J Fluoresc (2010) 20:421–424
DOI 10.1007/s10895-009-0560-1

SHORT COMMUNICATION

A Non-fluorescent Derivative from Derivatizing *trans*, *trans*-Muconic Acid with 2-(2-Naphthoxy)ethyl-2-(piperidino) ethanesulfonate

Tang-Chia Chung · Wung-Pung Su · Hwang-Shang Kou · Yu-Ting Lin · Min-Yuan Hung · Hsin-Lung Wu

Received: 20 August 2009 / Accepted: 12 October 2009 / Published online: 24 October 2009

Abstract *trans, trans*-Muconic acid (MA) is a polar metabolite of benzene and used as a biomarker for monitoring human exposure to benzene. Because MA is a trace metabolite, sensitive method is required for its detection. In addition, MA is a highly polar compound with dicarboxyl functions that could incur unfavorable adsorption on silica-based stationary phase usually used for separation. To address these problems, we planned to derivatize MA with a fluorescent reagent 2-(2-naphthoxy)ethyl-2-(piperidino)ethanesulfonate to give a naphthoxy derivative of MA for improving detection sensitivity and chromatographic properties. Surprisingly, the resulting derivative shows no fluorescent activity (λ_{ex}: 226 nm; λ_{em}: 350 nm). The negative results could be used as an instructive example for discussing on fluorescence quenching.

Keywords *trans*, *trans*-Muconic acid · 2-(2-naphthoxy) ethyl-2- (piperidino)ethanesulfonate · Derivatization · Fluorescence quenching

Introduction

Benzene widely exists in petrochemicals at varied levels [1, 2]. Due to the hematoxic and carcinogenic risks of benzene, human exposure to benzene is strictly controlled. *trans, trans*-Muconic acid (MA) is one of the urinary metabolites of benzene and frequently used as a biomarker for monitoring the extent of exposure to benzene [3–5]. For this purpose, several methods were developed for the analysis of MA, including GC [6, 7], LC [8–12] and hyphenated MS techniques [13–15]. Due to the high polarity of MA (Fig. 1), it is not easy to concentrate MA by conventional liquid–liquid extraction for enhancing a detection. Therefore, lengthy treatment including solid phase extraction (SPE) is often used to isolate and concentrate MA in various samples for a sensitive detection.

The purpose of this work is to improve the chemical property and detection sensitivity of MA by labeling with a lipophilic fluorophore/chromophore for better analysis of MA.

T.-C. Chung · W.-P. Su · H.-S. Kou · Y.-T. Lin · H.-L. Wu (✉)
Graduate Institute of Pharmaceutical Sciences,
Collage of Pharmacy, Kaohsiung Medical University,
Kaohsiung 807, Taiwan
e-mail: hlwu@kmu.edu.tw

M.-Y. Hung
Center for Resources Research and Development,
Kaohsiung Medical University,
Kaohsiung 807, Taiwan

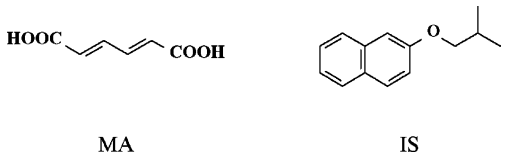
Experimental

HPLC conditions

A Waters LC system with a Model 510 pump, a Model 717 plus autosampler, a Model 2487 UV detector and a Merck Lichrospher C18 column (250×4 mm I.D., 5 μm) was used. A mixed solvent of methanol-water (85:15, *v*/*v*) was used at a flow rate of 1.0 mL/min. The column eluate was monitored by UV at 226 nm or by fluorimetry (λ_{ex}: 226 nm; λ_{em}: 350 nm). The peak-area ratios of the MA derivative to 2-isobutoxy-naphthalene (an internal standard; Fig. 1) were evaluated for optimizing the derivatization conditions.

Materials and reagent solution

MA (Sigma, St Louis, MO, USA), 18-crown-6 ether (18-crown-6) and 2-isobutoxy-naphthalene (internal standard; IS)



MA IS

Fig. 1 Structures of *trans, trans*-muconic acid (MA) and the internal standard (IS; 2-isobutoxynaphthalene) used in the study

(TCI, Tokyo, Japan), 2-(2-naphthoxy)ethyl-2-(piperidino) ethanesulfonate (NOEPES; synthesized at our laboratory) [16], Lichrospher RP18(e) column (250×4 mm I.D., 5 μm), potassium carbonate and potassium chloride (E. Merck, Darmstadt, Germany) were used without further treatment. All other chemicals were of analytical reagent grade. Solutions of MA, IS, 18-crown-6 and NOEPES each at various concentrations were prepared by dissolving the respective chemical in acetonitrile. Solution of sulfuric acid (1 M) was prepared in Milli-R Q (Waters) treated water.

Derivatization procedure

A 300-μL aliquot of MA solution was added to a series of 25-mL screw capped test tubes each containing 100 μL of IS (50 μM), about 30 mg of potassium carbonate, 100 μL of 18-crown-6 (20 mM) and 100 μL NOEPES (60 mM; the derivatizing reagent). The reactants were automatically shaken at 75 °C for 1.5 h in a thermostated shaker. After cooling, 500 μL of the reacted solution were taken to a test tube containing 500 μL H_2SO_4 (1 M) and then 0.1 g of KCl was added and mixed. After centrifugation (5,000 g, 3 min), the separated acetonitrile solution was used for HPLC with a sample injection of 15 μL.

GC-MS conditions

The separated derivative (from the section of Mass spectral analysis of the derivative) was analyzed by GC-MS with the following conditions: ThermoFinnigan GC with Finnigan PorisQ MS using a Rescek Rtx-5MS column (30 m, 0.25 mm, I.D., 0.25 μm df); carrier gas: He with the flow rate at 1.0 mL/min; injector, column and interface temperatures respectively as 200, 230 and 275 °C, and the ionization mode of EI with an acceleration energy of 70 eV.

Results and discussion

Optimizing the parameters for derivatization

For optimizing the conditions for derivatizing MA with an amount of 6 nmol (20 μM×300 μL), several parameters affecting the derivatization were studied, including the concentration of NOEPES and reaction activators, reaction temperature and reaction time. In assessing the effect of a test parameter, we kept other parameters constant as indicated in the section of the Derivatization procedure. The effect of the test parameter on the derivatization was evaluated by the peak-area ratios of the resulting MA derivative to the IS. The main results of the study are summarized as follows. The effects of the derivatizing reagent on the formation of MA derivative were studied by using NOEPES in the concentration range of 10–100 mM (100 μL for each level). Figure 2a shows the plateau formation of the derivative requires NOEPES at concentration ≥40 mM. The effects of alkaline potassium salt and 18-crown-6 on the derivatization of MA were studied by using

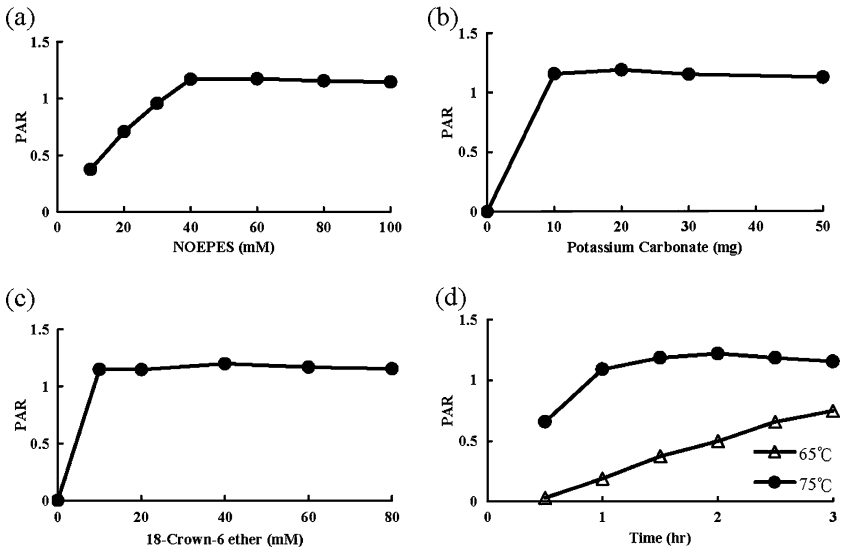


Fig. 2 Optimized conditions for derivatizing *trans, trans*-muconic acid (MA): **a** effect of concentration of 2-(2-naphthoxy)ethyl-2-(piperidino) ethanesulfonate (NOEPES) on the formation of the MA derivative; **b** effect of the amount of potassium carbonate on the formation of the MA derivative; **c** effect of the concentration of 18-crowm-6 on the formation of the MA derivative; **d** effect of reaction temperature and reaction time on the formation of *trans, trans*-muconic acid (MA) derivative

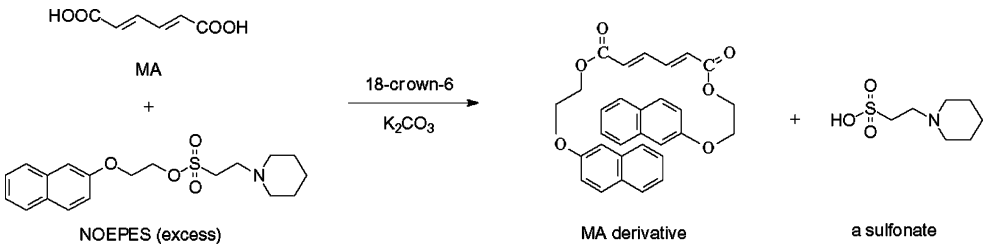


Fig. 3 A simplified nucleophilic substitution on derivatizing *trans, trans*-muconic acid (MA) with 2-(2-naphthoxy) ethyl-2-(piperidino) ethanesulfonate (NOEPES). The resulting MA derivative labeled with 2 chromophores is highly lipophilic. The sulfonate is a leaving group in the reaction

K_2CO_3, $KHCO_3$ or KF with a fixed level of 18-crown-6 (20 mM). The results indicated that K_2CO_3 or KF has better effect. Since K_2CO_3 is a routine chemical and selected for the reaction. The optimal amounts of K_2CO_3 and 18-crown-6 were further studied. The results indicated that using K_2CO_3 ≥10 mg (Fig. 2b) and 18-crown-6 ≥10 mM (Fig. 2c) is better for the optimal derivatization. In the absence of K_2CO_3 or 18-crown-6, the MA derivative was not observed. Both reagents are required to the derivatization of carboxyl function for improving its reactivity [17, 18].

The effects of reaction temperature and reaction time on the derivatization were studied. The results indicate that the equilibrium formation of MA derivative is attainable at 75 °C for 1.5 h (Fig. 2d). After derivatization, the reacted solution (in acetonitrile) was treated with aqueous H_2SO_4 (1 M) and then with KCl. The acid treatment is to protonate the excess reagent (NOEPES) for forming the ammonium salt (Fig. 3); and the subsequent addition of solid KCl leads to the demixing of acetonitrile in the system [19]. The separated acetonitrile solution was used for the LC analysis of MA that can minimize the interference from excess NOEPES. The stability of the MA derivative was observed at room temperature (about 25 °C) after derivatization over a period of 24 h at 2-h interval. The peak-area ratios of the derivative to the IS indicate no significant changes over the time studied. Thus, the derivative is sufficiently stable for the time required for the LC analysis.

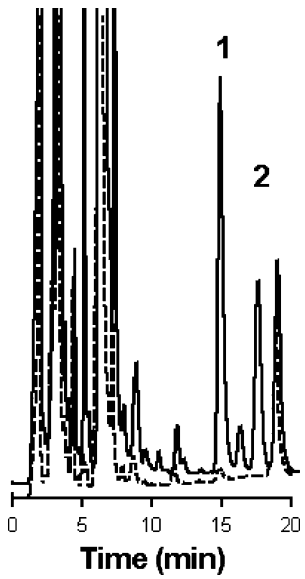


Fig. 4 Composite liquid chromatograms for the analysis of *trans, trans*-muconic acid (MA; 20 μM) with IS (50 μM) (*solid line*) and the reagent blank (*dotted line*). Peaks 1 for the internal standard and 2 for the MA derivative. See "HPLC conditions"

Mass spectral analysis of the derivative

The MA derivative was synthesized by scaling-up the amount of MA (1.0 mmol) used in the Derivatization procedure without adding IS. The resulting derivative was identified by MS after TLC separation with an Rf value of 0.26 on silica gel 60 F_{254} with the mobile phase of dichloromethane : n-hexane (1:1, *v*/*v*). The separated derivative exhibited a molecular ion peak at m/z 482 equivalent to MA labeled with two naphthoxy groups (Fig. 3). Other diagnostic ion peaks include m/z 144 (for naphthoxy fragment plus a migrated hydrogen) and m/z 216 for the fragment of 2-(2-naphthoxy)ethyleneoxycarbonyl plus a hydrogen. The retention time of peak 2 in Fig. 4 is identical to that of the confirmed MA derivative.

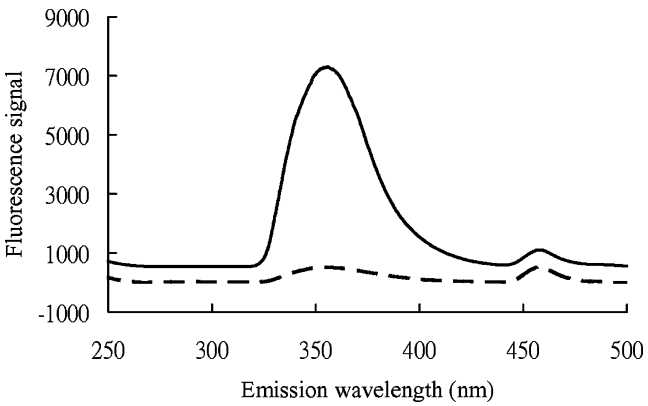


Fig. 5 Fluorescence spectra (with excitation at 226 nm) of 2-(2-naphthoxy)ethyl-2-(piperidino) ethanesulfonate (the derivatizing reagent; solid line) and the *trans, trans*-muconic acid derivative (*dashed line*) labeled with two naphthoxy fluorophores shows no fluorescence (see Fig. 3 for the structure). Each of the analyte was about 1 μM in methanol

Selectivity of the method

The selectivity of the method was briefly tested on the chromatographic separation of some organic acids existing in human urine including oxalic acid, uric acid, *o*-cresol, benzoic acid and hippuric acid each at 30 nmol (in 300 μL acetonitrile). The test solution was derivatized following the Derivatization procedure. All the test compounds are less retained than the MA derivative (with a retention time t_R of 16.8 min), including uric acid (t_R=3.6), hippuric acid (t_R= 4.0), benzoic acid (t_R=9.09) and oxalic acid (t_R=11.8). Because MA is labeled with 2 lipophilic naphthoxyethyl moieties, resulting in high retention. The same effect seemingly reflects on the derivative of oxalic acid with two carboxyl functions giving longer retention.

Fluorimetric detection of the MA derivative

We expected that the MA derivative labeled with two naphthoxy fluorophores (Fig. 3) should give strong fluorescence. Unfortunately, the derivative showed no fluorescence activity (with excitation at λ 226 nm and emission at λ 350 nm) (Fig. 5). The result differs entirely from our previous report [20] of derivatizing fatty acid with NOEPES giving high fluorescent activity. The unexpected phenomenon may arise from the close proximity of the two naphthoxy fluorophores of the derivative (Fig. 3) causing overlapping/collision interaction that results in the fluorescence quenching of the MA derivative [21, 22]. The results indicate that the derivatization of MA with NOEPES to enhance the sensitivity of the MA derivative is unattainable. Since the MA derivative contains two naphthoxy groups that are highly chromophoric. The derivative was preliminarily analyzed by LC-UV (Fig. 4) giving a lower quantitation limit of 0.3 μM and a detection limit of 0.02 μM MA (S/*N*=3; 15 μL injected). The results indicate that the MA derivative with two chromophores shows good sensitivity comparable to the reported GC-ECD and LC-UV methods.

Conclusion

In brief, analytical derivatization coupled with fluorimetric liquid chromatography was studied to improve the chemical properties and detection sensitivity of MA. The results indicate that derivatizing MA with NOEPES leads to a derivative labeled with two naphthoxyl groups showing no net fluorescence activity. However, the negative results could provide a good example of using the MA derivative as a model on discussing fluorescence quenching.

Acknowledgement H.-L.Wu is grateful to the National Science Council (Taiwan) for financial support of this work (NSC 96-2113-M-037-011-MY2).

References

1. Synder R, Hedli CC (1996) Eviron Health Perspect 104:1165–1171
2. Perbellini L, Faccini GB, Pasini F, Gazzoli F, Pistoia S, Rosellini R, Valsecchi M, Brugnone F (1988) Br J Ind Med 45:345–352
3. Medeiros AM, Bird MG, Witz G (1997) J Toxicol Environ Health 51:519–539
4. Amodio-Cocchier R, DelPrete U, Cirillo T, Agozzino E, Scarano G (2001) J Toxicol Environ Health 63:79–87
5. Valente T, Medico L, Chiapperini D, Bonsignore AD (2002) Ann Chim 92:911–917
6. Lin F-M, Wu H-L, Kou H-S, Lin S-J (2002) Anal Chim Acta 455:111–116
7. Yu R, Weisel CP (1996) J Toxicol Environ Health 48:453–477
8. Olmos V, Lenzken SC, Lopez CM, Villaamil EC (2006) J Anal Toxicol 30:258–261
9. Carrieri M, Bonfiglio E, Scapellato ML, Maccá I, Tranfo G, Faranda P, Paci E, Bartolucci GB (2006) Toxicol Lett 162:146–152
10. Lee B-L, Ong H-Y, Ong Y-B, Ong C-N (2005) J Chromatogr, B 818:277–283
11. Martins I, Siqueira MEPB (2002) RBCF, Rev Bras Ciênc Farm 38:205–212
12. Ducos P, Gaudin R, Robert A, Francin JM, Maire C (1990) Int Arch Occup Environ Health 62:529–534
13. Ruppert T, Scherer G, Tricker AR, Adlkofer F (1997) Int Arch Occup Environ Health 69:719–726
14. Manini P, De Palma G, Andreoli R, Poli D, Mozzoni P, Folesani G, Mutti A, Apostoli P (2006) Toxicol Lett 167:142–151
15. Tranfo G, Paci E, Sisto R, Pigini D (2008) J Chromatogr, B 867:26–31
16. Lu C-Y, Wu H-L, Chen S-H, Kou H-S (2000) Chromatographia 51:315–321
17. Starks M, Liotta C (1978) Phase transfer catalysis: principles and techniques. Academic, New York, pp 150–155
18. Toyo'oka T (1999) Modern derivatization methods for separation science. Wiley, Chichester, pp 122–123
19. Lingeman H, Underberg WM (1990) Detection-oriented derivatization techniques in liquid chromatography. Marcel Dekker, New York, p 253
20. Chung T-C, Chao M-C, Wu H-L (2007) J Chromatogr, A 1156:259–263
21. Epstein JR, Biran I, Walt DR (2002) Anal Chim Acta 469:3–36
22. Skoog DA, Holler FJ, Nieman TA (1998) Principles of instrumental analysis, 5th edn. Harcourt Brace, Philadelphia, pp 360–364