Journal of Chromatography A, 1377 (2015) 35–45

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of tranexamic acid in various matrices using microwave-assisted derivatization followed by dispersive liquid–liquid microextraction

Fang-Yi Liao[a], Ying-Chi Lin[b], Yen-Ling Chen[a], Chia-Hsien Feng[a,c,*]

[a] Department of Fragrance and Cosmetic Science, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[b] School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[c] Ph.D Program in Toxicology, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

ARTICLE INFO

Article history:
Received 5 August 2014
Received in revised form
13 November 2014
Accepted 5 December 2014
Available online 15 December 2014

Keywords:
Tranexamic acid
Derivatization
Cylinder-sampling method
Capillary liquid chromatography

ABSTRACT

Tranexamic acid (TA) is widely used to treat medical hemorrhagic conditions and as a whitening agent in cosmetic products. The aim of this study was to develop a micro-analytical method of determining TA in widely varying sample matrices, including pharmaceuticals, cosmetics, extracts of the stratum corneum, human keratinocyte cells, and human plasma. Using dansyl chloride as the derivatizing reagent in the pretreatment reduced the reaction time to within 4 min in combined microwave-assisted reaction. To prevent excess dansyl chloride and sample matrices from damaging the column, 4 μL chloroform was used to remove excess derivatizing agent and interference through dispersive liquid–liquid microextraction (DLLME) method. After pretreatment, 1 μL of sample solution was injected in capillary liquid chromatography coupled with ultraviolet detector (CapLC-UV). By coupling the proposed method with cylinder-sampling method, TA was successfully extracted from the stratum corneum. The calibration curves for the standard solution and human plasma were in the ranges of 0.1–50 μM and 5–500 μM, respectively. Both calibration curves had correlation coefficients (r^2) of 0.999. The limits of detection were 0.03 pmol in standard solution and 3 pmol in plasma. Compared to non-derivatized TA, the use of CapLC-UV for detecting derivatized TA provides a 1000-fold sensitivity improvement.

1. Introduction

Tranexamic acid (*trans*-4-aminomethylcyclohexanecarboxylic acid, TA), a synthetic derivative of lysine, prevents plasmin formation by binding to the lysine binding site of plasminogen and then inhibits fibrin degradation [1–3]. Due to its potent antifibrinolytic activity, TA is widely used to treat hemorrhagic conditions [4–8]. In cosmetics, TA is also used as a whitening agent by reducing the precursor of phospholipase A_2. Since phospholipase A_2 participates in arachidonic acid production, TA reduces melanin production by inhibiting inflammatory factors [9,10]. Because it has some structural similarity to tyrosine, TA competitively inhibits tyrosinase activity. Therefore, TA reduces melanin synthesis and pigment formation [11].

The various analytical methods proposed for determining the presence of TA in dosage forms and in biological fluids include high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD) [12], UV detection [13–15], fluorescence detection [16–19], chemiluminescence detection [20], or mass spectrometry (MS) [21–23]. Gas chromatography (GC) coupled with MS [24,25] and capillary electrophoresis (CE) coupled with UV detection [26] can be used for TA analysis. Although conventional HPLC is a robust and reproducible method, its limitations are its high solvent consumption and high waste liquid production. Although TA can be directly detected by ECD, the required instrument is not widely available in laboratories. Detection by ECD is also problematic in high electrolyte environments. Because of its high reliability and low cost, UV detection is the most common method. Many studies have used UV detection to measure TA through chromophore attachment. Fluorescence detection is more sensitive than UV, but it requires strict detection conditions that decrease its ease of use. Although MS is a powerful detection technique that enables analyte qualification and quantification, its drawbacks are its high instrument cost and the need for operator

* Corresponding author at: 100, Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan. Tel.: +886 7 3121101 2805; fax: +886 7 321 0683.
E-mail address: chfeng@kmu.edu.tw (C.-H. Feng).

http://dx.doi.org/10.1016/j.chroma.2014.12.020
0021-9673/

training. In GC analysis, the limited volatility of TA can be increased by forming derivatives to improve detection and chromatographic performance. A shortcoming of CE is the short optical path length of the capillary and the low sample loading, which reduce sensitivity.

Because TA does not have a good chromophore for UV detection, a derivatization reaction is needed to determine trace amounts of TA. The many derivatization reagents evaluated for use in detecting TA include phenyl isothiocyanate (PITC) [13,14,27], 2-hydroxy naphthaldehyde (2-HN) [15], fluorescamine [16], o-phthalaldehyde [17,20], naphthalene-2-3-dicarboxaldehyde (NDA) [18,19] and ofloxacin acyl chloride (OAC) [26]. Although PITC reacts with the primary amino group in TA, the reaction conditions are violent, and the derivatization time is long (20–30 min at 40–60 °C). When using 2-HN, 5 mL blood is required for derivatization with adequate sensitivity. Although fluorescamine and o-phthalaldehyde have good reactivity, both derivatives are unstable and unsuitable for pre-column derivatization. The fluorescamine and o-phthalaldehyde reaction are applicable for post-column derivatization because neither fluorescamine nor o-phthalaldehyde has inherent fluorescence properties [28]. Additional pumps are needed to deliver reagents in post-column derivatization. The NDA reaction can be performed at room temperature in only 2 min, but it requires 200 μL platelet-poor plasma, which complicates sampling. Application of OAC is inconvenient because the derivatizing reagent is not commercially available. All commercially available derivatizing reagents require biosamples ranging from 200 μL to 5 mL, which is problematic when biosamples are unique or limited.

The derivatization reagent selected in this study was dansyl chloride (DNS-Cl) because of its good reactivity and stable derivatives. Microwave-assisted derivatization (MAD) was used to reduce the reaction time and to enable faster derivatization compared to conventional heating [29]. Derivatization efficiency was maximized by adding excess DNS-Cl in the reaction. Since excess DNS-Cl increased interference and decreased column durability, excess DNS-Cl was removed by adding amino reagents as described in the literature [30–33]. However, the products of amino reagents and DNS-Cl were not removed and were injected into the chromatograph. Accordingly, this study used dispersive liquid–liquid microextraction (DLLME) [34] to avoid interference caused by excess DNS-Cl and sample matrices. Generally, DLLME is used to extract hydrophobic analytes in massive aqueous phase. This new application of DLLME provides a clean-up function for water-soluble analytes in aqueous samples. After pretreatment, capillary liquid chromatography combined with UV detector (CapLC-UV) was used to decrease solvent consumption and to separate TA from various samples.

To evaluate its medical research applications, this microanalytical method was used to determinate TA content in pharmaceuticals and in human plasma since TA is commonly used as a hemostat in surgery. A study of applications of TA in cosmetics by D. Li et al. showed that TA affects melanocyte function and inhibits melanin expression [11]. Melanocytes are located in the basal layer of the skin structure and keratinocytes are found in the outer epidermis and stratum corneum. This study compared the percutaneous effects of TA in different cosmetic formulations and explored whether TA penetrates keratinocyte cells when used as whitening agent applied to the skin during daytime. Cylinder-sampling method was used to determine the amount of TA in skin after application of cosmetics containing TA. Earlier studies have used cylinder-sampling method to sample bacterial flora in human skin [35,36]. P. Williamson et al. [35] used nonionic surfactants to sample bacterial flora in human skin in a quantitative investigation of cutaneous bacteria. The present study used ethanol, which is absorbed through the skin [37], as the solvent for extracting TA from the stratum corneum. Fig. 1 shows the details of the extraction procedure. To investigate whether TA can penetrate keratinocytes, the developed method was used to compare different amount of TA in human keratinocytes (HaCaT cell) with or without UV exposure. Potential applications of the developed method in cosmetic science include determining (1) the TA content in cosmeceuticals, (2) the penetration of TA into the skin after application of cosmetics, and (3) the effects of UV on TA in human keratinocytes.

The micro-scale analytical method established in this study determines TA after MAD and DLLME pretreatment. The CapLC-UV provides high resolution separation and effectively determines TA content in pharmaceuticals, cosmetics, extracts of the stratum corneum, human keratinocyte cells, and human plasma. To confirm a successful derivatization reaction in human plasma, the derivative was identified with an LTQ Orbitrap mass spectrometer.

2. Material and methods

2.1. Chemicals and reagents

The TA, DNS-Cl, caffeine, sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4) and disodium phosphate dodecahydrate (Na_2HPO_4) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Potassium hydroxide (KOH), potassium bicarbonate ($KHCO_3$), sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), acetonitrile, ethanol, chloroform (TCM), ethyl acetate (EA), formic acid (98%) and hydroxyl chloride (HCl) were purchased from Merck (Darmstadt, Germany). Ethyl paraben was obtained from AccuStandard (New Haven, CT, USA). The HaCaT cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose, 4 mM L-glutamine, without sodium pyruvate (Hyclone, Thermo Scientific, USA) and supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel) and 100 U/mL penicillin–streptomycin (Hyclone). The experiment was performed in DMEM alone (no FBS, no penicillin–streptomycin). Water used for the experiment and mobile phase was purified in a Millipore Milli-Q Labo system. Stock solution of TA (50 mM) and working solution were prepared in water. Caffeine (internal standard for all samples except plasma, 150 μM) was prepared in acetonitrile/water (2:3, v/v) solution. Ethyl paraben (internal standard for human plasma, 150 μM) was prepared in methanol/water (1:1, v/v) solution. The TA, caffeine and ethyl paraben solutions were then stored at 4 °C. The DNS-Cl (10 and 20 mM) was dissolved in acetonitrile before use. Phosphate buffered saline (PBS) solution was prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.15 g Na_2HPO_4 in 1 L water.

2.2. Derivatization and extraction

2.2.1. Aqueous solutions of TA

The derivitization and extraction conditions were optimized by adding 30 μL of TA standard solution, 10 μL of 20 mM KOH and 15 μL of 10 mM DNS-Cl acetonitrile solution to a 1.5-mL centrifuge tube. The MAD was performed in a microwave oven (Panasonic NN-ST677) at 400 W for 4 min. After the reaction, 15 μL of 20 mM KOH and 4 μL of TCM were added to the solution. The mixture was thoroughly vortexed for 30 s and centrifuged at 14,800 rpm for 3 min. The TCM layer was removed, and 35 μL of 10 mM HCl was added. After adding a 50-μL quantity of EA, the solution was vortexed for 30 s. The mixture was centrifuged at 14,800 rpm for 3 min. The organic layer was dried at 40 °C for 15 min and then dissolved in 15 μL of a 150-μM caffeine solution. A 1-μL quantity of the solution was then injected into the CapLC system.

2.2.2. Human plasma samples

For analysis of human plasma, a 20-μL aliquot of plasma was mixed with 20 μL acetonitrile in a 1.5-mL centrifuge tube. The