then dilute to volume with diluent.

Sample L-Valine (1.0 mg/mL): Transfer ~10 mg of *L*-Valine to a 10-mL vol. flask and add ~5mL of diluent. Sonicate the flask for 5 minutes with intermediate shaking, then add 2 mL of OPA-R and dilute to volume with diluent.

0.10% D-Valine Spike: Transfer ~10 mg of L-Valine to a 10-mL vol. flask and add ~5mL of diluent to dissolve the material. Add 1.0 mL of Intermediate Solution to the flask followed by 2 mL of OPA-R. Sonicate the flask for 5 minutes with intermediate shaking, then dilute to volume with diluent.

Chromatographic Conditions

The chromatographic system was set to the following conditions to perform the analytical method:

Column:	Chiralcel OD-3R, Part# 14824, 150- x 4.6-mm, 3-µm
Temperature:	40 °C
Flow Rate:	1.0 mL/min
Detection:	338nm (Bandwidth:10); Reference:390nm (Bandwidth:20)
Injection Volume:	5 µL
Run Time:	25.0 min
Needle Rinse:	On, MeCN
Blank:	Derivatization Blank

Gradient Profile

Time (min)	A (%)	B (%)
0	80	20
22.0	50	50
22.01	80	20
25.00	80	20

Equipment and software

The HPLC system employed was an Agilent 1100 series HPLC unit. The OD-3R chiral column was purchased from the Daicel Corporation Part Number 14824. The acquisition software utilized was Empower 2 licensed from the Waters Corporation.

Chromatographic Parameters

The chromatographic parameters (k', α, and Rs) of the separation for the final method are in Table 8, where k' is the capacity factor, α is the separation factor, and R_s is resolution. The R_s value between OPA D/L-Valine was provided by the software program Empower 2.

Table 8. Chromatographic Parameters for the Separation of OPA D/L-Valine Adducts.

Chromatographic Parameters of the Final Method			
Compound	k'	α	Rs
D-Valine	7.7	1.08	4.5
L-Valine	8.3		

Conclusions

A RP-HPLC method has been developed for the quantitation of *D*-Valine within *L*-Valine as their corresponding OPA adducts to the 0.05% chiral impurity level. The method provides baseline resolution of the enantiomers with a run time of 25 minutes which includes re-equilibration of the chiral column (Chiralcel® OD-3R). The method provides no resolution of the enantiomers of alanine; partial resolution of the enantiomers of Norvaline, Leucine, and *tert*-Leucine. However, baseline resolution was observed for the OPA enantiomers of Isoleucine. This analytical method fills in the gap of relying on the established optical rotation testing cited by most pharmacopoeias to test *L*-Valine for chiral purity.

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Wes A. Schafer and his team at Merck and Co. (Rahway Site NJ) are acknowledged for the initial column screening efforts. J. R. Denton would like to thank Roy Helmy (Rahway Site NJ) for his recommendations regarding this article.

Notes and references

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