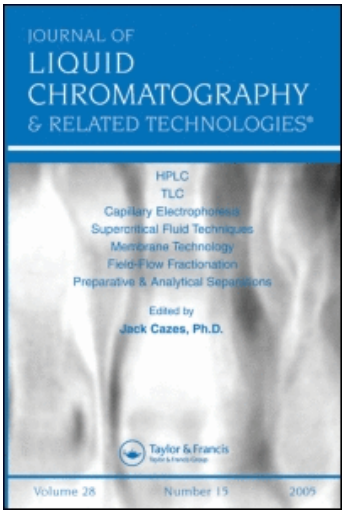

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Determination of Troxerutin in Troxerutin Tablets by Monolithic Capillary Electrochromatography

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Abstract: A new method to separate and determine the contents of troxerutin in troxerutin tablets by monolithic capillary electrochromatography (MLCEC) was established. The composition of the mobile phase, pH, its type and concentration, differently influenced the peak efficiency and resolution, and selectively modulated the interaction of analytes with the stationary phase. In the method, a poly-butyl methacrylate (PBMA) column (total length was 31.5 cm, effective length was 22.0 cm) was used. A mixture of 35 mM borax and acetonitrile (50/50) was used as the mobile phase. A pressure injection of 12 bar × 0.1 min of test solutions followed by an 8 bar × 0.1 min of the mobile phase was employed. The capillary temperature was 35°C and the operational voltage was 16 kV. The detection wavelength was set at 254 nm. Thiourea was used as the inner standard. The calibration curve showed good linearity over the range of $0.2022 \sim 0.8088\,\mathrm{mg \cdot mL^{-1}}$ ($r = 0.9991$); three level average recoveries were 100.9%, 98.9%, and 97.4% with RSD being less than 4.0%, respectively; LOD was $2.2\,\mu\mathrm{g \cdot mL^{-1}}$ and LOQ was $7.3\,\mu\mathrm{g \cdot mL^{-1}}$. The method was simple, rapid, accurate, reproducible with low consumption, and could be applied to the quality control of troxerutin tablets.

Keywords: Troxerutin, monolithic capillary electrochromatography, troxerutin tablets, determination

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INTRODUCTION

Hydroxyethylrutosides is a standardized mixture of semisynthetic flavonoids obtained by substituting hydroxyethyl groups in the naturally occurring flavonol rutin. It acts primarily on the microvascular endothelium to reduce the hyperpermeability and friability of micrangium, inhibit platelet agglutination and erythrocyte aggregation, prevent the thrombosis and angiosclerosis, and is commonly used for the relief of oedema and related symptoms in patients with chronic venous insufficiency.[1,2] Because there are four dissociation hydroxyls in rutin aglycone, some fifteen kinds of hydroxyethylrutins can be theoretically synthesized (four mono-, six di-, four tri- and one tetra-hydroxyethylrutosides); the proportion of the individual composition in hydroxyethylrutosides is related to the reaction conditions. Other hydroxyethylated constituents, such as tetra-hydroxyethyl-quercetin, in which the sugar moiety is absent, are also present in small amounts. Therefore, 7,3′,4′-hydroxyethylrutoside, namely 7,3′,4′-tris[*O*-(2-hydroxyethyl)]rutin (troxerutin, Figure 1) has the highest potency.

The good quality of the raw material of a drug and the finished product must include the related impurities in an analytical investigation, and this seemed particularly important to the quality control of troxerutin. Unfortunately, it is difficult to find the standards of the derivative impurities on the market, which sometimes makes the investigation impossible.

HPLC is the most commonly used method to control the quality of troxerutin and its preparations,[3–8] and even the concentration of troxerutin in serum.[9–12] Some other methods, such as TLC,[13] CE,[14] chemiluminescence,[15] ultraviolet spectrophotometry,[16–18] electrochemistry,[19] HPLC-MS,[20] CE-MS,[21] and coordination chemistry,[22,23] were also reported. Recently, the new development of the CE technology and instrumentation has allowed the use of CEC in the quality control of pharmaceuticals. CEC is the hybrid method that combines the advantages of both HPLC and CE, and owns characteristics of high performance, high selectivity, fast and low consumption. Together with hydrophobic interactions, the electrophoretic

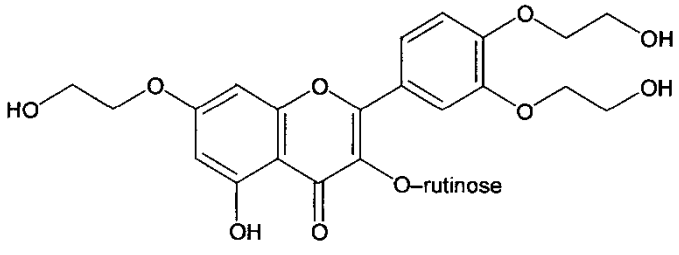

Figure 1. The chemical structure formula of troxerutin.

mobility of the analytes and several additional separation mechanisms, play an important role in the separation process. It is a powerful electrophoretic technique useful for the separation of substances belonging to different classes of compounds[24] and adapts to coupling with MS compared with CE. At present, the most widely used columns in CEC are packed with an alkyl-silica stationary phase similarly used in HPLC, but a drawback with this kind of column is that frits can result in the formation of bubbles, and additionally adsorb polar compounds, namely frit effects. Open tubular CEC columns are applicable to a rapid analysis of a mixture. Although no frits are used, the relatively low phase ratio in open tubular CEC restricts its further developments.

Recently, columns of MLCEC have attracted increasing attention because of their potential advantages,[25] which could be easily prepared by in situ polymerization,[26] and uniform porous organic polymeric continuous beds can be obtained. The monolithic columns have relatively high phase ratio and column capacity, no supporting frits and so there are no frit effects. In addition, the pore size of the stationary phase and its property could also be selected during the preparation procedure to obtain the optimal separation of various samples. Electroosmotic flow (EOF) is promoted by the incorporation of ionizable functional groups, such as acrylic acid, sulfonic acid, or ammonium monomers within the polymerized mixture. However, some shortcomings go with its advantages, such as that the swelling effect of the polymer can influence the stability and mechanical performance of the stationary beds and, unfortunately, the perfect uniform continuing beds are usually difficult to be obtained.

Papers reporting use of MLCEC were not found in the literature, though a few papers using CEC packed with ODS were reported.[27–30] In this paper, the possibility of using MLCEC to control the quality of troxerutin tablets was investigated. The suitability of MLCEC in the determination of the contents of troxerutin in troxerutin tablets was compared with that of HPLC.

EXPERIMENTAL

Reagents and Chemicals

Troxerutin standards were purchased from the College of Pharmacy, Shandong University (Shandong, China). n-Butyl methacrylate (BMA), ethylene dimethacrylate (EDMA), and γ-methacryloxypropyltrimethoxysiliane (γ-MAPS) were purchased from the Sigma Chemical Co. (St. Louis, MO). 1-Propanol, 1,4-butanediol, and azobisisobutyronitrile (AIBN) were obtained from the Fourth Shanghai Reagent Plant (Shanghai, China). All the chemicals used, such as thiourea, borax, etc. were of analytical grade,

and the solvents used, such as acetonitrile, all belong to the chromatographic grade (Dima Technology Inc, USA) without further purification. Water was distilled twice from a glass apparatus. Troxerutin tablets were purchased from the market.

Apparatus

All the experiments were performed on a HP^{3D} CE instrument (Agilent, Waldbronne, Germany), equipped with an on-column diode array UV absorbance detector and an autosampler. The instrument control and data acquisition were performed using ChemStation software for Windows 2000 on a Pentium 4 personal computer. The capillaries were cooled using a thermostated air bath. In the method, the MLCEC column (PBMA column, total length was 31.5 cm with effective length of 22.0 cm, 75 μm I.D. with 365 μm O.D.) was kindly supplied by Dalian Institute of Chemical Physics, the Chinese Academy of Sciences. The preparation of the monolithic polymer column can be referred to in the paper of Pin.[31]

Capillary Electrochromatography

A mixture of 35 mM borax and acetonitrile (50/50) was used as the mobile phase. The capillary conditioning was carried out with the mobile phase, applying 12 bar pressure accompanied by an electric voltage of 10 kV at the inlet end of the capillary. A stable current and a good baseline signal were monitored within about 20 min; a pressure injection of a 12 bar × 0.1 min of sample solutions, followed by an 8 bar × 0.1 min of the mobile phase was employed. The capillary temperature was maintained at 35°C. The operational voltage was 16 kV with 10 bar pressure at both ends of the capillary to prevent bubble formation. The detection wavelength was set at 254 nm. Thiourea was used as the internal standard.

Analytical Procedures

Standard Solutions

The troxerutin stock solution ($1.011\,mg \cdot mL^{-1}$) was prepared by dissolving the exactly weighed standard with water in a 10 mL volumetric flask. By dilution with water, a series of troxerutin solutions (concentration range of 0.2022 to $0.8088\,mg \cdot mL^{-1}$) were obtained. These solutions were used to control the linear correlation between absorbance values and concentrations.

Internal and Working Standard Solution

Thiourea standard solution was prepared by transferring 255.0 mg of thiourea, exactly weighed, in a 100 mL volumetric flask, dissolving and diluting up to the mark with water. Exactly 1 mL of thiourea and 5 mL of troxerutin solutions were transferred in a 10 mL volumetric flask and diluted up to the mark with water. Troxerutin ($0.5055\,mg \cdot mL^{-1}$) with $0.255\,mg \cdot mL^{-1}$ thiourea solution was obtained and used to point out the analytical conditions.

Analysis of Tablets

A tablet to be analyzed, containing 100 mg of hydroxyethylrutosides, had a middle weight of about 300 mg. The sugarcoating of the tablet was striped and twenty tablets were exactly weighed and accurately crushed. About 100 mg of the powdered material were weighed. This weighed powder was transferred into a 50 mL beaker, partially filled with about 15 mL of water. The mixture was put in an ultrasonic bath for 10 min. Afterward, 2.5 mL of the internal standard solution was added to the supernatant and transferred; the solution mixture was filtrated through a 0.45 μm filter membrane in a 25 mL volumetric flask. The extraction was repeated using about 8 mL of water, then was added to the volumetric flask. The solution was filled up to the mark with water and used for the analysis by MLCEC. The stability of the sample solution at ambient temperature was observed for 54 h and showed no evidence of decomposition (RSD was 4.3%). By comparing the migration times of peaks in the sample electropherograms with that of the standard, we identified the troxerutin peak.

RESULTS AND DISCUSSION

Investigation of the Mobile Phase

The analytical results were influenced more by the kinds of buffer electrolyte and their concentrations than various organic modifiers; with the increase of the proportion of the organic modifier in the mobile phase, as that in CE, the retention time was prolonged. This was likely to be the reason that the hydroxyethylrutosides had a weak interaction with PBMA, which had a shorter carbon chain. So, electrophoretic mechanism exerted the chief function to chromatographic mechanism in this MLCEC separation.

EOF in the MLCEC column mainly comes from the dissociation of the sulfonic group of AMPS, directed to the cathode. Because the dissociation

constant of the sulfonic group was far greater than that of the silica hydroxyl group, a bigger EOF could also be obtained at low pH. This MLCEC column is stable at the pH range of 2–12, and we could optimize the analytical conditions at a rather wider pH scope compared with the silica gel based solid phase.

When Tris was used as buffer electrolyte, hydroxyethylrutins couldn't make any separation; only a wide high peak could be observed. When phosphate and ammonium acetate were separately used as buffer electrolyte, the resolution of the peaks were restrictedly improved. When borax was used as buffer electrolyte, content resolution of the peaks could be obtained through optimizing analytical conditions. Borax ($Na_2B_4O_7 \cdot 10H_2O$) can hydrolyze in water to boracic acid [$B(OH)_3$], the dissociation of $B(OH)_3$ forms $[B(OH)_4]^-$, which can interact with the cis-orth- dioxyhydroxyl groups of hydroxyethylrutosides and can be profitable for the separation of troxerutin. For this reasons borax was used as buffer electrolyte in the experiment.

The pH of the mobile phase was adjusted to 3.0, 5.0, 7.0, 9.2, 10.0, and 11.0, respectively. There was nearly no peak resolution at pH 3.0 or 5.0. With the increasing of pH, the resolution increased but basically no change occurred when the pH value exceeded 9.0 for the troxerutin peak and its adjacent peak, and the retention time of the posterior peaks became longer and longer. This was because at the low pH, borax was hard to dissociate and difficult to form complexes with hydroxyethylrutosides, due to the similar apparent mobility and the weak interaction with the solid phase, the constituents in the hydroxyethylrutosides had nearly the same retention time and then caused a bad separation performance. With the increase of pH, the dissociation constant of borax increased and, hence, strengthened the action of $[B(OH)_4]^-$ with hydroxyethylrutosides. Thus, the constituents of hydroxyethylrutosides had different apparent mobility and their separation was achieved. Furthermore, the electronegative complexes made the retention time longer and longer. Borax solution (35 mM) was selected to form the mobile phase. Content resolution, shorter analytical time, and relatively simple operation could be gained.

Attempts were made to use methanol as an organic modifier, the peak shape was preferable, but some peaks could only show low resolution. The current and EOF could be strongly depressed when isopropanol was used as an organic modifier, the posterior peaks could obtain good resolution, but the peaks seriously extended with insupportable long analytical time, and the resolution of anterior peaks were apparently reduced. When acetonitrile was used as an organic modifier, shorter analytical time, content peak resolution, and column efficiency could be obtained by modulating the concentration of the borax solution and the proportion of acetonitrile in the mobile phase. In the end, a mixture of 35mM borax solution and acetonitrile (50/50) was chosen as the mobile phase.

Investigation of the Separation Voltage

The relationship of voltage (5 kV, 8 kV, 12 kV, 16 kV, and 20 kV) to corresponding current was investigated. The linear regression equation between them was as follows:

$$A = 1987.4V - 2.4413, \quad r = 0.9970$$

The result indicated the Joule heating effects could be neglected at the voltage of 5–20 kV. The better separation performance and shorter analytical time could be obtained when operational voltage was set at 16.0 kV.

Selection of Injection Mode

Three injection modes of electroosmosis, pressure, and pressure combined with electroosmosis were investigated under the same experimental conditions. When electroosmosis injection was used, the shapes and resolution of the peaks were very bad. The case could be improved when pressure combined with electroosmosis injection was used. But, lower column efficiency was observed in the above two injection modes. That the electronic discrimination in the electroosmotic injection caused the constituents in the injection band to distribute somewhat broadly and unevenly might be the reason for this result. The best shapes and resolution of the peaks were obtained when pressure injection was employed among them. The absolute sample size was difficult to control due to the external pressure above 1 bar not being able to be as precisely delivered as 50 mbar. To significantly reduce the injection-related imprecision,[32,33] an internal standard method was employed to correct the sample injection size, and a small band of the mobile phase was introduced to prevent the sample loss at voltage application, and therefore, the system precision was improved.

Influence of Column Temperature on Separation Performance

The compound distribution ratio between the mobile phase and stationary phase could be changed with the column temperature. The overall peaks resolution was increased with the heightening of column temperature, and a shorter analytical time could be obtained due to the decrease of the viscosity of the mobile phase. But, very low column efficiency and a discontent separation between the troxerutin peak and its preceding adjacent peak were observed at 40°C. The column temperature of 35°was selected. The influence of the temperature on the column efficiency and the electropherograms of the standard and sample were as Figures 2 and 3, respectively.

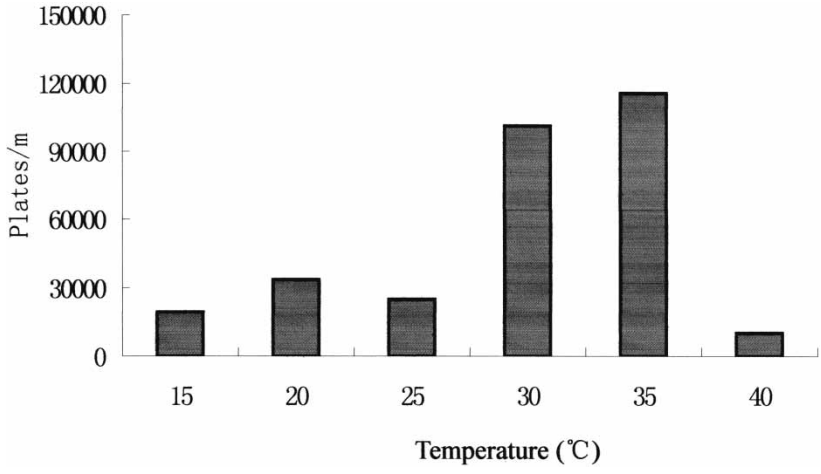

Figure 2. Influence of temperature on column efficiency.

Calibration Curve, Detection, and Quantity Limits

The calibration curve for troxerutin was set up on standard solutions in the suitable concentration range of 0.2022–0.8088 $mg \cdot mL^{-1}$. In the considered concentration ranges, good linearity was found. The calibration curve was obtained by plotting the peak area ratio of troxerutin to thiourea(Y) versus its concentration(X); the regression equation was as follows:

$$Y = 2.275X + 0.021; \quad r = 0.9991$$

The LOD ($S/N = 3$) and LOQ ($S/N = 10$) of troxerutin were 2.22 and 7.33 $\mu g \cdot mL^{-1}$, respectively.

Method Suitability Tests

The theoretical plate number exceeded 10^5 plates $\cdot m^{-1}$. The precision RSD of the developed method, in terms of peak area ratio of troxerutin to thiourea was 0.9% ($n = 6$); so, the precision was acceptable.

Method Reproducibility

Six replicate samples were prepared to be injected and to determine the contents of troxerutin. The RSD was 1.5%, which denoted that the method reproducibility was good.

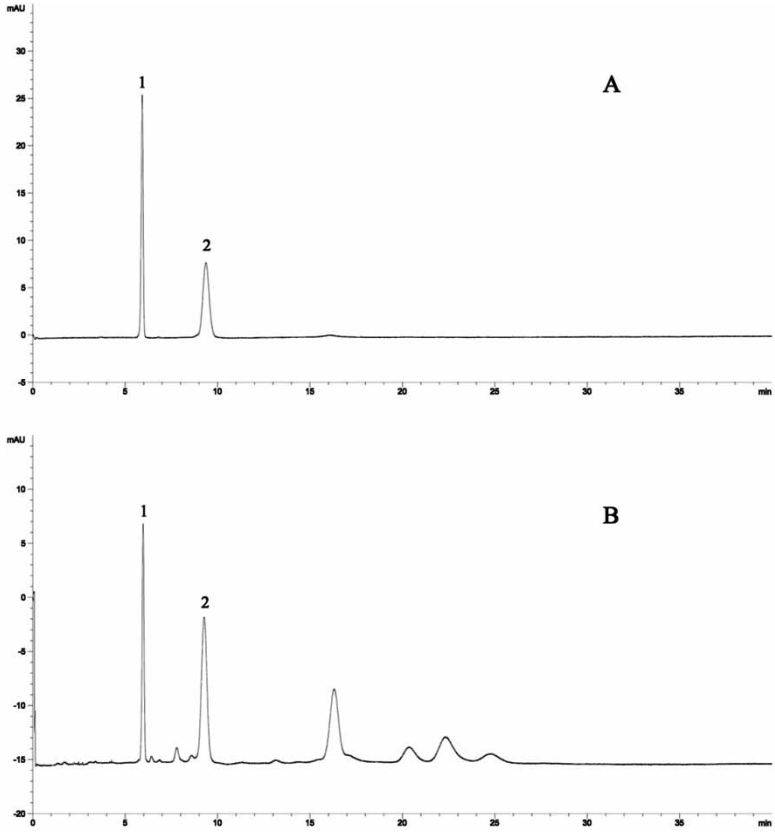

Figure 3. Electropherograms of standard and sample. A. Standard; B. Sample (1. Internal standard; 2. Troxerutin).

Recoveries

The proper amount of troxerutin was added to the corresponding prescription adjuvant to form 0.2022, 0.4044 and, 0.6066 $mg \cdot mL^{-1}$ solutions. The solutions were extracted and measured under the above experimental conditions. The recoveries of three levels from the model tablet were 100.9% (RSD 1.3%), 98.9% (RSD 3.4%), 97.4% (RSD 1.5%), respectively. The results indicated that the accuracy of the method was favorable.

Application to Troxerutin Tablets of Different Lots and Factories

The quantitative determination of the active compound in troxerutin tablets, made by MLCEC was summarized in Table 1, together with the HPLC

Table 1. Determination of the samples (mg/tablet)

Lot no.	CEC method	HPLC method
1	49.2	51.9
2	45.0	47.5
3	61.4	60.4
4	24.6	23.4
5	30.2	30.8
6	35.6	38.5
7	37.7	38.4

data.[34] The results of the two methods presented no significant difference, which was verified by the t test ($\alpha = 0.05$).

CONCLUSIONS

A method to separate and determine the contents of troxerutin in troxerutin tablets by MLCEC was established. If the reference substances of the other hydroxyethylrutosides could be obtained, further investigation could be implemented. Thus, we could deeply understand the quality of the product and the manufacturer could be guided and the curative effect might be improved.

The optimization of the MLCEC methods for the analysis of charged compounds particularly requires the careful investigation of different physicochemical parameters, e.g., the mobile phase composition, buffer type, pH, and concentration. The chromatographic process was weak in the paper, but the separation effect could be improved by optimizing the kind and ratio of the monomer in the preparation of the monolithic bed. And this also showed the flexibility of MLCEC. Our work showed that in the quality control of troxerutin, MLCEC provided the potential selectivity of HPLC and high efficiency of CE. MLCEC has received more and more attention because of its tempting potentials and broad prospects.

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