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β-Cyclodextrin enhanced on-line organic solvent field-amplified sample stacking in capillary zone electrophoresis for analysis of ambroxol in human plasma, following liquid–liquid extraction in the 96-well format

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ABSTRACT

A field-amplified sample stacking (FASS) and capillary zone electrophoresis (CZE) method is described for the quantification of ambroxol hydrochloride in human plasma, following liquid–liquid extraction in the 96-well format. The separation was carried out at 25 °C in a 31.2 cm × 75 μm fused-silica capillary with an applied voltage of 15 kV. The background electrolyte (BGE) was composed of 6.25 mM borate–25 mM phosphate (pH 3.0) and 1 mM β-cyclodextrin. The detection wavelength was 210 nm. Clean-up and preconcentration of plasma biosamples were developed by 96-well format liquid–liquid extraction (LLE). In this study, FASS in combination with β-cyclodextrin enhanced the sensitivity about 60–70 fold in total. The method was suitably validated with respect to stability, specificity, linearity, lower limit of quantitation, accuracy, precision, extraction recovery and robustness. The calibration graph was linear for ambroxol hydrochloride from 2 to 500 ng/ml. The lower limit of quantification was 2 ng/ml. The intra- and inter-day precisions of lowest limit of quantification (LLOQ) were 9.61 and 11.80%, respectively. The method developed was successfully applied to the evaluation of clinical pharmacokinetic study of ambroxol hydrochloride tablet after oral administration to 12 healthy volunteers.

1. Introduction

Ambroxol (trans-4-(2-amino-3, 5-dibromobenzyl)-aminocyclohexanol, Fig. 1), a metabolite of bromhexine [1], is an expectorant and mucolytic agent, which could reduce the bronchial hyper-reactivity, stimulate the cellular surfactant production and increase the amount of antibiotic penetration [2–6]. In addition to the mucolytic action, it has also been reported to have a cough-suppressing effect, antioxidant and anti-inflammatory action [1,7,8]. Ambroxol is administered as hydrochloride in a daily dose of 30–120 mg using oral, rectal, inhalation and intravenous routes, which produces good results in the treatment of chronic bronchitis [9].

In order to carry out clinical pharmacokinetic studies of ambroxol hydrochloride, a rapid and sensitive analytical method was needed, which could allow its determination in plasma. Common separation methods had been reported for the determination of ambroxol in various matrices based on HPLC determinations with UV [10], electrochemical [11,12] and mass spectrometric detection [13–16], and gas chromatography determinations with electron capture [17,18] and mass spectrometric detection [19]. Some of these methods suffered from inadequate sensitivity or long analysis time. Although LC–MS/MS method can provide excellent sensitivity and short run time, the apparatus is expensive and the matrix effects are difficult to overcome. Capillary electrophoresis (CE) is rapidly developing as an alternative analytical tool with different separation mechanism to HPLC, because of its high efficiency, rapid separation, extremely low solvent consumption compared with HPLC, and small sample volume requirement [20]. What is more, the cost of capillaries is far cheaper than that of HPLC columns. Up to date, only Pérez-Ruiz et al. reported two CE methods for clinical analysis of ambroxol [9,21]. Disadvantages

Abbreviations: CZE, capillary zone electrophoresis; CE, capillary electrophoresis; FASS, field-amplified sample stacking; BGE, background electrolyte; LLE, liquid–liquid extraction; EOF, electroosmotic flow; HD, hydrodynamic; EK, electrokinetic; LC–MS/MS, liquid chromatography tandem mass spectrometry; UV, ultraviolet; HPLC, high-performance liquid chromatography; LLOQ, lower limit of quantification; IS, internal standard; QC, quality control sample.

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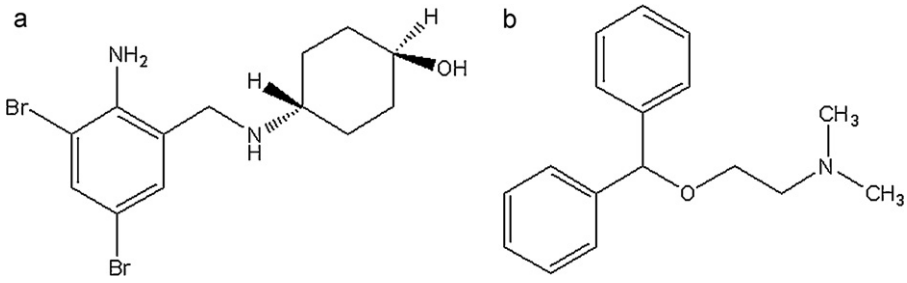

Fig. 1. Chemical structures of ambroxol (a) and internal standard diphenhydramine (b).

of these are tedious derivatization process [9] and low detection sensitivity and long analysis time [21], respectively.

To solve the problem of low sensitivity, field-amplified sample stacking (FASS) is usually employed as a simple and efficient technique in capillary zone electrophoresis (CZE), first introduced by Mikkers et al. [22,23]. It is based on a mismatch between the electric conductivity of the sample and that of the running buffer. When compared to injection from a nonstacking sample, conventional FASS provided a sensitivity enhancement of about 10- to 20-fold [24]. Shihabi [25] reported that some water-miscible organic solvents, especially acetonitrile and acetone, bring along greater degrees (~30 times) of stacking for the compounds compared to that for aqueous buffers or water. However, sometimes FASS could result in peak tailing and poor reproducibility, which is attributed to the introduction of matrix in large amount. Therefore, β-cyclodextrin which normally acts as carrier electrolyte additive in capillary electrophoretic (CE) techniques and chiral selector was reported to be used for improving peak shape and separation efficiency and obtaining good reproducibility of migration times [26].

In this paper, we tried to enhance sensitivity by means of organic solvent field-amplified sample stacking combined with β-cyclodextrin in capillary zone electrophoresis for the determination of ambroxol hydrochloride in human plasma. The method was evaluated in terms of selectivity, sensitivity, linearity, accuracy, precision and stability in accordance to the recommendations published by the FDA [27], and when combined with 96-well format liquid–liquid extraction, it was successfully applied to the analysis of ambroxol hydrochloride in clinical pharmacokinetic studies in 12 healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Ambroxol hydrochloride (99.4% purity) and the tablet formulation of ambroxol hydrochloride (30 mg, lot08030151) were from Jiangsu Hengrui Pharmaceutical Co. Ltd. (Jiangsu, PR China). Diphenhydramine hydrochloride (99.0% purity) was used as the internal standard (IS, Fig. 1) obtained from National Institute for the Control of Pharmaceutical and Biological Product (Beijing, PR China). $Na_2B_4O_7$, NaH_2PO_4 and β-cyclodextrin were acquired from China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, PR China). Human control plasma (sodium heparin as an anticoagulant) was obtained from Kunming General Hospital of Chengdu Military Command (Yunnan, PR China). Water was deionized and purified by using a Milli-Q system (Millipore, Milford, MA, USA) and was used to prepare all aqueous solutions.

2.2. Instrumentation

The employed CE system consisted of a Beckman P/ACE MDQ instrument (Beckman Coulter, Brea, CA) equipped with a photodiode array detection detector (PDA) and P/ACE System MDQ Software. Detection was performed at 210 nm, where ambroxol had the maximum absorption. Fused-silica capillaries (31.2 cm × 75 μm i.d., effective length 21 cm) were obtained from Hebei Yongnian Optical Fiber Factory (Hebei, China). 96-Well plate refrigerated centrifuge (Model SC210A, Thermo Electron, USA) was also used. The 2.0 ml Oasis® 96-well plates were purchased from Waters Corporation (Milford, USA).

2.3. Capillary electrophoretic conditions

The background electrolyte (BGE) used in this study was composed of 25 mM borate–25 mM phosphate (pH 3.0) and 1 mM β-cyclodextrin. It was prepared by accurately weighing 0.39 g phosphate and 0.24 g borate and making up to 100 ml with deionized water. After that, buffer pH was adjusted to 3.0 with 10% phosphoric acid. Then 0.1135 g β-CD was weighed and added into the borate–phosphate buffer. The capillary temperature was maintained at 25 °C and the separation voltage was 15 kV with the current of about 70 μA. The sample was introduced by using electrokinetic (7.5 kV × 15 s) injection modes. BGE were prepared freshly every day and filtered through a 0.45 μm hydrophilic cellulose membrane filter prior to use. A new capillary was conditioned by rinsing with 1 M NaOH, 0.1 M NaOH, H_2O and 0.1 M HCl (30 min each) sequentially. Daily conditioning before start-up was water (2 min), 0.1 M NaOH (10 min), water (2 min), and running buffer (10 min) in regular sequences. Between runs, the capillary was rinsed with 0.01 M NaOH, H_2O and separating buffer (2 min each) sequentially.

2.4. Preparation of stock solutions, calibration samples and quality control samples

Stock solutions of ambroxol hydrochloride and the IS were prepared in methanol at concentrations of 2 mg/ml and 1 mg/ml, respectively. The ambroxol hydrochloride stock solution was diluted with distilled water to working solutions ranging from 20 to 5000 ng/ml. A 250 ng/ml IS working solution was obtained by diluting the stock solution of IS with distilled water. All described solutions were protected from light, stored at 4 °C.

Calibration samples were obtained by diluting standard working solutions (20 μl) with drug-free human control plasma (180 μl), to span a calibration standard range of 2–500 ng/ml (2, 5, 10, 20, 50, 100, 200, and 500 ng/ml). Quality control (QC) samples (5, 20, 400 ng/ml) were independently prepared by spiking appropriate amount of the working standard solution in drug-free human control plasma.

2.5. Sample preparation

Samples were prepared using LLE in 96-well format plates. An eight-channel 300 μl electronic pipetting tool and an eight-channel 1200 μl electronic pipetting tool (Eppendorf Xplorer®, Eppendorf AG, Hamburg, Germany) were used for liquid transfer steps. Subject plasma samples were thawed at room temperature. 200 μl of