

Application Note

► Determination of trans,trans-muconic acid in urine samples

Category	Clinical
Matrix	Urine extract
Method	UHPLC
Keywords	Trans,trans-muconic acid (tt-MA), benzene exposure, benzene metabolite, biomarker, urine
Analytes ID	Trans,trans-muconic acid (tt-MA) VCL0004N, 10/10



PLATIN blue

Summary

This application note describes a gradient method using a sub-2 μm column for the determination of the benzene metabolite, trans,trans-muconic acid (tt-MA) in urine samples. The separation of the target compound from its complex urine matrix could be carried out in less than 6 minutes using the KNAUER PLATINblue UHPLC system. The high speed and reliability of the method make it well-suited for routine analysis in clinical diagnostics. The retention time of tt-MA applying a classical HPLC separation is in the range of approximately 16 min. With the new developed UHPLC method tt-MA elutes after 2.4 min from the column.

Introduction

The ubiquity of benzene in the environment due to its occurrence in mineral oil and combustion processes results in health issues. Caused by the mutagenic and carcinogenic properties of the compound, the International Agency for Research on Cancer (IARC, 1989) classified benzene as a group 1 carcinogen. Therefore monitoring of this substance is of paramount importance.¹ Not only the environmental benzene concentration is of interest, but also the control of human benzene exposure, especially at work places. It is important to establish a method that is sensitive enough to detect human exposure to very low benzene concentrations as it is already toxic in low doses.²

Human uptake of benzene from the environment occurs mainly via inhalation. Benzene is metabolized in the liver to a series of ring-hydroxylated and conjugated metabolites as well as ring-opened products which are excreted in urine. Additionally to phenol and S-phenylmercapturic acid (S-PMA), tt-MA is one of the well-known benzene metabolites excreted in urine (see fig. 1). These three substances can be seen as potential biomarker candidates for benzene exposure. On average, 0.11 % of an inhaled benzene dose is excreted as S-PMA and 3.9 % as tt-MA. Although phenol is excreted even in a much higher rate, this substance is not a reliable biomarker for benzene exposure below 5 ppm, because it is also found in lower rates in urine of people without benzene exposure.^{2,3}

Determination of the biomarker tt-MA can be carried out by highly sensitive GC-MS and LC-MS methods. But for routine analyses in clinical diagnostics these are not suitable.²

In this application note, we demonstrate a fast and reliable UHPLC method in combination with UV detection that is suitable for high throughput analyses in clinical routine.

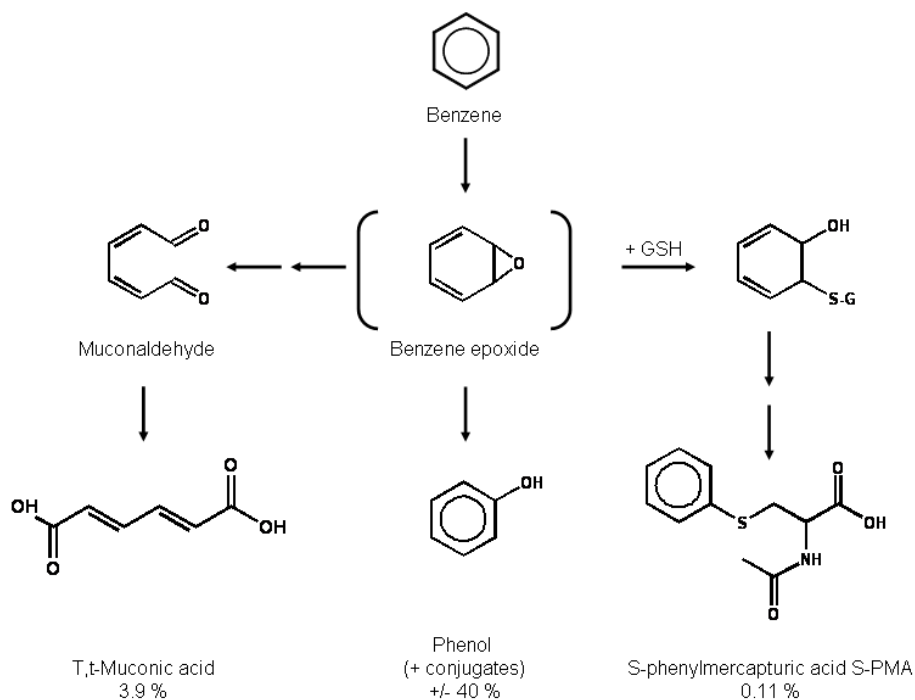


Fig. 1

Some metabolic pathways of benzene and urinary excretion of benzene metabolites³

Experimental: sample preparation

Metabolites like tt-MA can be easily extracted from urine matrices using liquid-liquid extraction techniques with ethyl acetate for example.³ In this work, urinary extracts were obtained by means of ion chromatography. An aliquot of the urine sample was applied on a column and eluted with 10 % of acetic acid. After dilution 1:1 with 1 % acetic acid the blank urine extract was ready for analysis by UHPLC.

Experimental: preparation of standard solution

The standard solution was prepared by adding 50 µl of a 2 mg/l tt-MA stock solution in deionized water to 50 µl of a blank urine extract prepared as described above. After adding 100 µl of 1 % acetic acid, the standard solution could be analyzed by UHPLC.

Method parameters

Column	fused core C18 1.7 µm, 100 x 2 mm		
Eluent A	H ₂ O (1 % HAc)		
Eluent B	Methanol		
Gradient	Time [min]	% A	% B
	0.00	95	5
	4.18	75	25
	4.20	10	90
	5.20	10	90
	5.22	95	5
	7.00	95	5
Flow rate	0.4 ml/min		
Injection volume	10 µl		
Column temperature	30 °C		
System pressure	approx. 720 bar		
Detection	UV at 259 nm (50 Hz, 0.01 s)		
Run time	7.00 min (Analysis time 4.50 min)		

Results

Fig. 2

Chromatograms of a blank urine extract (green) and urine extract spiked with tt-MA (blue)

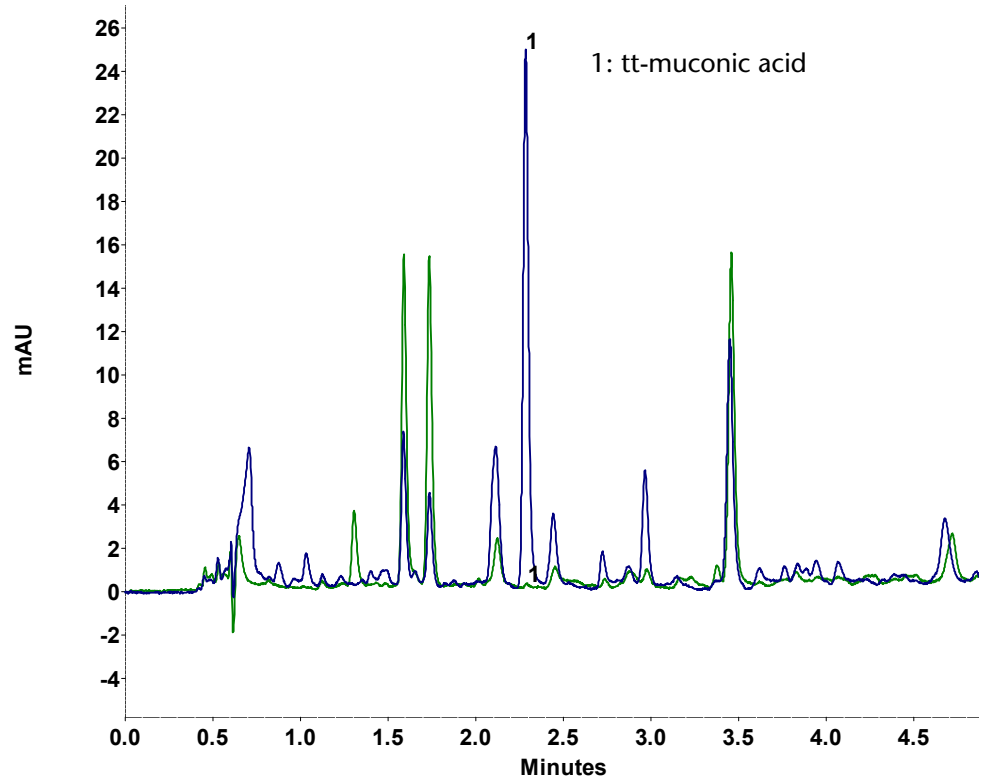
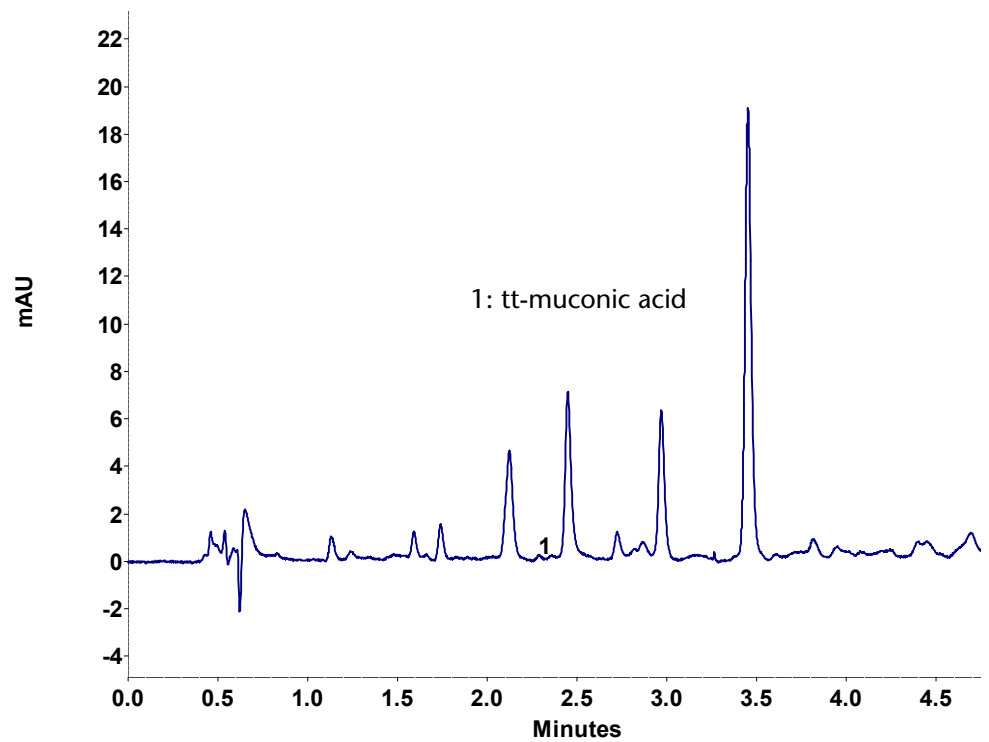


Fig. 3

Chromatogram of an unknown urine extract sample



A chromatogram of the blank urine extract (green) and urine extract spiked with tt-MA (blue) is shown in fig. 2. The substance of interest is baseline separated from other matrix compounds with good resolution. The limit of detection (LOD) lies in the range of 0.017 mg/l tt-MA (S/N = 3). Further differences in the chromatograms in fig. 2 result from

Results (continued)

the use of different urine extract samples and the higher dilution factor for the spiked extract.

In fig. 3 the chromatogram of an unknown urine extract sample is shown where no tt-MA has to be recovered under the listed method parameters. It becomes obvious that no tt-MA could be found as it is naturally expected.

Method performance

Limit of detection	0.017 mg/L (S/N = 3)
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Conclusion

A fast and reliable method for the analysis of the benzene metabolite tt-MA is highly desirable to better protect humans from negative health effects caused by benzene exposure, because it allows more frequent testing. Using the KNAUER PLATINblue UHPLC system equipped with a high pressure gradient using methanol, tt-MA could be easily separated from the complex urine extract matrix in less than 5 minutes (fig. 2), which is about 5 times faster than a conventional HPLC gradient method. Moreover, eluent consumption per sample was reduced by more than 90 % compared to the HPLC method. Sensitive UV detection of tt-MA concentrations in the range of 0.017 mg/l can be realized by applying the KNAUER PLATINblue detector PDA-1.

Using UHPLC and its advantages, long equilibration and analysis times can be avoided. This does not only save time but also money and additionally protects the environment. All these facts make this method well suited for analyses in clinical routine.

References

1. Tharnpoophasiam, P. et al.; Southeast Asian J Trop Med Public Health, Vol. 35, No. 3, 717-723 (September 2004)
2. Hu, X. et al.; Biomed Environ Sci, Vol. 19, 292-296 (2006)
3. Boogard, P. J. et al.; Environ Health Perspectives; Vol. 104, Supplement 6, 1151-1157 (December 1996)

Physical properties of recommended column



Stationary phase	fused core C18 1.7 µm, 100 x 2 mm
USP code	L1
Pore size	x
Pore volume	x
Specific surface area	x
Particle size	x
Form	spherical
Surface area	x
% C	x
Endcapping	x
Dimensions	100 x 2 mm
Order number	upon request

Recommended instrumentation



This application requires the PLATINblue binary high pressure gradient UHPLC system equipped with degasser, autosampler, column thermostat, and PDA detector. Other configurations are also available. Please contact KNAUER to configure a system that is perfect for your needs.

Description	Order No.
PLATINblue UHPLC System	A69420
PLATINblue Pump P-1	
PLATINblue Pump P-1 with Degasser	
PLATINblue Autosampler AS-1	
PLATINblue Column Thermostat T-1 Basic	
PLATINblue Detector PDA-1	
PDA-1 flow cell (10 mm, 2 µl)	
PLATINblue modular eluent tray	
PLATINblue CG Data system	
PLATINblue CG PDA license	
PLATINblue stainless steel capillary kit	
+ PDA-1 flow cell (50 mm)	A64151

Authors

Silvia Marten, Head of Columns and Applications Department, KNAUER
Mareike Naguschewski, Columns and Applications Department, KNAUER

Contact information

Wissenschaftliche Gerätebau
 Dr. Ing. Herbert Knauer GmbH
 Hegauer Weg 38
 14163 Berlin, Germany

Tel: +49 (0)30 / 809727-0
 Fax: +49 (0)30 / 8015010
 E-Mail: info@knauer.net
 Internet: www.knauer.net

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by Knauer