N-Acetyltaurine – preliminary work to prove its potential as a marker of alcohol consumption

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Aims: In both, forensic and clinical settings, highly specific and long-term markers of alcohol consumption are still warranted. Metabolomics in rats following administration of ethanol revealed an increase in urinary excretion of N-acetyltaurine (NAT) with alcohol dose [1]. **Methods:** NAT has been synthesized using a method similar to that of Johnson et al. [2]. Pyridine and acetic anhydride were added to taurine dissolved in water. The reaction mixture was stirred at 4°C, and left overnight at -20°C. The crude reaction product was cleaned up via a silica gel elution column. Each fraction was checked by LC/MS/MS in negative ionization mode at the following transitions: m/z 166→166, 166→124, 166→107. The internal standard N-propionyltaurine (NPT) was synthesized in the same way substituting acetic anhydride by propionic anhydride; fractions were checked by the following transitions: m/z 180→180, 180→124, 180→107. Subsequently, separation of taurine, NAT and NPT was optimized [3]. Preparation of 100 µL urine aliquots was by dilution and centrifugation. The method was validated and applied to urine specimens obtained from teetotalers and social drinkers. **Results and Discussion:** Yields of about 30% for both NAT and NPT were in line with that for NAT reported by Johnson et al. [2]. However, the proposed solvent clean up proved insufficient. Run time was 7 min to separate NAT and NPT from taurine being present in authentic urine specimens. Concentrations of NAT in teetotalers significantly differed from those in social drinkers. **Conclusion:** Both, NAT and NPT can be synthesized in sufficient yield and chromatographic purity following clean up by silica gel. Sample preparation and quantitation by LC/MS/MS using NPT as the internal standard is rapid and promising to be applied to a larger quantity of specimens.

1. Introduction

Clinicians, resident physicians and public agencies all use biomarkers as an objective measure to assess a subject's alcohol use. Currently used alcohol markers are not without restriction of any kind; priority is given to markers with a high specificity and long-term detectability. At present, routinely applied direct markers in urine such as ethyl glucuronide and sulfate have a rather short window of detection. Metabolomics in rats following administration of ethanol revealed an increase in urinary excretion of NAT with increasing alcohol dose [1]. The aim of our present study was a) to synthesize NAT and an appropriate internal standard and b) to develop and validate an assay in urine thus enabling a prospective study of NAT as a marker of drinking activity and alcohol toxicity in man.

2. Materials and Methods

2.1. Materials

Urine samples were collected from 5 subjects with regular alcohol consumption as well as from 5 teetotalers. Informed consent was obtained from the subjects prior to urine sampling.

The samples were stored at -80°C until analysis. Methanol and acetonitrile (both HPLC grade) were obtained from Roth (Karlsruhe); all other solvents and chemicals were of the highest purity grade available.

2.2. Synthesis

NAT has been synthesized using a method similar to that of Johnson *et al.* [2] (Fig. 1). Firstly, 0.5 g taurine was dissolved in 7 ml water. Then, 2.5 ml pyridine and 2 ml acetic anhydride were added dropwise. The reaction mixture was stirred at 4°C for 2 h, and left overnight at -20°C. The solvent was removed under vacuum; then, the crude reaction product was dissolved in q.s. ethanol:dichlormethane (1:1 vol%) and was cleaned up via a silica gel column. The fractions were dried under nitrogen (1 h at 40°C) and then dissolved in methanol. Each fraction was checked by LC/MS/MS in negative ionization mode at the following transitions: m/z 166→166, 166→124, 166→107 (Fig. 2). The internal standard NPT was synthesized in the same way substituting acetic anhydride by propionic anhydride; fractions were checked by the following transitions: m/z 180→180, 180→124, 180→107.

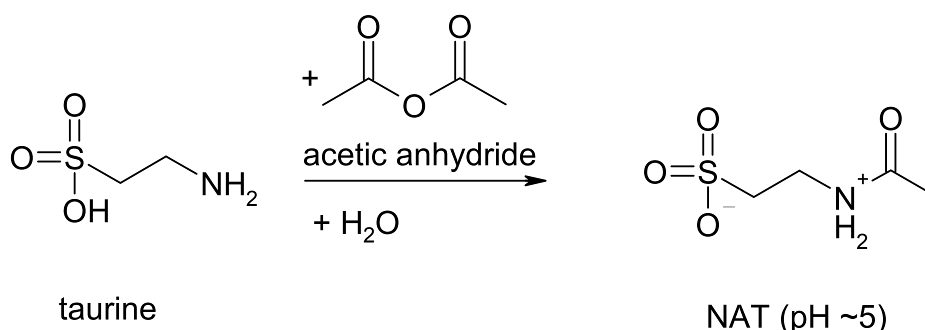

Fig. 1. Synthesis of N-acetyltaurine.

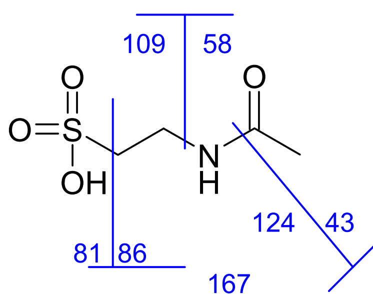

Fig. 2. Expected fragmentation of N-acetyltaurine: m/z = 167, 124, 109, 86, 81, 58, 43.

2.3. Sample preparation

Frozen urine samples were thawed just prior to analysis. Preparation of 100 µL urine aliquots was by dilution in 400 µL mobile phase (10 mM ammonium acetate buffer pH 9.3/MeOH/acetonitrile, 95/1/4 vol%) and adding 5 µL of the internal standard NPT (500 ng/mL). The samples were centrifuged (10 min, 4300•g) and 10 µL of the supernatant were injected for analysis.

2.4. Instrumentation

Determination of the concentration of NAT was improved [3] and performed on an API 4000 triple-quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany), with a Turbo ioniza-

tion source operated in the negative ionization mode, interfaced to two HPLC pumps and an auto sampler (HP 1100 series, Agilent, Waldbronn, Germany) controlled by Analyst 1.4 software. Separation was achieved on a Hypercarb column (100 x 2.1 mm; 5 µm particle size; Thermo Scientific, Dreieich, Germany), ahead a guard column, with 10 mM ammonium acetate buffer (wich was adjusted to pH 9.3 with ammonia)/methanol/acetonitrile (95/1/4 vol%) as the mobile phase at a flow rate of 200 µL/min. To increase the ionization rate of NAT, isopropanol was infused post-column at a constant flow of 25 µL/min.

The following settings were used: temperature, 620 °C; ion spray voltage, −4000 V; entrance potential, -10 V; declustering potential -50V. Data were acquired in multiple reaction-monitoring mode, using the same three transitions for NAT and the same two for NPT specified above. The transition m/z 166→107 was used for quantification of NAT.

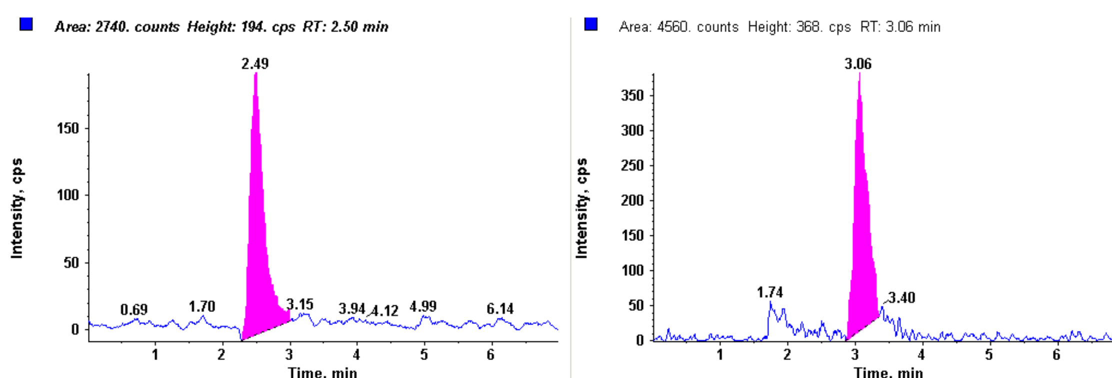

Fig. 3. Chromatogram of an authentic urine sample. Left: NAT m/z 166→107, right: NPT m/z 180→107, RT: retention time.

2.5. Evaluation

Validation experiments were carried out according to the guidelines of the GFTCh [4]. Following validation, the assay was applied to urine specimens obtained from teetotalers and social drinkers.

3. Results and Discussion

3.1. Synthesis

Yields of about 30% for both NAT and NPT were in line with that for NAT reported by Johnson *et al.* [2]. However, the proposed solvent clean up proved insufficient. Clean up of the crude reaction mixture via a silica gel column resulted in a product unadulterated with taurine.

3.2. Analysis

The total run time was 7 min to separate NAT and NPT from taurine being present in authentic urine specimens. Concentrations of NAT in teetotalers significantly differ from those obtained following alcohol use (Tab. 1).

3.3. Validation

Validation results were in accordance with the GTFCh guideline [4]. Table 2 summarizes the validation data for the LC/MS/MS assay of NAT in urine after sample preparation. All values were in acceptable ranges.

Tab. 1. NAT concentrations (normalized to 100 mg creatinine/100 mL urine); drinkers versus teetotalers.

	Drinker		Teetotaler
	NAT [ng/mL] normalized		NAT [ng/mL] normalized
1	109973	1	200
2	20538	2	188
3	32110	3	114
4	6500	4	95
5	732	5	84

Tab. 2. Validation results of the LC/MS/MS assay for the determination of NAT in urine. LOD: limit of detection, LOQ: limit of quantification.

Validation parameter	Concentration [ng/mL]	Urine		
Linearity [ng/mL]: 100-1000; Correlation coefficient R = 1.00				
LOD [ng/mL]		11.23		
LOQ [ng/mL]		42.53		
Inaccuracy [Bias %]	100	0.3		
	750	0		
Within-run imprecision [RSD %]	100	0.6		
	750	0.8		
Between-run imprecision [RSD %]	100	0		
	750	0		
Processed sample stability [residual content %]			hour	
	100	104	0	
		99.1	24	
		98.6	48	
	750	99.7	0	
		99.6	24	
		100	48	
Freeze/thaw stability [residual content %]	100	98.7 (day 0)		
		97.8 (day 3)		
	750	100 (day 0)		
		99.7 (day 3)		
Long-term stability [residual content %]		4°C	-20°C	day
	100	101	101	0
		100	101	7
		103	99.3	14
		98.5	102	28
	750	100	100	0
		100	99.9	7
		99.6	100	14
		99.6	99.7	28
Process efficiency [%]	750	99.2		

4. Conclusion

Both, NAT and NPT can be synthesized in sufficient yield and chromatographic purity following clean up by silica gel.

Sample preparation and quantitation by LC/MS/MS using NPT as the internal standard is rapid and promising to be applied to a larger quantity of specimens. First results also indicated a greater window of detection compared to EtG. Further work is in progress.

5. References

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