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Selective Derivatization of Penicillamine Using 5,5′-Dithiobis(2-Nitrobenzoic Acid): Improved Matrix Stability and LC-MS/MS Sensitivity

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Overview

Purpose

- To present a highly selective thiol derivatization reaction for penicillamine, a compound which undergoes rapid oxidation in plasma.

Method

- Multiple derivatizing reagents were screened, including 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB). Derivatization recovery, selectivity, sensitivity and peak shape were evaluated for each reagent.

Results

- Derivatization of the penicillamine thiol functional group with DTNB was highly selective and sensitive.
- DTNB-derivatized penicillamine was stable in plasma for three freeze-thaw cycles with short-term and long-term stabilities of 24 hours (4 °C) and 8 days (-80 °C), respectively; post-reconstituted extracts demonstrated 71 hours of stability (4 °C).
- Precision and accuracy data for three batches over an analytical range of 100 – 8,000 ng/mL met acceptance criteria without matrix effect or specificity issues for eight different plasma donors, including lipemic and hemolyzed matrix.

Introduction

Compounds containing thiol functional groups are recognized as unstable due to rapid auto-oxidation or ion-catalyzed-oxidation in biological matrices. It is therefore often necessary to derivatize the thiol functionality to improve matrix stability. However, some compounds, such as penicillamine, also have competing functional groups which can cross-react with the more commonly used derivatizing agents for thiol, resulting in multiple reaction products.

Attempts to develop a high-throughput and thiol-selective derivatization procedure eventually led to the testing of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). DTNB, also known as Ellman's reagent, is a low-cost readily available compound used to derivatize thiols via cleavage and liberation of a single TNB moiety with subsequent disulphide bond formation with reactant species (Figure 1).

Derivatization of penicillamine with DTNB not only circumvented cross-reactivity with other functional groups, but also conferred improved plasma stability and LC-MS/MS sensitivity under ESI conditions. The research reported herein examines the characteristics of penicillamine derivatization with DTNB and its applicability for regulated quantitative bioanalysis by LC-MS/MS.

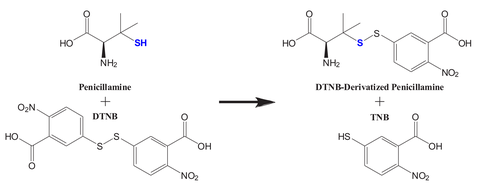

Figure 1. Derivatization reaction of penicillamine with DTNB.

Methods

Sample Extraction

An aliquot of 3.6 mL of DTNB (0.64%, w/v in alkaline buffer) was added to 300 µL of preservative-treated plasma containing penicillamine, mixed and incubated for a minimum of 15 minutes at 4 °C. Samples were then fortified with internal standard and buffer followed by protein precipitation with perchloric acid; the supernatant was diluted 1:1 with acetonitrile.

Chromatography

Penicillamine and internal standard were chromatographed on a C_{18} column (30 x 2.1 mm, 3.5 µm) using mobile phases composed of aqueous heptafluorobutyric acid (HFBA) and ACN at a flow rate of 700 µL/min.

Extended Chromatography

A 50 x 2.1mm, 1.7 µm C18 column was used with mobile phases composed of aqueous heptafluorobutyric acid and ACN at a flow rate of 700 µL/min. A Nexera X2 UPLC system from Shimadzu was used to generate a low slope gradient of 5% to 20% organic over 8 minutes.

DTNB-Derivatized Penicillamine Detection

Derivatized penicillamine and D3-internal standard were monitored using a SCIEX API 5500 operated in positive ESI-MRM mode for the transitions *m/z* 347 > *m/z* 268 and *m/z* 350 > *m/z* 271.

Results and Discussion

Underivatized Penicillamine

The mass spectrometric properties of underivatized penicillamine were characterized by poor MS/MS efficiency, with the most sensitive fragments either containing an interference or resulting in high chemical background noise, thereby compromising S/N ratio. In fact, a S/N of only 165:1 was obtained for un-derivatized penicillamine at high QC concentration (6000 ng/mL) when monitoring the MRM transition *m/z* 150 > *m/z* 55, translating to insufficient sensitivity for the targeted LOQ of 100 ng/mL. Penicillamine was also extremely unstable in biological matrices, such as whole blood and plasma. Although anticoagulant and blood preservatives extended penicillamine stability, degradation was still observed in harvested plasma (Table 1).

Table 1. Kinetics of un-derivatized penicillamine in K_2EDTA human plasma.

Kinetic Time Point (min)	Low QC (300 ng/mL)				High QC (6000 ng/mL)			
	Ratio	Average	%CV	% Deviation	Ratio	Average	%CV	% Deviation
0	0.1509 0.1623 0.1589	0.1574	3.7	N/AP	3.0747 2.9379 2.8905	2.9677	3.2	N/AP
5	0.1739 0.1497 0.1525	0.1587	8.4	0.8	2.8815 2.8996 2.8873	2.8895	0.3	-2.6
15	0.1547 0.1499 0.1489	0.1512	2.1	-4.0	2.9552 2.7924 2.8303	2.8593	3.0	-3.7
30	0.1589 0.1539 0.1507	0.1545	2.7	-1.8	2.7913 2.7995 2.8987	2.8298	2.1	-4.6
60	0.1491 0.1432 0.1454	0.1459	2.0	-7.3	2.7287 2.7288 2.7319	2.7298	0.1	-8.0
120	0.1236 0.1401 0.1226	0.1288	7.6	-18.2	2.4424 2.3885 2.3698	2.4002	1.6	-19.1
240	0.0888 0.0921 0.0927	0.0912	2.3	-42.0	1.8210 1.8613 1.9036	1.8619	2.2	-37.3

Results and Discussion (Continued)

Derivatization Tests

Multiple derivatization reactions were evaluated in an attempt to improve LC-MS/MS sensitivity and matrix stability. Screened derivatizing reagents such as 2-bromo-3′-methoxyacetophenone, N-ethylmaleimide or pentafluorobenzyl bromide all resulted in multiple products and poorly controlled reactions due to cross-reactivity with other penicillamine functional groups.

Derivatization with DTNB resulted in a single chromatographic peak with high sensitivity (S/N ≥ 10:1 at LOQ coinciding to 6.3 pg on-column). A Q1 scan (Figure 2) confirmed the presence of a derivatized compound with *m/z* 347, while a product ion scan confirmed selective derivatization of the thiol with no evidence of derivatized amine (Figure 3). The derivatization yield, evaluated using a DTNB-penicillamine reference standard, was > 95% after only 15 minutes incubation time at 4 °C, suggesting a controlled reaction with little or no cross-reactivity. Further, a product ion scan of DTNB-penicillamine reference standard demonstrated spectral similarity with that of penicillamine derivatized *in-situ* (Figure 3), confirming selective formation of S-derivatized compound.

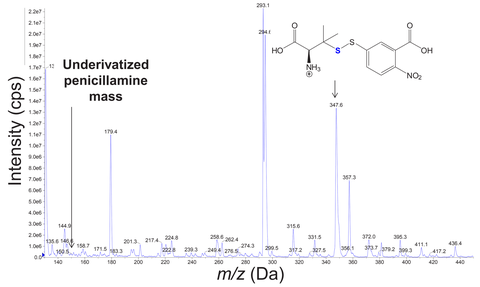

Figure 2. Q1 scan of DTNB-derivatized penicillamine (*m/z* 347) demonstrating the absence of un-derivatized penicillamine (*m/z* 150).

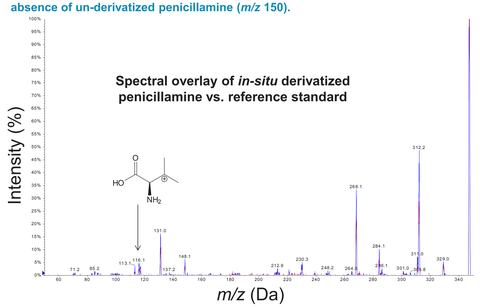

Figure 3. Product ion scan comparison for penicillamine derivatized with DTNB in-situ (blue) vs. DTNB-penicillamine reference standard (red) at 20 eV collision energy.

Results and Discussion (Continued)

Extended Chromatography

To determine if other derivatization by-products co-eluted with DTNB-penicillamine, an extended gradient was applied. Only one peak was obtained (Figure 4), suggesting that penicillamine was the predominant reaction product.

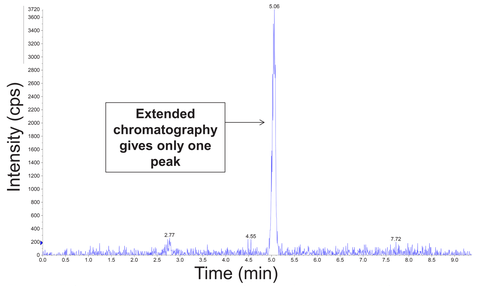

Figure 4. Extended chromatography conditions; only one peak is obtained.

DTNB-Derivatized Penicillamine Method Stressing

The derivatization of penicillamine with DTNB was implemented in a human plasma assay supporting an analytical range of 100 ng/mL – 8,000 ng/mL. Precision and accuracy data for three batches (Table 2) met acceptance criteria (% CV and % bias ±15.0) without matrix effect (Table 3) or specificity issues observed for 8 different plasma lots, including lipemic and hemolyzed matrix.

Table 2. Inter-batch precision and accuracy.

Batch Number	Concentration (ng/mL)			
	LOQ QC 100.00	Low QC 300.00	Mid QC 1500.00	High QC 6000.00
1	98.17	281.97	1542.70	6375.50
	98.48	296.90	1519.41	5827.45
	90.84	296.17	1573.51	6291.72
	94.33	299.25	1493.17	5890.00
	98.47	308.35	1525.22	5903.59
	95.62	316.04	1465.60	6146.40
2	100.01	278.82	1384.63	5502.76
	104.25	287.23	1449.81	5261.70
	103.05	318.62	1428.32	5405.20
	101.50	297.39	1448.30	5876.06
	110.41	292.73	1433.01	5989.45
	106.50	301.60	1470.98	6349.58
3	100.13	287.66	1482.18	5886.94
	93.43	291.64	1499.21	6017.62
	95.62	296.16	1520.35	5972.09
	94.04	290.33	1498.66	5837.40
	87.22	299.09	1481.77	6048.29
	101.15	297.19	1447.34	5959.52
Mean	98.51	296.51	1481.34	5918.96
% C.V.	5.8	3.5	3.1	5.0
% Nominal	98.5	98.8	98.8	98.6

Results and Discussion (Continued)

Table 3. Matrix effect evaluation with QCs spiked in 8 lots.

Matrix Lot	Low QC (300.00 ng/mL)				High QC (6000.00 ng/mL)			
	Peak Area Drug	Peak Area IS	Ratio	% Dev.	Peak Area Drug	Peak Area IS	Ratio	% Dev.
1	10913	234449	0.0465	-1.1	212483	236087	0.9000	-2.6
	10420	233494	0.0446	-5.3	209826	228618	0.9178	-0.7
	10653	225102	0.0473	0.5	216910	237237	0.9143	-1.1
2	10714	233592	0.0459	-2.6	214289	242861	0.8823	-4.5
	10715	236122	0.0454	-3.7	213439	233154	0.9154	-0.9
	10679	231316	0.0462	-2.0	212087	237237	0.8940	-3.3
3	11123	233201	0.0477	1.3	204809	229656	0.8918	-3.5
	10577	236869	0.0447	-5.2	214698	234330	0.9162	-0.9
	10672	236296	0.0452	-4.1	208685	238298	0.8757	-5.2
4	11013	236651	0.0465	-1.2	214516	238602	0.8991	-2.7
	11026	239686	0.0460	-2.3	215589	235415	0.9158	-0.9
	10579	233822	0.0452	-4.0	215691	234531	0.9197	-0.5
5	10562	230726	0.0458	-2.8	216800	239703	0.9045	-2.1
	10705	235666	0.0454	-3.6	215193	236540	0.9098	-1.6
	10515	232600	0.0452	-4.0	210360	232215	0.9059	-2.0
6	10360	231227	0.0448	-4.9	214216	237860	0.9006	-2.5
	10657	233178	0.0457	-3.0	210116	240882	0.8723	-5.6
	11216	233977	0.0479	1.9	217218	232088	0.9359	1.3
Lipemic	10796	232110	0.0465	-1.2	214560	227875	0.9416	1.9
	10528	233813	0.0450	-4.4	216816	236200	0.9179	-0.7
	10902	240393	0.0453	-3.7	214579	237452	0.9037	-2.2
Hemolyzed	10413	221409	0.0470	-0.1	210104	225406	0.9321	0.9
	10205	214789	0.0475	1.0	204762	228727	0.8952	-3.1
	10312	223325	0.0462	-1.9	201723	221856	0.9093	-1.6
Mean	10677	232242	0.0460		212478	234285	0.9071	
% C.V.	2.4	2.5	2.1		2.0	2.2	1.9	

DTNB-Derivatized Penicillamine Stabilities

DTNB-derivatized penicillamine was stable in plasma for three freeze-thaw cycles with short and long-term stabilities of 24 hours (4 °C) and 8 days (-80 °C), respectively; post-reconstituted extracts demonstrated 71 hours of stability (Table 4).

Table 4. DTNB-derivatized penicillamine stabilities.

Stability Evaluation	Low QC		High QC	
	Concentration (ng/mL)	Dev. %	Concentration (ng/mL)	Dev. %
Freeze-Thaw (3 cycles)	341.68	13.9	6424.53	7.1
	320.46	6.8	6180.69	3.0
	309.64	3.2	6113.55	1.9
Short-Term (23.7 hours at 4°C)	321.26	7.1	6524.90	8.7
	320.50	6.8	6104.24	1.7
	285.68	-4.8	6078.07	1.3
Long-Term (8 days at -80°C)	324.00	8.0	5962.80	-0.6
	305.62	1.9	6319.43	5.3
	325.68	8.6	6037.87	0.6
Autosampler (70.9 hours at 4°C)	300.84	-1.0	6470.71	1.2
	314.38	-11.5	6344.47	-2.2
	296.26	1.9	6336.59	-2.0

Conclusion

In designing a highly selective thiol derivatization reaction for penicillamine, sensitivity and stability were markedly improved, leading to a validatable, high-throughput and robust LC-MS/MS quantitation method.

Acknowledgements

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