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Short communication

Determination of monohydroxyethylrutoside in heart tissue by high-performance liquid chromatography with electrochemical detection

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Abstract

7-Monohydroxyethylrutoside (monoHER) is one of the components of the registered drug Venoruton. It showed a good protection against the cardiotoxic effects of doxorubicin. The analysis of monoHER was developed to study the pharmacokinetic profile of the drug in heart tissue. MonoHER was extracted from heart tissue homogenate with methanol. The supernatant was diluted 1:1 (v/v) with 25 m*M* phosphate buffer and injected onto a reversed-phase ODS column. The mobile phase consisted of 49% methanol and 51% of an aqueous solution containing 10 m*M* sodium dihydrogenphosphate (pH 3.4), 10 m*M* acetic acid and 36 μ*M* EDTA. The retention time of monoHER was about 5.2 min and no endogenous peaks were interfering. The lower limit of quantification was 0.072 nmol g^{-1} wet heart tissue. The calibration line was linear up to 24 nmol g^{-1}. The within-day accuracy and precision of the quality controls (0.12, 1.2 and 12.0 nmol g^{-1}) were smaller than 17 and 19%, respectively. The between-day accuracy and precision were better than 6 and 11%, respectively. The recovery of monoHER from heart tissue ranged from 104.1 to 114.3% and was concentration independent. MonoHER was stable in heart tissue when stored at −80°C for 6 months. Repeated injection of monoHER from aliquots of 7.2 nmol g^{-1} placed on the sample tray at 4°C for 24 h showed a decrease in the concentration of 30.3%. Analyzing sample duplicates in a mirror image sequence could compensate for the influence of this gradual decrease. The small sample volume allowed one to measure monoHER in the hearts of mice.

Keywords: Monohydroxyethylrutoside

1. Introduction

7-Monohydroxyethylrutoside (monoHER), a semi-synthetic flavonoid, is one of the components of the hydroxyethylated rutoside (HER) mixture constituting Venoruton. It protected against doxorubicin-induced cardiotoxicity without interfering with the antitumor effect of doxorubicin [1]. Because mice are used as a model to study doxorubicin-induced cardiotoxicity, the study of the pharmacokinetics of monoHER in heart tissue of mice is necessary to understand its mode of action. For that reason a sensitive and selective method is required to measure the levels of monoHER in heart tissue.

Because of their wide range of therapeutic and pharmacological activities [2], HERs were measured by different methods, e.g., thin-layer chromatography

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(TLC), high-performance liquid chromatography (HPLC) and circular dichroism to study its metabolism and pharmacokinetics in man and animals [3]. Few studies measured the levels of HERs in the tissues of mice [4,5]. More studies measured the levels of HERs in the body fluids of various animal species [6–8]. Methods used were based on measuring the radioactivity or fluorescence of HERs after separation with HPLC or TLC. The drawbacks of these methods are the low sensitivity and selectivity and the lack of simplicity.

More sensitive methods were developed to measure the levels of other flavonoids. Quercetin levels were measured in human plasma using HPLC with diode array detection [9]. Flavopiridole, a synthetic flavonoid used as an anticancer drug, was measured in the bile and liver perfusates of the rat using HPLC with UV detection [10]. [H^3]α-Naphthoflavone metabolism, by liver microsomes of the rat, was also studied using HPLC separation with subsequent radioactivity measurement [11]. Although these methods were more sensitive than the previously mentioned procedures, they were not sensitive enough to study the pharmacokinetics of monoHER in the hearts of mice.

So far, no sensitive assay for measuring non-radioactively labeled monoHER in tissues was developed. To study the pharmacokinetics of monoHER and a possible pharmacokinetic interaction between monoHER and doxorubicin in heart tissue of mice a sensitive and specific assay had to be developed. Therefore, the objective of this study was to develop and validate a sensitive and selective method for the determination of monoHER in heart tissue.

2. Materials and methods

2.1. Chemicals

7-Monohydroxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). Acetic acid, acetone, *o*-phosphoric acid (85%), potassium chloride, sodium bisulfite, silver chloride and sodium dihydrogenphosphate monohydrate were purchased from Merck (Amsterdam, The Netherlands). EDTA was from Sigma–Aldrich (Zwijndrecht, The Netherlands). Methanol, HPLC grade was from J.T. Baker (Deventer, The Netherlands).

2.2. Instrumentation

The HPLC system consisted of a Gynkotec solvent delivery system 300, a Spark Basic Marathon autosampler with a cooled sample tray (4°C) and a degasser GT-103 (Separations, H.I. Ambacht, The Netherlands). A Decade electrochemical detection (ECD) system was used provided with an integrated thermostat for the column and the cell, a glassy carbon-working electrode and an Ag/AgCl reference electrode (Antec Leyden, Leiden, The Netherlands). The electrochemical detector was set at +0.7 V vs. Ag/AgCl. The thermostat was set at 35°C. The chromatographic data were stored and handled by a Dell Dimension XPS p166s computer (Dell, Amsterdam, The Netherlands) provided with a Gynkotec Chromeleon chromatography data system (Separations).

The separation was performed with a YMC ODS-AQ, 3 μm, 150×4.6 mm I.D. reversed-phase analytical column (Bester, Amsterdam, The Netherlands) protected by a Chrompack, SS 10×2 mm, C_{18} guard column (Chrompack, Bergen op Zoom, The Netherlands). The mobile phase, consisting of 49% methanol and 51% of an aqueous solution containing 10 m*M* sodium dihydrogenphosphate, 10 m*M* acetic acid and 36 μ*M* EDTA (v/v/v), was used with a flow-rate of 0.7 ml min^{-1}.

2.3. Sample preparation

Two stock solutions of 0.2 m*M* monoHER (one for the standards and one for the quality control samples) were freshly prepared in a methanol–25 m*M* phosphate buffer (4:1, v/v) mixture, pH 3.33. Bovine heart was dismembranated and tissue powder was dissolved in 25 m*M* phosphate buffer (pH 3.33) containing 0.4% sodium bisulfite to obtain a homogenate of 125 mg tissue ml^{-1}. This homogenate was used to prepare the samples. Because of the reactivity of monoHER as an antioxidant, the samples had to be prepared freshly before each chromatographic run, while keeping all samples and

solutions on ice during handling. Standards (0.07, 0.24, 0.72, 2.4, 7.2 and 24 nmol g^{-1}) and quality control samples (0.12, 1.2 and 12.0 nmol g^{-1}) were prepared by mixing the appropriate amounts of tissue homogenate with the corresponding stock solution.

The samples were processed in duplicate by deproteinizing 77 μl of sample with 123 μl methanol. After 15 min of shaking at 4°C the samples were centrifuged (3 min, 1°C, 13 500 *g*) and 130 μl supernatant of each sample was transferred to a polypropylene micro test tube (1.5 ml, Eppendorf) containing 130 μl (25 m*M*) phosphate buffer, pH 3.33. To be sure that all possible precipitate had been spun down the sample was centrifuged again (3 min, 1°C, 13 500 *g*) and the supernatant was transferred to a new polypropylene micro test tube. The calibration samples and the quality control samples were then placed on the cooled sample tray and injected onto the HPLC system.

For recovery experiments, quality control samples were freshly prepared in tissue homogenate and in the MeOH–phosphate buffer mixture and analyzed in duplicate.

2.4. Stability of the samples and the detector

The stability of the samples during a run was tested by the repeated injection of aliquots of a freshly prepared methanol extract of a tissue homogenate containing 7.2 nmol g^{-1} monoHER. The autosampler (4°C) was adjusted to inject one sample per hour for 24 h.

Because some fresh and 6-month-stored hearts of mice obtained shortly after administration of monoHER were available, we could obtain an indication about the stability of monoHER in heart tissue stored at −80°C. Four hearts, freshly taken from mice at 0.5 ($n=2$) and 1 h ($n=2$) after the intraperitoneal (i.p.) administration of 500 mg kg^{-1} monoHER, were analyzed immediately. Two hearts, obtained at the same times, were analyzed after 6 months of storage at −80°C.

2.5. Sample analysis

To correct for a possible gradual decrease of the detector signal during a run, either caused by a drift of the detector signal or the instability of the samples on the cooled tray, the calibration samples (C), quality control samples (QC) and samples (S) were analyzed in duplicate in the following reverse (mirror image) order: $C_1, \ldots, C_n$, $QC_1, \ldots, QC_3$, $S_1, \ldots, S_n | S_n, \ldots, S_1$, $QC_3, \ldots, QC_1$, $C_n, \ldots, C_1$. In this way the mean of the duplicates compensated for a possible gradual decrease of the detector signal and the stability of the samples.

The concentrations of the samples were calculated by interpolating the peak areas of the samples (mV min) on the calibration line obtained by linear regression using a weighting factor of $1/y$.

3. Results and discussion

3.1. Analysis

Representative chromatograms of monoHER in heart tissue of mice obtained at 30 min after i.p. treatment with 500 mg kg^{-1} monoHER and bovine heart tissue spiked with 7.2 nmol g^{-1} monoHER in comparison to blank bovine heart are shown in Fig. 1. The retention time of monoHER was about 5.2 min and no endogenous peaks were interfering.

The lower limit of quantification (LLQ), the lowest concentration that could be analyzed with an acceptable accuracy and precision, i.e., less than 20%, was 0.072 nmol g^{-1}. The within-day accuracy

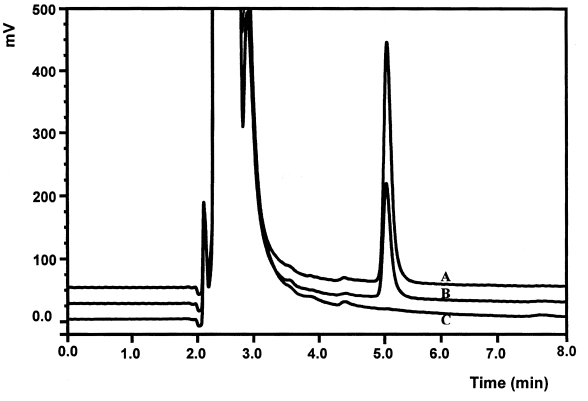

Fig. 1. Representative chromatograms of monoHER in (A) heart tissue of mice taken at 30 min after i.p. treatment with 500 mg kg^{-1} monoHER, (B) bovine heart spiked with 7.2 nmol g^{-1} monoHER and (C) blank bovine heart.

and precision of this sample were 90.3 and 14.3%, respectively.

The LLQ obtained with our procedure was much lower than that obtained in the past for flavonoids in tissue and body fluids. Autoradiography and direct counting of $^{14}CO_2$, produced by combustion of liver, kidney, spleen and lung tissue [4,5], were used to quantify radiolabeled mono- and triHERs in mice. This method lacked the specificity to differentiate between the intact HER and its metabolites. A more sensitive and selective HPLC method was developed to measure the levels of HERs in human serum [12]. With this method di- and triHERs were measured with a UV detector and tetraHER with a fluorescence detector. The detection limits were 1.4, 1.3 and 0.13 μ*M* for di-, tri- and tetraHERs, respectively.

Quercetin was recently determined in human plasma using HPLC with diode array detection. The disadvantages of this method were the low sensitivity, 300 n*M*, and the time consuming extraction procedure of the plasma samples prior to HPLC analysis [9]. Such an extraction procedure cannot be used for monoHER, because it could be oxidized during extraction. In another procedure, using HPLC with UV detection, dihydroquercetin was measured in urine and plasma of the rat [13]. The extraction process was simple, but the sensitivity was low, i.e., the detection limits in urine and plasma were 1.6 and 0.7 μ*M*, respectively.

The higher sensitivity and selectivity achieved by our method, is principally obtained by the use of the electrochemical detector.

3.2. Calibrators and quality control samples

Fig. 2 shows a representative calibration line of monoHER in heart tissue, which was obtained by linear regression using a weighting factor $1/y$. The peak area increased linearly with the concentration in the dynamic range of 0.072–24.0 nmol g^{-1}. The correlation coefficient was never less than 0.996 and the offset was always smaller than 0.37 mV min. Between days, a gradual decrease in the slope of the calibration lines (sensitivity) was observed, which was restored by cleaning the detector. The mean between-day accuracy ($n=6$) of the calibration samples was less than 11.6% of the nominal values (Table 1). The accuracy was concentration dependent and ranged from 94.1 to 123.1% for the LLQ and from 97.0 to 99.5% for the highest concentration (24.0 nmol g^{-1}).

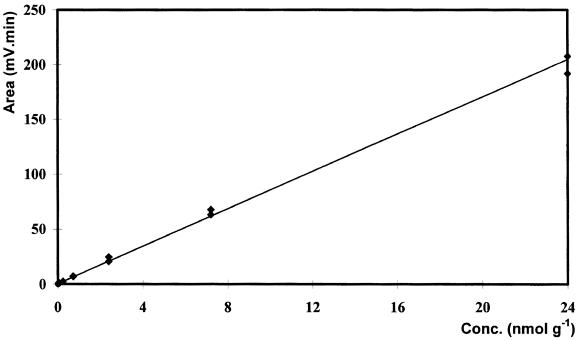

Fig. 2. A representative calibration line of monoHER in heart tissue calculated by weighted linear regression ($1/y$).

The mean within- ($n=6$) and between-day ($n=6$) accuracy and precision of the quality control samples are summarized in Table 2. The accuracy ranged from 6.5 to 16.1% and from 0.8 to 5.6% for the within- and between-day analyses, respectively. The precision ranged from 7.5 to 18.2% and from 8.4 to 10.8% for the within- and between-day analyses, respectively. The accuracy and precision of the within- and between-day analyses were concentration independent.

Comparable precisions were obtained for the within- and between-day analyses of other flavonoids in the body fluids of human and rat [9,13]. Although the precisions were comparable, they were however related to higher concentrations.

The recoveries of monoHER from heart tissue homogenate at the concentration levels of the quality control samples are also summarized in Table 2. The recovery of monoHER was concentration indepen-

Table 1
Summary of the between-day accuracy (mean±SD, $n=6$) of the calibration samples of monoHER in heart tissue

Concentration (nmol g^{-1})	Mean (%)	Range (%)
0.072	108.6±14.5	94.1–123.1
0.24	111.6±12.2	99.3–123.8
0.72	108.9±8.5	100.4–117.4
2.4	108.3±6.1	102.1–114.4
7.2	103.9±4.1	99.8–108.0
24.0	98.2±1.2	97.0–99.5

Table 2
Summary of the recovery of monoHER and the within- ($n=6$) and between-day ($n=6$) accuracy and precision (mean±SD) of quality control samples of monoHER in heart tissue

Concentration (nmol g^{-1})	Recovery (%)	Within-day		Between-day	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
0.12	104.7	111.4	18.2	103.6	10.8
1.2	114.3	116.1	7.5	105.6	10.8
12.0	104.1	106.5	9.0	99.2	8.4

dent with a mean overall recovery of 107.7±5.7%. In general, lower recoveries were obtained for other flavonoids from body fluids of man and rat [12,13], which may be attributed to the long lasting extraction procedure prior to the HPLC analysis.

3.3. Stability of the samples and the detector response

Fig. 3 shows that the peak area of monoHER, in aliquots of processed samples, decreased when stored at 4°C in the cooled autosampler tray for 24 h. This decrease might be caused by either the stability of the ECD system and/or the stability of monoHER in the autosampler. To discriminate between these two possibilities a monoHER extract was prepared and split into two series. The first series was analyzed immediately over a period of 24 h. During this period the other series was stored at −80°C and then analyzed by HPLC. The peak area of the second series started at about the same initial value as the first sample of the first series and then decreased at the same rate during the run of the whole series. These results indicate that the decrease in peak area within the HPLC run could principally be attributed to the stability of monoHER in the autosampler (4°C) and not to a decrease in the sensitivity of the detector. The sensitivity of the detector was maintained by cleaning the working electrode with acetone before each run to avoid a decrease in the detector signal due to film formation and memory effects [14]. The decrease in monoHER level may be caused by enzymes in the tissue extract or, because monoHER is an antioxidant [15], the presence of oxidants like molecular oxygen. These interactions may also explain the observed decrease in quercetin levels when stored in human plasma at different temperature [9].

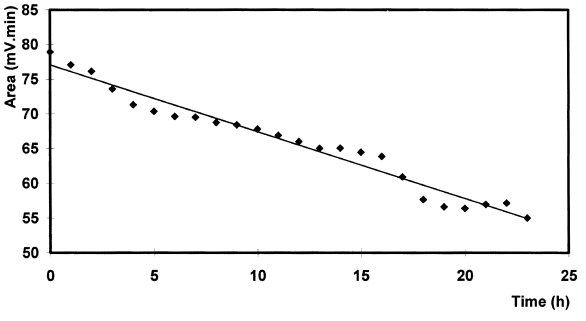

Fig. 3. The stability of monoHER in heart tissue extract in the tray of the HPLC system during 24 h at 4°C.

To correct for the decrease in monoHER stability during a run sample duplicates were arranged in a mirror image sequence as indicated earlier by our group [16]. Therefore, a high accuracy could always be obtained especially for the highest concentrations.

3.4. monoHER in heart tissue and its stability upon storage at −80°C

monoHER concentrations in freshly analyzed hearts of mice were 15.6 and 34.9 nmol g^{-1} at 0.5 h and 8.7 and 11.8 nmol g^{-1} at 1.0 h after i.p. administration of monoHER. After 6 months of storage at −80°C, the concentrations of monoHER found in hearts taken at 0.5 and 1.0 h of administration were 34.4 and 13.4 nmol g^{-1}, respectively. These values were comparable to the highest values found for the fresh samples indicating that the samples were stable during storage at −80°C for 6 months. The levels of monoHER in heart tissue at 0.5 and 1.0 h after administration could easily be measured, after the proper dilution, within the dynamic range of our method. The high recovery and

sensitivity of this method allowed the measurement of monoHER in the heart tissue of mice for up to 8 h after i.p. and intravenous (i.v.) administration of 500 mg kg^{-1} (unpublished data).

4. Conclusion

monoHER could be analyzed in heart tissue by HPLC without interference of endogenous compounds. Although a gradual decrease of the peak area occurred during a run the mirror image-analyzed duplicates resulted in a good accuracy and precision for the analysis of monoHER in heart tissue.

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