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Short communication

Determination of 3-amino-5-mercapto-1,2,4-triazole in serum

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Abstract

A high performance liquid chromatography (HPLC) method using fluorescence detection to determine 3-amino-5-mercapto-1,2,4-triazole (AMT) levels in serum has been developed. Sample preparation involved treatment with tributylphosphine (TBP) to reduce disulfides formed during storage, precipitation of proteins with acetonitrile (ACN), and precolumn derivatization using the thiol reactive fluorescent probe monobromobimane (MBB). The conjugate (AMT-MBB) was resolved by gradient elution from a C_{18} reversed-phase column. The assay method was linear over a concentration range of 0.78–50 μg/ml and had a limit of detection (LOD) of 0.05 μg/ml AMT (10 μl injection). This method provides a sensitive and specific tool for the determination of AMT in serum and may have potential industrial hygiene application.

Keyword: 3-Amino-5-mercapto-1,2,4-triazole

1. Introduction

The chemical, 3-amino-5-mercapto-1,2,4-triazole (AMT) is used in a number of commercial applications including pesticide synthesis, processing of silver halide photographic materials, as a viscosity index improver, dispersant, and as an antioxidant for lubricating oils and aluminum [1].

There are few reports concerning AMT toxicity and no published reports on its environmental fate or occupational exposure. One study reported AMT to be a goitrogen, inhibiting thyroid peroxidase in rats [2]. AMT is also structurally related to, and is a metabolite of the herbicide amitrol (3-amino-1,2,4-triazole) [3]. Amitrol, a well-known goitrogen [4–6] exhibits potent antithyroid activity in rats [2].

A sensitive and selective analytical method is required in order to carry out toxicokinetic or biomonitoring studies of AMT. This paper describes the development of a method suitable for the detection of AMT in serum. High performance liquid chromatography (HPLC) with fluorescent detection of a highly fluorescent species (AMT-MBB) obtained from the conjugation of AMT to the thiol specific probe, monobromobimane (MBB) was used (Fig. 1). Applicability of this method was demonstrated by the determination of AMT levels in the serum of dosed rats.

2. Experimental

2.1. Chemicals and reagents

3-Amino-5-mercapto-1,2,4-triazole, tributylphosphine (TBP), acetonitrile (ACN), dimethyl sulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF) were obtained from Aldrich (Milwaukee, WI). Monobromobimane was purchased from Molecular Probes (Eugene, OR). Frozen human serum was from Sigma (St. Louis, MO). HPLC grade methanol was acquired from Fisher Scientific (Pittsburgh, PA). Distilled water used throughout this research was purified by a Millipore MilliQ system (Molshiem, France). All solvents used for HPLC were filtered (0.22 μm membrane) and degassed by sonication under vacuum for 10 min at ambient temperature. Monobromobimane was prepared as a 74 mmol l^{-1} solution in acetonitrile. Tributylphosphine (TBP) was prepared as an 800 mmol l^{-1} solution in methanol.

2.2. Preparation of calibration standards and AMT spiked samples

AMT stock solutions (1000 μg/ml) were prepared in either ACN/water (50%, v/v), DMSO/water (10%, v/v) or

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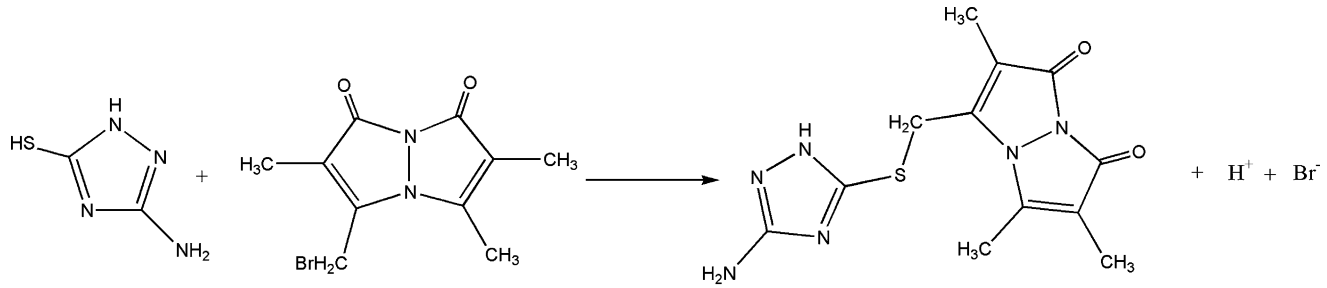

Fig. 1. Reaction of AMT with MBB.

DMF/water (10%, v/v). Working standard solutions used for fluorescent and UV detection were prepared by the appropriate dilutions with human serum or water to yield final AMT concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 μg/ml. An appropriate volume of stock solution was added to give final AMT concentrations of 1, 10, 20, 25 and 50 μg/ml in the spiked samples.

2.3. Determination of AMT in serum and water

Each standard/sample (600 μl) was treated with methanolic TBP (6 μl) and incubated at 50 °C for 1 h. ACN (600 μl) was added to serum samples and corresponding standards only, mixed thoroughly then centrifuged at 10,000 × *g* for 5 min to remove protein. Aliquots of each supernatant fluid (600 μl) were treated with MBB (6 μl) and after mixing were incubated overnight at 4 °C in the dark. Derivatized standards were filtered (0.2 μm polypropylene) prior to HPLC analysis.

2.4. High performance liquid chromatography

HPLC analysis was performed on a Shimadzu system consisting of two LC-600 pumps, SIL-10AD autoinjector, LPI-6B interface, RF-551 Fluorescence HPLC monitor (1.5 s response time) and an SPD-M10A Diode Array Detector. An injection volume of 10 μl was used and separations were performed on a 5 μm particle size, 250 mm × 4.6 mm Supelco Discovery C_{18} column (Bellefonte, PA) at a flow rate of 1 ml/min. Excitation and emission wavelengths of 380 and 480 nm, respectively, were used for the spectrofluorometric detection of the AMT-MBB derivative. The elution gradient profile started at 27% methanol/water, where it was held for 11 min before being increased at a rate of 10.6%/min for 5 min to a final 80% methanol/water concentration. An absorbance at 250 nm and a 2% methanol/water isocratic mobile phase were used for the UV detection of non-derivatized AMT.

2.5. Assay validation

Accuracy, intra- and interday precisions of the method were determined by analyzing five replicate standard samples in human serum at four different AMT concentrations (1, 10, 20 and 50 μg/ml) on three separate days. These quality control samples were prepared independently of the calibration standards. The intra- and interday precision was calculated as the coefficients of variation. Accuracy was calculated by back calculation of the calibration standards and comparison to the known spiked concentration.

2.6. Animals

Male Fisher 344 rats (weight 200–225 g) were obtained from Charles River Laboratories (Wilmington, MA) and were housed in an American Association for Accreditation of Laboratory Animal Care approved facility for 2 weeks before use. Food and water were provided ad libitum. Rats were anesthetized (40 mg/kg pentobarbital) then dosed intratracheally, with 200 μl of 5.4 mg/ml AMT in DMSO/phosphate buffered saline (10% v/v). One hour following the dosing blood was collected in a terminal procedure from the vena cava. The blood was centrifuged at 1000 × *g* for 10 min at 4 °C. The serum was then assayed as described in Section 2.3.

3. Results and discussion

Initial work was carried out using aqueous AMT samples. AMT has a strong UV absorbance from 200 to 280 nm, with a maximum absorbance at 250 nm and local minima around 220 nm. Monitoring of both wavelengths proved useful with respect to establishing peak purity in a complex matrix. Despite good sensitivity (LOD 0.05 μg/ml) and linear standard plots obtained, UV detection of AMT was not suitable for the analysis of AMT because of its poor retention on C_{18}. A retention time of 4.3 min ($k' = 0.43$) was observed while running an isocratic mobile phase of 2% methanol/water. The retention of AMT on C_{18} was further diminished by the presence of a serum matrix effect resulting in a retention time of 3.4 min (approximately a 70% decrease in retention). This suggested direct analysis of AMT using HPLC with UV detection is inappropriate due to both sample matrix effects and poor chromatographic retention.

To overcome this limitation a reverse-phase HPLC-fluorescent method was devised which takes advantage of

the free thiol present in AMT. The free thiol was conjugated to the probe, MBB, resulting in a highly fluorescent stable species with a retention time of 8.2 ($k' = 1.7$) minutes using the present gradient elution. MBB is commonly used for the detection of free thiol containing compounds in various biological materials. The method for the detection of AMT in serum involved four distinct steps: (i) reduction of oxidized AMT to its free form with TBP; (ii) protein removal with acetonitrile; (iii) precolumn derivatization using thiol reactive MBB; and (iv) separation of the AMT-MBB conjugate by reverse phase chromatography with fluorescence detection.

Storage studies were undertaken to develop an efficient sample preparation procedure that minimized AMT loss prior to HPLC analysis. The addition of a reduction step was essential as results on non-derivatized AMT containing serum samples showed significant loss (>55%) when stored at −20 °C overnight. Excellent recovery (>95%) was achieved when the stored samples were treated with TBP. The addition of TBP prior to freezing the samples also prevented significant oxidation of AMT. Protein binding studies indicated no significant loss of AMT due to protein binding (data not shown).

In the development of the analytical procedure different approaches to facilitate protein removal and possible AMT concentration were investigated. In preliminary work a solid phase extraction (SPE) procedure using C_{18} cartridges was used to concentrate and separate AMT-MBB from serum protein. Problems associated with the apparent instability of MBB hydrolysis products that gave rise to secondary break down products, one of which could not be separated from the AMT-MBB conjugate precluded use of SPE with AMT-MBB. A suitable stationary phase that would allow the concentration of AMT prior to derivatization with MBB could not be found. Of the many packing materials tested only charcoal provided suitable retention of AMT, but it could not be recovered once bound. Other packing materials including C_2, C_8, alumina, aminopropyl, cyclohexyl, cyanopropyl, diol, and phenyl all resulted in poor and inconsistent AMT retention.

The failure of SPE columns to concentrate AMT and remove protein led to the use of trichloroacetic acid (∼10% sample volume) to effect protein removal from serum. However, the poor reproducibility that was observed suggested AMT was lost in the precipitation process. This resulted in the use of acetonitrile despite an incurred loss in sensitivity caused by sample dilution by the volume required.

Reaction of the AMT thiol functionality with MBB was significantly slower when compared to reaction times reported for other thiol containing compounds. Derivatization was complete in ∼3 h at room temperature (or for convenience overnight at 4 °C) compared to a matter of minutes for the thiol containing compounds cysteine and glutathione [7].

The AMT-MBB derivative in human serum was separated from all main contaminants and the presence of endogenous thiol containing compounds did not interfere with the conjugation of AMT or detection of its derivative. Results of the HPLC method validation studies are reported in Table 1. Good accuracy and precision of the method was found. Intraday precision of AMT ranged from 0.5 to 10.1% while interday precision ranged from approximately 2.2 to 8.3%. The back calculated concentrations of AMT were within 10% of the nominal value. Standard curves from 0.78 to 50 μg/ml AMT were linear and always gave correlation coefficients higher than 0.999 as determined by least squares analysis. The limit of detection determined at a signal to noise ratio of 3, was 0.05 μg/ml AMT for a 10 μl injection. The relative recovery of the overall method was investigated by the comparison of six human serum samples spiked with known concentrations of AMT to that of corresponding standards in DMF/water. Good recoveries were obtained with values always exceeding 90%.

Table 1
Accuracy and precision data for the determination of AMT in human serum

Spiked concentration (μg/ml)	Back calculated concentration ± S.D.	% CV	% bias[a]
Intraday[b] ($n = 5$)			
1	1.1 ± 0.1	10.1	8.0
10	9.8 ± 0.3	2.3	2.0
20	20.1 ± 0.4	2.0	0.3
50	50.5 ± 0.3	0.5	1.0
Interday[b] ($n = 3$)			
1	1.1 ± 0.1	8.3	10.0
10	9.5 ± 0.3	3.6	5.2
20	19.2 ± 0.8	4.3	4.2
50	49.9 ± 1.1	2.2	0.1

[a] Bias = (observed concentration − nominal concentration)/nominal concentration.
[b] Intra- and interday-precision defined in the text.

Human sera samples from individuals with known documented exposure were not available. Rats were dosed with AMT to demonstrate the applicability of the described assay. Rats were dosed with 200 μl of a 5.4 mg/ml AMT solution and after 1 h serum samples were obtained. No interference of the analyte peak was observed (Fig. 2) and results from the assay are given in Table 2. Good reproducibility was observed between the duplicate samples analyzed. AMT toxicokinetics are not known. The 1 hr time point may not have been optimal, but the results obtained illustrate the

Table 2
Determination of AMT levels in rat serum

Animal	Calculated AMT concentration (μg/ml) from duplicate samples	
Dosed		
A	2.23	2.21
B	3.11	3.14
Control		
C	n/d	n/d
D	n/d	n/d

n/d: not detected (<0.05 μg/ml AMT).

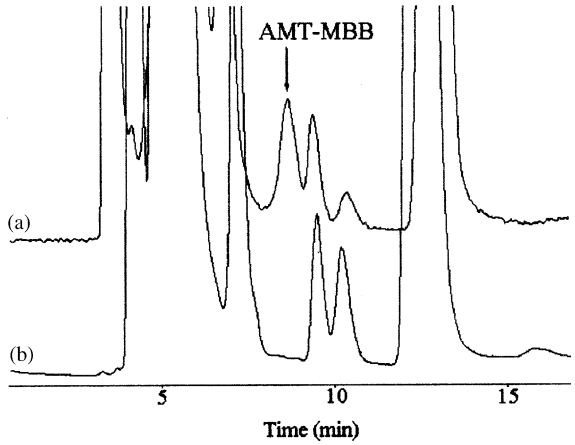

Fig. 2. Reverse-phase HPLC chromatograms of (a) rat serum obtained 1 h after dosing with 200 μl of 5.4 mg/ml AMT and derivatized with MBB (determined AMT level 3.11 μg/ml serum) and (b) rat serum blank treated with MBB.

suitability of this method for analysis of AMT in human blood as a potential marker of exposure.

AMT has been reported to be a metabolite of amitrol which was detected in the urine of rats [3]. Preliminary work on AMT spiked urine samples with the omission of the protein removal step indicate that this method could readily be adapted to determine AMT levels in urine samples. Linear standard curves ($r^2 > 0.999$) within the concentration range 0.78–50 μg/ml (67–4305 pmole on column) were obtained. The similarity of the standard plots between AMT spiked urine and the corresponding aqueous standards appear to suggest the absence of any matrix influence.

The AMT-MBB conjugate was shown to be sufficiently stable when in solution. Samples stored at 4 °C for 4 days or at −20 °C for 10 days showed no significant change in fluorescence intensity.

4. Conclusion

An HPLC method using fluorescence detection was developed for the analysis of AMT in serum. The method involves the reduction of any oxidized AMT using TBP, precolumn derivatization of free thiol with MBB, separation and quantitation of the AMT adduct using reversed-phase chromatography with fluorescence detection. The assay was found to be simple, specific and sensitive. It should provide a suitable tool for the determination of AMT in serum and may also have potential industrial hygiene application.

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