

# Quantitative Determination of Unchanged Hydralazine in Human Whole Blood Using LC/MS/MS

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## INTRODUCTION

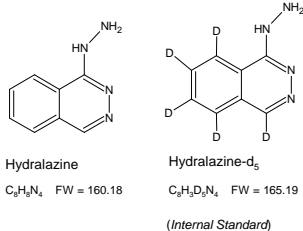
Hydralazine is a potent vasodilator used in the treatment of hypertension. It is a highly reactive phthalazine that rapidly forms hydrazones with endogenous  $\alpha$ -keto acids, such as pyruvic acid. To determine unchanged hydralazine, the analyte must be derivatized to a stable form immediately upon blood sample collection.

Herein we describe the development and validation of a rapid, selective, and sensitive LC/MS/MS assay for the determination of hydralazine in human whole blood. The validation data presented demonstrate that the assay meets the performance requirements needed to support its use for clinical studies.

## OBJECTIVE

To develop and validate a rapid, selective, and sensitive bioanalytical assay for the determination of unchanged hydralazine in human whole blood.

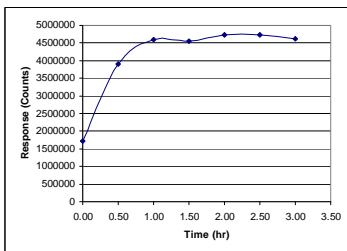
Figure 1. Chemical Structures



## DERIVATIZATION OPTIMIZATION

An experiment was performed to evaluate the optimal length of time needed for derivatization. The 2,4-pentanedione derivatization agent was added to whole blood, which was then fortified with hydralazine at a concentration of 122 ng/mL, and allowed to mix continuously at room temperature. Three aliquots were removed at 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 hr time points and extracted. The 0 hr time point was not truly 0 hr due to the unavoidable amount of time (several minutes) that passed prior to extraction. The mean response of the hydralazine derivative was plotted versus the length of derivatization time. As shown in the plot, the reaction is near completion after 1 hour. Therefore a 2-hour derivatization was chosen for validation.

Figure 2. Derivatization Time Profile



## METHOD SUMMARY

### Validation Samples

Matrix blanks, calibration standards, and quality controls were prepared in human whole blood, containing dipotassium EDTA and 2,4-pentanedione. Calibrators were made at eight concentration levels spanning the quantitation range of 0.163 to 163 ng/mL. Quality control (QC) pools were prepared at the lower limit of quantitation and five additional concentrations spanning the quantitation range for the evaluation of assay performance, stability, and recovery.

### Extraction Method

Previously derivatized, matrix aliquots (100  $\mu$ L) were fortified with 1.00 mL of 1.0 ng/mL derivatized working internal standard solution. Analytes were isolated through liquid-liquid extraction with 3.0 mL of methyl t-butyl ether. The organic layer was transferred and evaporated, and samples were reconstituted with 500  $\mu$ L of acetonitrile.

### Chromatographic and

### Mass Spectrometric Conditions

HPLC separation was achieved using column switching with two Thermo Betasil Silica-100 columns (precolumn: 10 mm x 2.1 mm; analytical: 100 mm x 3.0 mm) with a mobile phase of 0.8 mM ammonium formate and 0.005% formic acid in 80:15:5 acetonitrile / methanol / water, v/v/v and a flow rate of 0.40 mL/min. The precolumn was back-flushed with 0.8 mM ammonium formate and 0.005% formic acid in 50:15:35 acetonitrile / methanol / water, v/v/v to remove phospholipids.

Analytes were detected on a Sciex API4000 in MRM mode using positive ion TurbolonSpray. Retention times for hydralazine and its internal standard were approximately 1.95 min and the total analysis time was approximately 5 min.

## RESULTS

Calibration curves were constructed using the chromatographic peak area ratios of the analyte and internal standard from the calibration samples by applying a quadratic, 1/concentration squared (1/c<sup>2</sup>) weighted regression algorithm. Analyte concentrations in unknown samples were then calculated from their peak area ratios versus the calibration curve.

The assay was validated for hydralazine over a concentration range of 0.163 to 163 ng/mL. Linearity over the calibration range was demonstrated by an average correlation coefficient from three validation runs of 0.9992.

Evaluation of unstabilized hydralazine in fresh whole blood held at room temperature showed a loss of approximately 26% within 15 minutes of fortification. In contrast, the hydralazine derivative showed good stability in matrix, under three-cycle freeze/thaw and 24-hour room temperature thawed conditions. Post preparative extract stability was also established for 72-hour storage at 4  $^{\circ}$ C.

Table 1. Intra-Assay Precision and Accuracy

Sample	Concentration (ng/mL)	%CV	%Bias	n
IA LOQ	0.163	3.11	-2.17	6
IA 1	0.488	2.73	-0.630	6
IA 2	1.22	1.62	-0.895	6
IA 3	4.90	2.21	-2.18	6
IA 4	20.4	1.54	-1.41	6
IA 5	122	3.76	0.320	6

Table 2. Inter-Assay Precision and Accuracy

Sample	Concentration (ng/mL)	%CV	%Bias	n
LLOQ	0.163	6.77	2.21	18
QC 1	0.488	7.13	2.81	18
QC 2	1.22	6.00	0.697	18
QC 3	4.90	5.90	1.10	18
QC 4	20.4	6.53	1.66	18
QC 5	122	5.48	2.25	18

Table 3. Stability Summary

Stability Conditions	Minimum Stability
Freeze/Thaw Stability	3 Freeze/Thaw Cycles
Extract Stability	72 hr at Approximately 4 $^{\circ}$ C
Thawed Matrix Stability	24 hr at Room Temperature

Figure 3. Derivatization Reaction

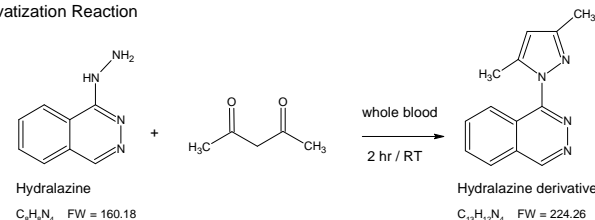


Figure 4. Plasma Blank with Internal Standard

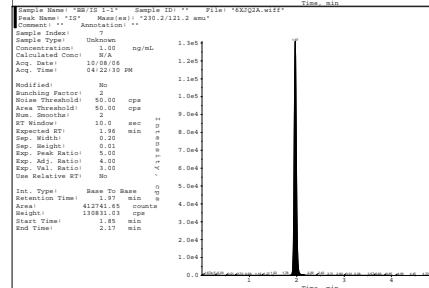
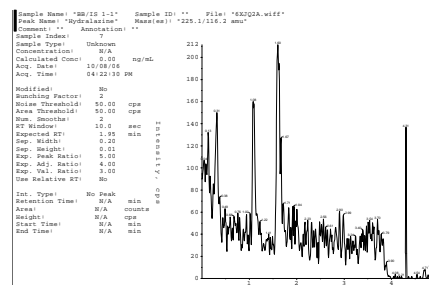
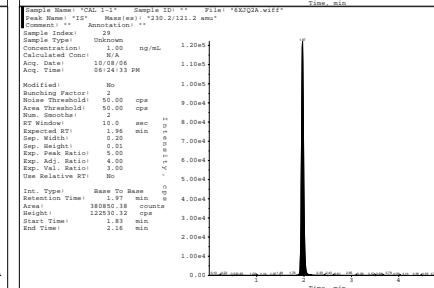
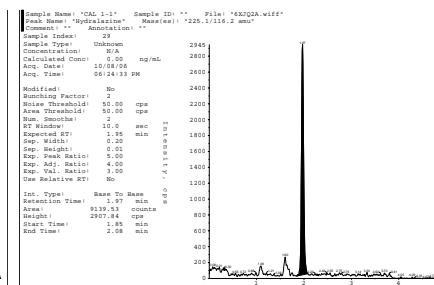


Figure 5. Lower Limit of Quantitation, 0.163 ng/mL



## CONCLUSIONS

An LC/MS/MS assay has been successfully developed and validated for the determination of hydralazine in human whole blood containing dipotassium EDTA. Results of the validation demonstrated the efficacy of applying derivatization immediately upon sample collection to stabilize a highly unstable analyte. The assay is suitable for the analysis of clinical study samples as demonstrated by its specificity, precision, accuracy, recovery, and stability characteristics.

## ACKNOWLEDGMENTS

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