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Process clearance of dithiothreitol monitored by reversed-phase high-performance liquid chromatography with fluorescence detection Assay development and validation

Donald O. O'Keefe

Department of Animal Drug Evaluation, Merck Research Laboratories, P.O. Box 2000, R80V, Rahway, NJ 07065, USA

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Abstract

A sensitive method to measure residual dithiothreitol (DTT) in downstream process intermediates of a biopharmaceutical was developed. Initial sample treatment with sodium borohydride ensured that all DTT species were present in the reduced form. The reduced DTT was then labeled with the thiol-specific fluorescent probe monobromobimane (mBBr). mBBr-DTT was resolved from other labeled products by reversed-phase HPLC with fluorescence detection and quantitated against a reference standard. Validation of the method demonstrated it was quantitative from 5 to 500 pmol DTT. The method's detection limit of 0.5 pmol was three orders of magnitude lower than a previously reported method that relied on UV absorbance detection. Intra- and inter-assay precision were $<10\%$ while intra- and inter-assay accuracy ranged from 85% to 115%. The method documented the removal of DTT to a level of less than 1.5 pg/μl from downstream process intermediates of a biopharmaceutical. © 1997 Elsevier Science B.V.

Keywords: Derivatization, LC; Validation; Pharmaceutical analysis; Dithiothreitol; Dihydroxydithiane; Monobromobimane

1. Introduction

Recombinant DNA technology has catalyzed significant advances in protein research and its applications. Proteins that were heretofore unavailable, or only available in limiting amounts, can now be obtained in large enough quantities to permit extensive studies or to exploit therapeutically. But, in order to take full advantage of the these benefits, the protein often needs to be purified from its recombinant source. Protein purification is not always straightforward. The initial step of cell breakage can liberate unwanted proteolytic enzymes that can degrade the recombinant protein if appropriate inhibitors are not added. The presence of certain metals can also promote protein degradation and therefore make the addition of specific chelators essential. Also, recombinant proteins from bacterial sources can be present in inclusion bodies that require chaotropic agents and often reducing agents for solubilization. All these process additives need to be removed, or reduced to very low levels, from the final product to diminish their effect in subsequent studies. More importantly, however, if the recombinant protein has therapeutic potential, regulatory authorities can dictate monitoring downstream intermediates for the removal of process additives before final drug approval. Therefore, valid assays are needed to measure residual amounts of additives in process samples.

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Dithiothreitol (DTT) is a potent disulfide reducing agent that, along with chaotropic agents, is routinely used to solubilize recombinant proteins from inclusion bodies early in a purification process [1]. Reduction of disulfide bonds by DTT produces thiols in the target molecule and *trans*-4,5-dihydroxy-1,2-dithiane, the oxidized form of DTT [2]. In solution, the oxidation of DTT is not only dependent on the redox potential of other thiols and oxidants, but it is also dependent on time, pH, temperature and concentration [3,4]. These factors increase the possibility that *trans*-4,5-dihydroxy-1,2-dithiane is present in solutions containing DTT. Therefore, any chromatographic assay that quantitates residual levels of the reducing agent must either measure both DTT and *trans*-4,5-dihydroxy-1,2-dithiane or incorporate a procedure for converting one form to the other and then measuring the latter.

Current chromatographic methods for measuring DTT and oxidized DTT do so separately. For DTT and its oxidized counterpart, methods utilizing HPLC with UV absorbance detection have detection limits of 960 pmol and 273 pmol, respectively [3]. When monitoring purification processes for the clearance of additives it is often desirable to have assays that achieve low pg/μl levels of detection. In this respect, the high pmol detection limits for the two species of DTT obtained by UV absorbance can be insufficient for monitoring process clearance of the reducing agent. Greater clearance might be demonstrated if fluorescence detection was employed because this detection method is often more sensitive than that of UV absorbance.

Monobromobimane (mBBr) is a thiol-specific fluorescent probe that is detectable at low pmol levels [5]. Small thiols, such as DTT, are readily labeled with mBBr and easily resolved by HPLC [6,7]. Furthermore, the fluorescent adducts produced after mBBr alkylation are stable to light [5]. All these properties make mBBr an attractive agent for pre-column derivatization and low level detection of DTT. A fluorescence-based HPLC method with a low limit of detection was developed for monitoring process clearance of DTT. The method first used sodium borohydride to convert oxidized DTT to the reduced species and then the reduced species was labeled with mBBr prior to analysis by reversed-phase HPLC. The method was validated and used to demonstrate the clearance of DTT to less than 1.5 pg/μl for one biopharmaceutical process.

2. Experimental

2.1. Materials

HPLC-grade acetonitrile, HPLC-grade KH_2PO_4 and disodium ethylenediamine tetraacetate (EDTA) were acquired from Fisher. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Boehringer Mannheim. DTT was acquired from Pierce. mBBr was purchased from Molecular Probes (Eugene, OR, USA). The remaining reagents were purchased from Sigma. All solutions were made in Milli-Q water (Millipore). DTT, cysteine and $NaBH_4$ solutions were made fresh prior to use. All samples in this study were obtained from product streams derived from the downstream processing of a biopharmaceutical.

2.2. Preparation of the reference standard

A 1.2 m*M* DTT solution was prepared in 235 m*M* Tris–HCl, pH 8.0, containing 3.5 m*M* EDTA. mBBr was added to a final concentration of 5.6 m*M* from a 100 m*M* stock solution in acetonitrile. The mixture was incubated for 2 min away from direct light at room temperature before the reaction was terminated by the addition of cysteine to 20 m*M*. The reference standard was prepared fresh before each use and diluted in phosphate–buffered saline containing 0.1% (w/v) sodium dodecyl sulfate before injection onto the HPLC column.

2.3. Pre-column derivatization

A 80-μl volume of 0.5 *M* $NaBH_4$ was mixed with an equal volume of the sample to be tested. After 10 min, 45 μl of the mBBr labeling solution was added and after gentle mixing the sample was incubated for 2 min away from direct light. The mBBr labeling solution consisted of 1 part 100 m*M* mBBr in acetonitrile, 5 parts 2.0 *M* Tris–HCl, pH 8.0 and 5 parts 30 m*M* EDTA, pH 8.0. Sample derivatization was stopped by adding one-tenth volume of cysteine to a final concentration of 10 m*M*. $NaBH_4$ remaining

in the sample was destroyed by adding HCl to a final concentration of 0.15 *M* and mixing the sample vigorously. The final pH of the sample was adjusted to approximately 4.7 by the addition of 7.5 μl of 2.0 *M* NaOH.

2.4. Chromatography

mBBr-labeled samples (150-μl injections) were chromatographed on a VYDAC C_{18} column (15× 0.46 cm I.D., 5 μm particle size, 300 Å pore size) using a biphasic linear gradient at 1.0 ml/min. The gradient was 88–75% solvent A for the first 10 min followed by 75–65% solvent A for the remaining 16.4 min. Solvent A was 20 m*M* KH_2PO_4, pH 4.5 and solvent B was 20 m*M* KH_2PO_4, pH 4.5–acetonitrile (25:75, v/v). The chromatography system consisted of a Waters Model 712 autosampler and two Waters Model M-6000 HPLC pumps controlled by a Waters Model 680 Automated Gradient Controller. A Spectro Vision FD-200 fluorescence detector was in-line to monitor the column effluent. Fluorescence emitted at greater than 418 nm was detected after excitation at 382 nm. The chromatograph was integrated through a PE Nelson 900 Series Interface with a MicroVAX 3100-40 computer running the Access*Chrom GC–LC Data System from Perkin-Elmer Nelson Systems. The resulting peak heights were used in all subsequent analyses.

2.5. Method validation

The reports of Buick et al. [8] and Karnes et al. [9] were source documents for the validation procedures described here.

3. Results and discussion

3.1. Method development

DTT reacts rapidly and completely with small millimolar excesses of mBBr [6]. Under the conditions described here, greater than 95% of the DTT was labeled at both thiols. The dilabeled species had a retention time of approximately 20.3 min (Fig. 1A) while the monolabeled species had a retention time of 8.1 min. The oxidized form of DTT was refractory

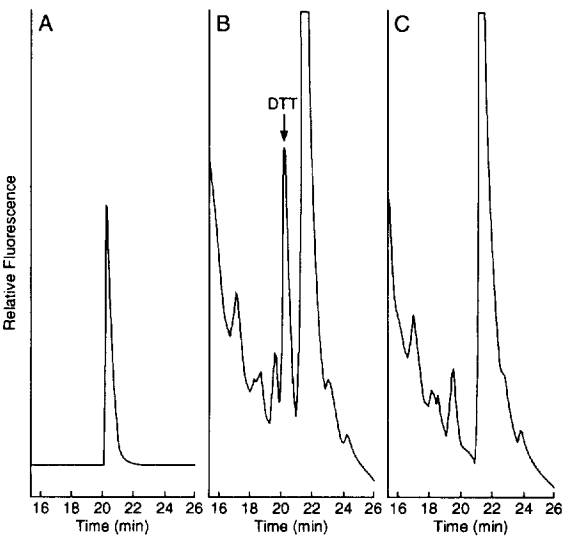

Fig. 1. Chromatograms of mBBr-DTT and pre-column derivatized samples of downstream intermediates. (A) 100 pmol of DTT; (B) a derivatized downstream intermediate spiked with 100 pmol of DTT; (C) the same derivatized downstream intermediate without a spike of DTT.

to mBBr labeling due to the absence of thiols (data not shown). Oxidized DTT was made amenable to mBBr labeling after reduction with $NaBH_4$. The kinetics of $NaBH_4$ reduction were fast. Greater than 99% of *trans*-4,5-dihydroxy-1,2-dithiane (0.5 m*M*) was converted to DTT within 5 min (data not shown). Extended treatment with $NaBH_4$ for up to 1 h did not alter DTT labeling by mBBr. Further experiments demonstrated that 0.25 *M* $NaBH_4$ was sufficient to reduce all the oxidized DTT under the conditions described here (data not shown).

3.2. Method validation

3.2.1. Selectivity

A valid assay for monitoring the removal of DTT from downstream process intermediates must distinguish between the reducing agent and all other endogenous compounds [8]. The selectivity of the method for this downstream process is demonstrated in Fig. 1 which shows representative chromatograms of mBBr-DTT, a derivatized downstream intermediate spiked with DTT and the same derivatized downstream intermediate alone. In greater than 98% of all the downstream intermediates analyzed, the

DTT peak was distinguishable from peaks representing endogenous compounds in the sample.

3.2.2. Linearity

Two criteria were used to determine the linearity of the method. First, the logarithm of the fluorescence response had to be directly proportional to the logarithm of the amount of DTT. Ideally, the slope of this line should be 1.0 [10]. Second, the correlation coefficient of the corresponding curve determined by least squares analysis had to be ≥0.99. In multiple analyses, DTT in the range of 1–800 pmol showed the desired proportionality with an average correlation coefficient equal to 0.992 The average slope of 0.98 was within the acceptable range [10]. Hence, the method was linear from 1 to 800 pmol DTT.

3.2.3. Limit of detection

The average peak height from nine injections of 0.5 pmol DTT was six-fold greater than the average noise from nine blank injections and also greater than seven times the standard deviation of the blank injections. The blank consisted of all the derivatization reagents excluding DTT. Hence, 0.5 pmol DTT was the method's limit of detection according to established criteria [10,11]. This detection limit is 1900-fold less than that of a previously reported method for detecting DTT that relied solely on UV absorbance [3].

3.2.4. Quantitative range

The interval over which the method has acceptable inter-assay accuracy and precision (see Sections 3.2.5 and 3.2.6) is defined as the quantitative range. Acceptable criteria depend on the intended use of the method and can be arbitrary. For the purposes of this method, the upper and lower limits of the method's quantitative range were defined as having inter-assay precision of ≤15% and inter-assay accuracy between 85–115%. According to the data in Table 1, the quantitative range of the method spanned two orders of magnitude from 5 to 500 pmol DTT. This one hundred-fold range minimizes the preparation of numerous sample dilutions prior to analysis. The lower limit of 5 pmol DTT also defined the method's limit of quantitation.

Table 1
Intra-assay and inter-assay accuracy and precision

Theoretical quantity (pmol DTT)	Experimental[a] quantity (pmol DTT)	Precision[b] (%)	Accuracy[c] (%)
Intra-assay (n=6)			
2	1.4±0.3	21.4	70.0
5	4.9±0.1	2.0	98.0
10	9.5±0.1	1.0	95.0
20	19.8±0.3	1.5	99.0
100	109.7±2.0	1.8	109.7
400	393.0±1.9	0.5	98.2
500	462.0±10.2	2.2	92.4
Inter-assay (n=3)			
2	2.0±0.5	25.0	100.0
5	4.9±0.1	2.0	98.0
10	9.1±0.6	6.6	91.0
20	21.1±1.1	5.2	105.5
100	103.2±6.2	6.0	103.2
400	369.6±27.6	7.5	92.4
500	429.1±39.2	9.1	85.8

[a] The experimental quantities of DTT were interpolated from standard curves and are expressed as the mean±the standard deviation.
[b] Precision is defined as (standard deviation/mean DTT quantity)·100%.
[c] Accuracy is defined as (determined DTT quantity/actual DTT quantity)·100%.

3.2.5. Assay precision

The intra-assay precision, i.e., the variability of replicate determinations within an assay, was calculated from six replicate injections of the reference standard. The amount of DTT corresponding to each of the resulting peak heights was interpolated from a standard curve constructed on the same day of the assay. As shown in Table 1, intra-assay precision was generally less than 3% throughout the method's quantitative range. Inter-assay precision was determined by repeating the previous analysis on three separate days. The mean quantities of DTT established on each day were then used to calculate the inter-assay precision which was less than 10% within the method's quantitative range (Table 1).

3.2.6. Assay accuracy

The method's accuracy is the difference between the determined amount of DTT in a sample and the actual amount in that sample. Both intra-assay accuracy and inter-assay accuracy were determined by the same procedures used for precision. Table 1 demonstrates that intra-assay accuracy was between 92 and 110% throughout the method's quantitative range while inter-assay accuracy was between 85 and 106% over the same range.

3.2.7. Recovery

Three different amounts of either DTT or oxidized DTT were spiked individually into identical samples of a downstream intermediate prior to undergoing pre-column derivatization. The peak height from the fluorescently-labeled DTT in these samples was compared to that of identical amounts of a labeled reference standard. Duplicate samples were prepared and analyzed for each. The results in Table 2 demonstrate that both forms of DTT were recovered in sufficient quantities from the downstream intermediate throughout the quantitative range and that recovery was not concentration dependent. The reduced recovery of DTT at 100 and 500 pmol is acceptable based on the accuracy of the assay reported for these levels (Table 1). The difference in the recovery of DTT versus oxidized DTT at these low levels might be explained by differences in the adhesion of the standards to either the reaction vessel or to the pipetter in the absence of a process sample. For example, higher adhesion by oxidized DTT in the standard would decrease the amount available for reduction and labeling compared to the oxidized DTT spiked into the process intermediate. Such a condition would lead to an apparent recovery in excess of 100%.

Table 2
Recovery of DTT from process intermediates of a biopharmaceutical[a]

DTT (pmol)	Recovery (%)	
	DTT[b]	Oxidized DTT[b]
20	104.7	84.8
100	80.7	130.8
500	78.8	138.3
Mean	88.1	118.0

[a] Percent recovery=(peak height of spiked DTT/peak height of standard DTT)·100%.
[b] The value of each replicate differed from the average by <3%.

3.2.8. Ruggedness

The ruggedness of a method establishes its performance under various operating conditions. During the course of a method's implementation, factors influencing its performance might include operation by different analysts, hardware changes, protocol changes and time between analyses. The method's ruggedness was assessed by determining the linearity of the method under different conditions. The two most probable conditions that would change over the course of several analyses are the HPLC column and the shape of the gradient. The former parameter changes due to the limited life of chromatography columns while the latter parameter changes when new endogenous compounds are found in process samples that interfere with the analyte peak and therefore alter the assay's selectivity. The method's linearity was unchanged when the gradient was modified such that the mBBr-DTT peak eluted approximately 1–2 min earlier or later than that found with the original gradient. Identical results were also observed when a Nova-Pak C_{18} column (15×0.39 cm I.D., 4 μm particle size, 60 Å pore size, Waters, Milford, MA, USA) was used with the gradient described in Section 2.4. The method's linearity was also unaltered when several analyses were performed months apart. Therefore, under these different conditions the method proved rugged.

Table 3
Process clearance of DTT from the product stream of a biopharmaceutical

Process step	DTT detected[a] (pmol)	DTT level[b] (pg/μl)	Clearance[c]
A	89.9	$9.0 \cdot 10^5$	1.0
B	12.8	39.8	$2.3 \cdot 10^4$
C	<1.0	<3.1	$>2.9 \cdot 10^5$
D	<1.0	<3.1	$>2.9 \cdot 10^5$
E	<0.5	<1.5	$>5.8 \cdot 10^5$

[a] Refers to the amount of DTT detected in the chromatogram of the sample and does not reflect any sample dilutions.
[b] Refers to the level of DTT calculated to be in the sample after adjustments were made for sample dilutions.
[c] Clearance is equal to the quotient of the DTT concentration (pg/μl) contained in the sample from step A divided by the concentration of DTT contained in the in-process sample. Adjustments were made for sample dilutions.

3.3. Process clearance

The validated method was used to monitor the clearance of DTT from several downstream intermediates from a biopharmaceutical. Pre-column derivatization of the intermediates, followed by HPLC analysis, produced chromatograms similar to those seen in panels B and C of Fig. 1. The height of the peak in these chromatograms that had the same retention time as mBBr-DTT was used to interpolate the quantity of DTT in the sample from a calibration curve of the reference standard. The results from one set of analyses are presented in Table 3. From a step shortly after the addition of DTT (Process Step A) to the final intermediate in this analysis (Process Step E), the process effectively cleared DTT from the product stream by approximately six orders of magnitude. The level of DTT in the final intermediate examined was less than 1.5 pg/μl.

4. Conclusions

Monitoring the clearance of additives from the downstream process of biopharmaceuticals often involves the use of highly sensitive and valid methods. This paper described one method for process monitoring that met these two criteria. The method employed a highly-sensitive thiol-specific fluorescent probe to label residual DTT in process samples prior to reversed-phase HPLC. Similar methods involving pre-column derivatization with fluorescent labels specific for other groups, such as amines, alcohols, carbonyl or carboxyl groups [12], are likely to be useful for monitoring the clearance of entities bearing these chemical components from other pharmaceutical processes.

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