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Short communication

Determination of a novel xanthine derivative, MKS 213-492, in plasma by high-performance liquid chromatography with electrochemical detection

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Abstract

A reversed-phase liquid chromatographic method with coulometric detection has been developed for the determination of free MKS 213-492 (a novel xanthine derivative) in plasma. This method is based on a simple and rapid plasma extraction procedure using precolumn-switching. The oxidation of this pharmacologically active xanthine derivative was optimized with respect to applied potential, pH of mobile and rinsing phase and rinsing time. The detection limit for MKS 213-492 was found to be 54 pg/injection.

1. Introduction

The substance MKS 213-492 (I) is a xanthine derivative and therefore pharmacologically active, blocking phosphodiesterase and enhancing membrane permeability for calcium ions. Substances containing this purine structure are used in the treatment of asthma, cor pulmonale and cardiac insufficiency.

Analysis of biological samples for pharmacokinetic investigations involves time-consuming sample preparation and chromatographic separation and detection. Therefore, a liquid–solid extraction with a precolumn switching system for fully automated sample extraction should increase the number of separations per unit time.

Electrochemical detectors have been demonstrated to be very useful in high-performance liquid chromatography. For compounds with a redox center, the combination of HPLC with electrochemical detection (ED) offers excellent sensitivity and selectivity.

A number of organic cyclic systems containing nitrogen atoms has already been studied using oxidative HPLC-ED; these include indole derivatives, hydroxyindoles [1–3] and phenothiazines [4–6]. Purine structures [7] are also known to be electrochemically active. Thus, it was only logical to apply electrochemical detection for the quantitation of I in plasma.

2. Experimental

2.1. Chemicals and solutions

All chemicals were of analytical grade. Metha-

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nol was purchased from Loba (Vienna, Austria) and had to be distilled before use to eliminate impurities that could influence the quality of the detection by causing peaks and high background currents.

The water for eluents and solutions was processed with a nanopure ultrafiltration unit. Sodium dihydrogenphosphate and disodium hydrogenphosphate, phosphoric acid as well as citric acid were obtained from E. Merck (Darmstadt, Germany).

Compound I was obtained from Sandoz (Basle, Switzerland). This substance represents the physiologically active *R*(+)-enantiomer.

Preparation of the rinsing phases: A 0.1 *M* citric acid solution was added to a 0.02 *M* disodium hydrogenphosphate solution until the pH of the solution indicated the desired concentration of protons. Degassing of the rinsing phase was not necessary.

Preparation of solutions of I: As the analyte is only slightly soluble in water, I was dissolved in the mobile phase.

Before the plasma samples were injected onto the chromatographic system, they were diluted with different amounts of water.

Spiked plasma samples were prepared by mixing human plasma with 100 μl of solutions of I with different concentrations. After vortex-mixing for 30 s, the samples were centrifuged for 5 min at 2000 rpm.

Quantitation of I was performed by external standardization via a calibration curve. Concentrations between 54 pg and 50 ng were used.

2.2. Chromatography

To determine I, two chromatographic systems, the analytical and the washing system, were linked by a switching valve.

Analytical system: A Perkin-Elmer Series 10 solvent delivery system fitted with a Rheodyne 7125 injection valve equipped with different loops (20 to 500 μl) was used for the separation of plasma samples in conjunction with an analytical Brownlee Spheri-5 column, 100 × 4.6 mm I.D., packed with RP 8, 5-μm particle size.

The mobile phase consisted of methanol–0.02 *M* phosphate buffer (a 0.02 *M* sodium dihydrogenphosphate solution adjusted with phosphoric acid to pH 4.5) (50:50, v/v), maintained at a flow-rate of 1.0 ml/min.

A Swagelok Bourdon tube was used to dampen pulsation. The detector was an ESA Coulochem 5100A with a 5020 guard cell and a 5010 analytical cell from Environmental Sciences Associates (MA, USA). The analytical cell contained palladium reference electrodes.

Rinsing system: The rinsing phase, a 0.02 *M* phosphate–citrate buffer, was delivered by a LCD Milton Roy minimetric pump II at a flow-rate of 1.0 ml/min. No high-efficiency pulsation dampener was necessary for this system.

The precolumn (40 × 4.0 mm I.D.) used for the on-line extraction of the analyte was packed with Perisorb RP 18, 30-μm particle size.

The central switching valve, valve 2, common to both systems and used for the actual precolumn switching, was a 7100 Rheodyne 6-port valve.

2.3. Hydrodynamic voltammogram

The voltammetric characteristics of the compound were determined under the chromatographic conditions by repeatedly injecting the sample at different working electrode potentials. The initial injection was made at a low potential applied between working and auxiliary electrode. The potential was increased in 100-mV steps. The background current was allowed to stabilize before each injection.

To obtain a hydrodynamic voltammogram of I, the measured peak heights were plotted against applied potentials.

2.4. Sample preparation

As direct injection of plasma samples of I would contaminate the stationary phase of the analytical column, the samples were purified on-line after injection by solid-phase adsorption on a C_{18} precolumn. This technique allows the application of plasma samples, diluted 1:1 with water, to the chromatographic system up to a volume of 500 μl.

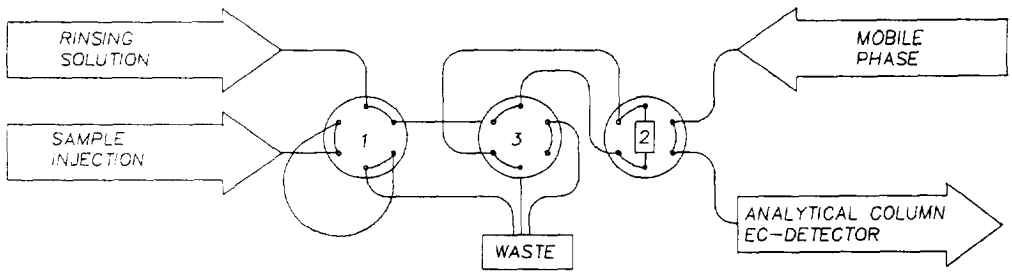

Fig. 1. Switching system for electrochemical detection of I in plasma.

2.5. Schedule of precolumn switching

The procedure for the determination of I may be divided into four steps (Fig. 1).

Step 1: 1.0 ml of the spiked plasma sample is diluted with 1.0 ml of water. This mixture is injected into the 200-μl loop of the injection valve (valve 1).

Step 2: Valve 1 is turned to transfer the plasma sample via the rinsing phase to the precolumn. There, the analyte is retained while plasma components are eluted. Following a 2-min rinsing phase, valve 3 is turned to inverse the rinsing flow for 2 min.

Step 3: After this 4-min rinsing phase, the precolumn is switched into the analytical system by turning valve 2. The analyte is eluted from the precolumn by the mobile phase and, hence, transferred to the analytical column.

Step 4: After the chromatographic separation of I from plasma by-products, the substance is oxidized in the coulometric cell at an operating potential of +0.4 V.

The total run time is about 15 min.

3. Results and discussion

3.1. Electrochemical activity

As presumed, I showed electrochemical activity. The structure responsible for this activity is assumed to be the nitrogen atom 7 in the xanthine molecule. The optimal detection potential was found by plotting a hydrodynamic voltammogram versus a palladium reference electrode. Fig. 2 shows the dependence of detector current on the detector working electrode potential. A maximum current (transport limited) is obtained at potentials above +0.4 volts.

The low quantitation potential compared with that given by Metrohm for similar xanthines [8] is caused by the palladium reference electrode and by the substituents of this molecule.

3.2. Optimization of rinsing phase

The rinsing system is also a chromatographic system using differences in polarity to separate I from plasma components, which if present would make sensitive electrochemical detection almost impossible. pH optimization from pH 3.5 to 8.0 showed a response maximum at pH 4.5. Fig. 3 depicts the dependence of signal intensity on pH in the range of pH 3.5 to 5.5. Using pH 4.5

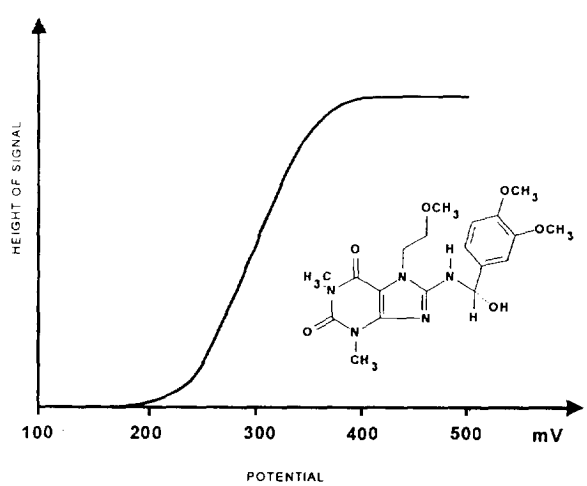

Fig. 2. Structure and hydrodynamic voltammogram of I.

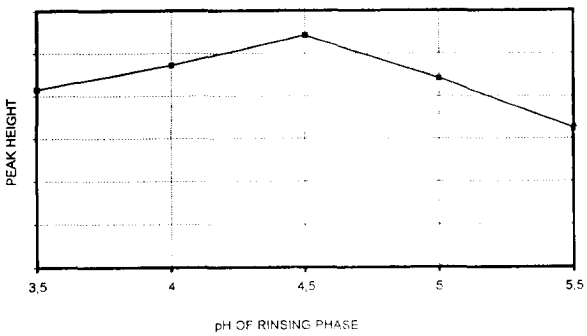

Fig. 3. Dependence of sensitivity of electrochemical detection on pH of rinsing phase.

produced an improvement of about 35% compared to pH 5.5.

As can be seen from Fig. 1, the switching system consists of three valves. The great advantage of this technique is that turning valve 3 changes the flow direction of the rinsing solution. Particles which despite centrifugation of the plasma samples may be injected onto the rinsing system are adsorbed on the sieve of the precolumn. Using inverse flow direction, these particles are directly eluted to waste and so do not impair chromatographic performance. This procedure did not influence peak shape.

Comparing RP 2, RP 8 and RP 18 as precolumn materials and using different rinsing times from 1 to 30 min, the optimal rinsing time turned out to be 4 min with an RP 18 precolumn. Under the latter conditions, most plasma components are washed out without loss of analyte.

3.3. Optimization of HPLC system

The HPLC system used for this kind of multidimensional column switching is shown in Fig. 1. It consists of two independent chromatographic systems, the analytical and the rinsing component. Detailed information of the complete analytical procedure including extraction, separation and detection is given in the Experimental section.

As desorption of I from the precolumn and its separation on the analytical column are connected inseparably in this system by the eluent, the nature of the mobile phase had to be evaluated. Three different ratios of methanol–buffer (1:4, 1:1, 4:1, v/v) were tested with respect to optimal sensitivity and selectivity. Using a transfer time of 3 min, the ratio of 1:4 leads to the elution of only 52% of the analyte from the precolumn. The ratio of 4:1 provides to little polarity for satisfactory separation. The methanol–buffer (1:1, v/v) showed the best results with a t_R of 4.5 min for I and was used throughout this work. On one hand, this mobile phase eluted the analyte quantitatively from the precolumn within the transfer time and on the other hand allowed its separation from the remaining plasma by-products not washed out by the rinsing phase.

Generally the flow-rate was 1.0 ml/min. The flow of mobile phase was seen to be limited by the resistance of the whole system. In addition to HPLC components commonly used, the guard cell and the analytical cells as well as the security graphite filters in the electrochemical detection system cause higher resistance from the beginning of analysis. Furthermore, the pulse dampening system, the column for precolumn switching and the repetitive injections of plasma samples increase the pressure resistance of the chromatographic system. Thus, flow-rates higher than 1.0 ml/min, which would be advantageous for series analyses, are inapplicable.

3.4. Optimization of detection

To enhance selectivity, detection was carried out at cell 2 of the analytical detection unit using a quantitation potential of +0.4 V. Cell 1 was set at +0.2 V, a potential where no oxidation of the analyte occurs, but impurities with half-wave potentials less than that of the compound of interest are preoxidized. This means that these impurities cannot increase the background current.

Apart from the technical basis for successful electrochemical detection of I (e.g. pulse dampening system, grounding etc.), the pH of the mobile phase turned out to be a very important parameter, because both the working potential

and the response depend on it. As shown by Surmann [9], changing of pH can increase sensitivity of electrochemical detection by a factor of 20 or more. Unfortunately, this influence is hardly predictable, and so pH has to be optimized separately for each analytical system. This was done using eluents with different pHs ranging from 3.5 to 8.0; pH 4.5 proved to yield the highest response.

The electrolyte used for these electrochemical investigations was a phosphate buffer. Phosphate ions seem to be something like a standard electrolyte in electrochemical detection. Our work confirmed the fact that this supporting electrolyte improved signal intensity by 10% compared to lithium perchlorate.

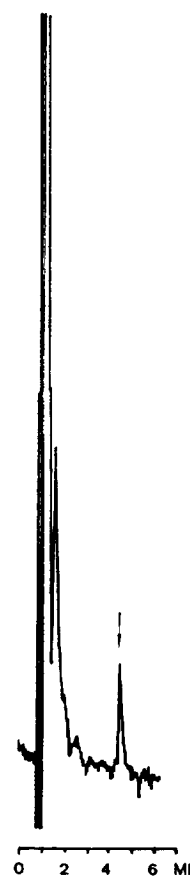

Fig. 4. Injection of 54 pg of I.

3.5. Stability of the chromatographic system

Approximately 7 ml of plasma, corresponding to 70 samples, can be injected without adverse effects on extraction quality. No decrease in peak shape quality and analytical column performance was observed after about 300 injections. Within this period the guard cell and the analytical cells did not require maintenance.

3.6. Statistical data

Recovery of I from plasma was estimated by comparing the peak height after an injection of pure I solution with the peak height after the injection of spiked plasma samples containing the same dose of I ($n = 8$). The recovery was 100.5% (C.V. 1.02%) using a concentration of 500 ng/200 μl and 5.1% at a concentration of 50 ng/200 μl. Linearity is obtained from 50 pg up to 100 ng per injection, slope: 37.4 ($\pm$ 2.0), intercept: 14.0 ($\pm$ 0.9). The correlation coefficients were between 0.998 and 0.999. The limit of detection was 54 pg/injection (Fig. 4).

4. Conclusions

The major advantage of this method is the avoidance of time-consuming sample preparation when plasma samples are extracted before injection.

The method developed is practical, simple, time saving and reproducible. Furthermore, this technique allows detection of I both sensitively and selectively and can be used in a fully automated system for series analysis.

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