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High-performance liquid chromatography with electrochemical detection for the determination of 7-monohydroxyethylrutoside in plasma

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Abstract

MonoHER (7-monohydroxyethyl rutoside) is a semisynthetic flavonoid, which can be used as a modulator for doxorubicin-induced cardiotoxicity. To study the pharmacokinetics of monoHER in mice and human an HPLC procedure was developed to measure the level of monoHER in plasma. After extraction of monoHER with methanol, the supernatant was equally diluted (v/v) with 25 m*M* phosphate buffer (pH 3.33). This solution was analysed by HPLC, using a reversed-phase ODS column, with a mobile phase consisting of 49% methanol and 51% of an aqueous solution containing 10 m*M* sodium dihydrogen phosphate (pH 3.4), 10 m*M* acetic acid and 36μ*M* EDTA. The retention time of monoHER was about 5.2 min. The lower limit of quantification of monoHER was set at 0.3 μ*M* and the calibration line was linear up to 75 μ*M*. The within-day accuracy and precision of the quality control samples (0.45, 1.0, 10 and 40 μ*M*) were better than 15 and 13%, respectively. The between-day accuracy and precision were less than 3, 20%, respectively. The recovery of monoHER (using quality control concentrations) was concentration independent and ranged from 90.5 to 95.3% except for the lowest quality control, 0.45 μ*M*, of which the recovery was 85%. The concentration of monoHER in plasma decreased with 10% when stored at −80°C for one month and with 20% when stored at −20°C for 3 weeks. The repeated injection of monoHER in aliquots of 10 μ*M*, stored in the autosampler tray (4°C), showed a consistent decrease during a run: 15% over 24 h. To compensate for this decrease, sample duplicates were analysed in a mirror image sequence. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: 7-Monohydroxyethylrutoside

1. Introduction

MonoHER, a semisynthetic hydroxyethylated rutoside (HER), is the best antioxidant in the registered flavonoid mixture Venoruton® [1,2]. Recently, it was reported that monoHER protected against doxorubicin-induced cardiotoxicity in mice and did not influence the antitumor activity of doxorubicin neither in vitro nor in vivo [3]. In order to obtain reliable information about the pharmacokinetics of monoHER in human and mice, a sensitive and selective method is required to quantify monoHER levels in plasma.

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Because of their wide range of biological and therapeutic effects [4–6], great attention has been paid to the analysis of HERs in biological fluids. The radioactivity of ^{14}C-labelled HERs was measured in body fluids of various animals after separation with different chromatographic procedures [7–9]. A number of procedures was developed to determine unlabelled HERs and their metabolites in plasma of animals and human, such as TLC, circular dichromism, HPLC and spectrofluorimetry [10–13]. These methods were not sensitive and specific enough to give accurate and reliable pharmacokinetic data.

More reliable methods were developed to measure the level of different flavonoids in various body fluids of human and animals. The levels of different HERs were measured in human serum using HPLC with UV or fluorescence detection [14]. Dihydroquercetin was measured in urine and plasma of the rat using HPLC with UV detection [15]. In a more recent study, quercetin levels were measured in human plasma using HPLC with diode array detection [16]. Although these methods were more sensitive than the previously mentioned procedures, they were either unsuitable or insensitive to study the pharmacokinetics of monoHER after different routes of administration. Therefore, the purpose of this study was to develop and validate [17] a sensitive and selective method for the determination of monoHER in plasma.

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, silver chloride and sodium dihydrogen phosphate monohydrate were purchased from Merck (Amsterdam, The Netherlands), EDTA from Sigma–Aldrich Chemie (Zwijndrecht, The Netherlands) and methanol, HPLC grade, 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 detector (ECD) 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, H. I. Ambacht, The Netherlands).

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 dihydrogen phosphate, 10 m*M* acetic acid and 36 μ*M* EDTA, was used with a flow-rate of 0.7 ml/min.

2.3. Sample preparation

Two stock solutions of 2.0 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 mixture pH 3.33 (4:1, v/v). Heparinised plasma from healthy volunteers was stored at −20°C. This plasma 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 and kept on ice during handling. Standards (0.3, 0.6, 1.5, 4.5, 15, 45 and 75 μ*M*) and quality control samples (QC) (0.45, 1.0, 10 and 40 μ*M*) were prepared by mixing appropriate amounts of plasma with dilutions of the respective stock solutions.

The samples were processed in duplicate by deproteinising 77 μl of sample with 123 μl methanol. After careful shaking, the samples were centrifuged (3 min, 1°C, 9000 rpm) and 130 μl supernatant of each sample was transferred into 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 recentrifuged (3 min, 1°C, 9000 rpm) and the supernatant was transferred into 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. Unknown samples were processed accordingly.

For recovery experiments, quality control samples were freshly prepared in plasma and in the MeOH/phosphate buffer mixture and analysed in duplicate.

2.4. Linearity and lower limit of quantification (LLQ)

The LLQ was determined by the analysis of a calibration curve in three-fold beginning with an extra standard sample of 0.15 μ*M*. Of each standard sample the concentration was calculated from the calibration line obtained from the means of the whole set of standard samples. When the accuracy of the lowest concentration was higher than 25% this standard samples was not accepted and the calculations were repeated without this standard sample until an accuracy of better than 25% was achieved for the lowest concentration used. That lowest concentration was defined as the lowest limit of quantification (LLQ).

The linearity of each calibration line was detected by calculating the correlation coefficient (r^2) of each line by means of the least squares method. Only calibration lines with $r^2>0.99$ and with randomly divided signs of the residuals were considered to be linear.

2.5. Within- and between-day analysis

For the determination of the within-day accuracy and precision, 6 replicates of the QC samples were analysed at the same day together with a freshly prepared set of standard samples. The accuracy and the precision of the between-day analysis were determined by analysing the QC samples in duplicate on 6 different days. The results obtained for the first duplicate value of each QC concentration used for the within-day validation were also used as the first data set in the between-day validation.

2.6. 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 plasma containing 10 μ*M* monoHER. The autosampler (4°C) was adjusted to inject one sample per h for 24 h.

The stability of monoHER in plasma stored at −20 and −80°C was also estimated. Quality control samples with concentrations of 0.45, 1.0, 10 and 40 μ*M* were prepared in plasma and divided into two parts. The first part was stored at −20°C for 3 and 23 days. The second part was stored at −80°C for 5, 13 and 33 days. These stability samples were analysed in a run together with freshly prepared quality control samples.

2.7. Detection of monoHER in mice plasma after i.v. administration

Balb/c mice (20–30 g) were maintained in cages of 4–6 mice on regular chow and water ad libitum for 4 days before use. Mice received 500 mg/kg monoHER i.v. as a bolus injection via the tail vein. Blood samples were collected at regular times after administration. At each time point three mice were sacrificed. Samples were processed together with freshly prepared standard samples.

2.8. 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 unknown samples (S) were analyzed in duplicate in the following reverse (mirror image) order: $C_1, \ldots C_n$, $QC_1, \ldots QC_4$, $S_1, \ldots S_n \mid S_n, \ldots S_1$, $QC_4, \ldots QC_1$, $C_n, \ldots C_1$. In this way the mean of the duplicates compensated for a possible gradual decrease of the detector signal.

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/X.

3. Results and discussion

3.1. Analysis

The chromatograms of monoHER in plasma of mice obtained at 10 min after i.v. treatment with 500 mg/kg monoHER and human plasma spiked with 15 μ*M* monoHER in comparison to blank plasma of a mouse and a human are shown in Fig. 1. The retention time of monoHER under the described chromatographic conditions was about 5.2 min. In human plasma no endogenous peaks were interfering. However, in blank plasma of mice a very small interfering peak frequently appeared in front of the peak of monoHER. The peak did not correspond to the structurally related flavonoid rutin, which abundantly occurs in animal chow, nor to other commercially available flavonoids like quercetin. Drugs which will be co-administered with monoHER, like anthracyclines, did not interfere with the peak of monoHER. The small interfering endogenous peak, in the plasma of mice, limited the lower limit of quantification (LLQ) to 0.3 μ*M*, which was about three times higher than the apparent concentration of the mean interfering peak. Nevertheless, this LLQ was lower than those reported in the literature. Various other HERs were quantified by TLC and paper chromatography measuring radioactivity, UV absorption or fluorescence (after complexation with boric acid) [7,18,19]. The sensitivity and selectivity of these methods were low e.g. the detection limit of monoHER measured by UV absorption was only about 7.5 μ*M* [18]. However, for tetra-HER (which has fluorescence on its own) a better detection limit of 0.13 μ*M* was found using HPLC with fluorescence detection [14].

Recently, quercetin was determined in human plasma with a detection limit of 0.3 μ*M* using HPLC with diode array detection. However, the disadvantages of this method are the large sample volume used and the time consuming extraction procedure prior to HPLC analysis [16]. Such a long extraction procedure can not be used for monoHER, because of its instability as an antioxidant. A simple extraction procedure was used for the measurement of dihydroquercetin in urine and plasma of the rat, but the detection limits in urine and plasma were 1.6 and 0.7 μ*M*, respectively [15]. The better sensitivity and selectivity, achieved by our method, can basically be attributed to the use of the electrochemical detector.

3.2. Calibrators and quality control samples

Calibration lines were calculated with different weighting factors (1, 1/X or 1/X^2). The best fit, i.e. possessing the highest correlation coefficient and randomly distributed signs of the residuals, were obtained with a weighting factor of 1/X. A representative calibration line of monoHER in plasma is shown in Fig. 2. The peak area increased linearly with the concentration in the dynamic range of 0.3–75 μ*M*. The correlation coefficient was never less than 0.994, the offset was always smaller than 0.08 mV. min. Between days, a gradual decrease in the slope of the calibration lines was observed which was restored by cleaning the detector. The mean

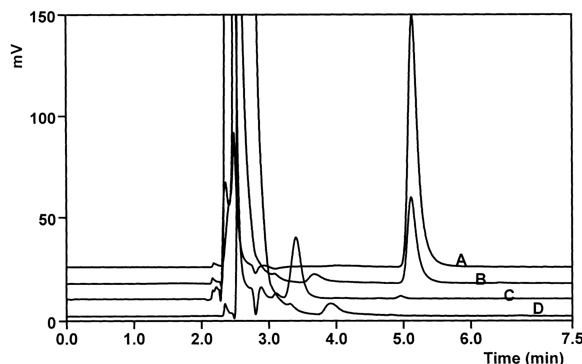

Fig. 1. Representative chromatograms of monoHER in (A) plasma of mice taken at 10 min after i.v. treatment with 500 mg/kg monoHER, (B) human plasma spiked with 15 μ*M* monoHER, (C) blank plasma of a mouse and (D) blank human plasma.

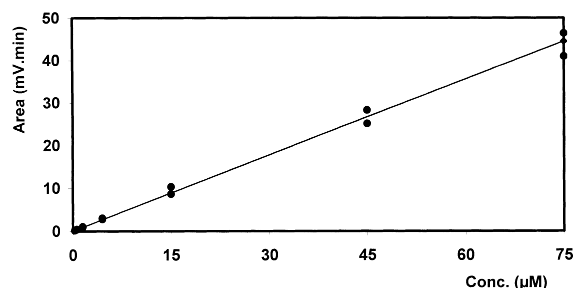

Fig. 2. A representative calibration line of monoHER in plasma calculated by weighted linear regression (1/x).

Table 1
Between-day ($n=6$) accuracy of the calibration samples of monoHER in plasma

Conc. (μM)	Mean (%)	Range (%)
0.3	88.4±10.4	78.0–98.8
0.6	97.7±3.2	94.4–100.9
1.5	104.8±5.6	99.3–110.4
4.5	104.0±6.9	97.0–110.9
15.0	106.8±2.7	104.1–109.5
45.0	100.9±1.2	99.6–102.1
75.0	97.4±0.5	96.9–98.0

between-day accuracy ($n=6$) of the calibration samples was less than 12% of the nominal values (Table 1). The accuracy was concentration independent, ranging between 94.4 and 110.9% for all concentrations except for the LLQ, which ranged from 78 to 98.8%.

The within ($n=6$) and between-day ($n=6$) accuracy and precision of the quality control samples are summarised in Table 2. The accuracy ranged from 2.7 to 14.9% and from 0.6 to 2.6% for the within and between-day analysis, respectively. The precision ranged from 2.4 to 12.4% and from 5.2 to 19.5% for the within and between-day analysis, respectively. Although the analysis of the lowest quality control was less accurate and precise than the other quality control samples in the within and between-day analysis, no further concentration dependency was observed.

Comparable precisions were obtained for the within and between-day analysis of quercetin in human plasma [16]. Dihydroquercetin was more preciously measured in the body fluids of rat [15] but it concerned concentrations higher than 0.7 μM. The accuracy was not clearly mentioned in the two studies.

The recoveries of monoHER from the quality control samples are summarized in Table 2 as well. The recovery of monoHER from the lowest quality control sample was 84.8%. For the higher concentrations the recovery ranged from 90.5 to 95.3 and was concentration independent. The decrease of the recovery especially in the lowest concentration may be attributed to co-precipitation of monoHER with the plasma proteins during methanol extraction. A lower recovery of HERs was obtained from human serum with another procedure [14]. This may be due to the long lasting extraction procedure used prior to the HPLC analysis. A comparable recovery of dihydroquercetin was only obtained from the body fluids of the rat when simultaneously extracted and deproteinised with acidified methanol [15].

3.1. Stability of the samples and the detector response

Fig. 3 shows that the peak area of monoHER, in aliquots of a processed sample, decreased steadily while standing in the cooled autosampler tray for 24 h. This decrease may be the result of either the stability of the ECD and/or the stability of monoHER in the autosampler. The sensitivity of the detector between the runs was maintained by cleaning the working electrode with acetone before each analysis to avoid decrease in the detection signal due to film formation and memory effects [20]. To correct for the gradual decrease (Fig. 3) in the peak area during a run, standard, quality control and test sample duplicates were arranged in a mirror image sequence as indicated earlier by our group [21]. In

Table 2
Recovery, within ($n=6$) and between-day ($n=6$) accuracy and precision of the quality control samples of monoHER in plasma

Conc. (μM)	Recovery (%)	Within-day		Between-day	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
0.45	84.8	85.1	12.4	100.7	19.5
1.0	90.5	97.2	3.7	100.6	7.3
10	95.3	103.5	3.3	102.6	5.2
45	92.5	97.3	2.4	99.3	5.5

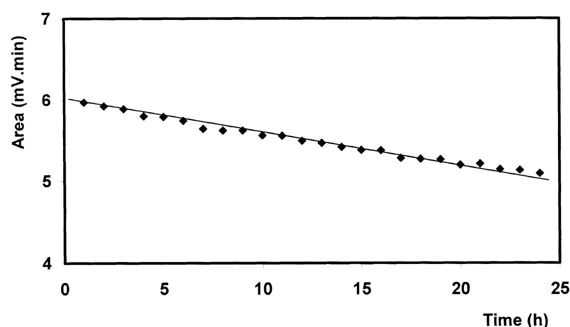

Fig. 3. Peak areas obtained by repetitive injection of a monoHER extract from plasma standing in the tray of the HPLC system during 24 h at 4°C.

this way a series consisting of 20 samples, 7 standards and 4 quality control samples could be analyzed in duplicate with a high accuracy.

3.2. Stability upon storage at −20 and −80°C

The level of monoHER in plasma decreased with 20% when stored at −20°C for 23 days. When stored at −80°C, monoHER level decreased with 10% after 33 days. This temperature–dependent decrease may still be attributed to an interaction of monoHER, being an antioxidant [22], with molecular oxygen or other surrounding oxidants. This may also support the idea that the decrease in the peak area during a run at 4°C is mainly due to a decrease in monoHER stability and not to a decrease in the detector sensitivity. This interaction may also explain the observed decrease in quercetin levels in human plasma when stored at different temperatures [16].

3.3. Applicability of the method

The use of a small sample volume and the high recovery and sensitivity of our method allowed us to determine monoHER in plasma samples of mice receiving an i.v. dose of 500 mg/kg. Plasma samples were collected during a period of 24 h after monoHER administration. Immediately after administration, a peak concentration of 2.0 m*M* appeared in the plasma. MonoHER levels dropped quickly and could be detected for two h after the i.v. administration of monoHER. The method will also be applied to study the pharmacokinetics of monoHER in mice after various routes of administration and in coming clinical studies.

4. Conclusion

An accurate and precise procedure was developed to measure monoHER in plasma of mice and human. The procedure was applied for the analysis of monoHER in plasma samples of mice. It will also be applied for studying the pharmacokinetics of monoHER in patients.

References

[1] S.A.B.E. van Acker, R. Towart, B.C.P. Husken, J.de Jong, W.J.F. van der Vijgh, A. Bast, Phlebology Suppl. 1 (1993) 31.
[2] G.R.M.M. Haenen, F.P. Jansen, A. Bast, Phlebology Suppl. 1 (1993) 10.
[3] S.A.B.E. van Acker, E. Boven, K. Kuiper, D. van den Berg, J.A. Grimbergen, K. Kramer, A. Bast, W.J.F. van der Vijgh, Clin. Cancer Res. 3 (1997) 1747.
[4] E. Middleton, C. Kandaswami, The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer, in: J.B. Harborne (Ed.), The Flavonoids: Advances in Research since 1986, Chapman and Hall, London, 1994, p. 619.
[5] W. Voelter, G. Jung (Eds.), *O*-(β-Hydroxyethyl)-rutoside, Experimentelle und klinische Ergebnisse, Springer, Berlin, 1978.
[6] L. Farkas, M. Gabor, F. Kallay (Eds.), Flavonoids and Bioflavonoids, Elsevier, Amsterdam, 1977.
[7] A. Barrow, L.A. Griffiths, Xenobiotica 4 (1974) 743.
[8] A.M. Hackett, L.A. Griffiths, Eur. J. Drug. Metab. Pharmacokin. 4 (1979) 207.
[9] A. Barrow, L.A. Griffiths, Xenobiotica 2 (1972) 575.
[10] V. Wienert, W. Gahlen, Der Hautarzt 21 (1970) 278.
[11] G. Jung, M. Ottnad, W. Voelter, Europ. J. Drug. Metab. Pharmacokin. 3 (1977) 131.
[12] W. Kuhnz, G. Jung, W. Voelter, in: W. Voelter, G. Jung (Eds.), Hochdruck flussigchromatographische Bestimmung von O-(β-Hydroxyethyl)-rutosiden in humanserum. In: *O*-(β-Hydroxyethyl)-rutoside: neue Egebnisse in Experiment und klinik, Springer-Verlag, Berlin, 1983, p. 25.
[13] H.S.I. Tan, P.J. Mowery, W.A. Ritschel, C. Neu, J. Pharm. Sci. 67 (1978) 1142.
[14] W. Kuhnz, K. Zech, R. Lupp, G. Jung, W. Voelter, J. Chromatogr. 272 (1983) 333.
[15] S.V. Geodakyan, I.V. Voskoboinikova, J.A. Kolesnik, N.A. Tjukavkina, V.I. Litvinenko, V.I. Glyzin, J. Chromatogr. 577 (1992) 371.

[16] B. Liu, D. Anderson, D.R. Ferry, L.W. Seymour, P.G. de Takats, D.J. Kerr, J. Chromatogr. B 666 (1995) 149.
[17] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilvery, J.P. Skelly, A. Yacobi, T. Layoff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pitman, S. Spector, Pharm. Res. 9 (1992) 588.
[18] A. Barrow, L.A. Griffiths, Xenobiotica 4 (1974) 1.
[19] A.M. Hackett, P. Niebes, L.A. Griffiths, J. Chromatogr. 148 (1978) 469.
[20] W.F. Hodnick, E.B. Milosavljevic, J.H. Nelson, R.S. Pardini, Biochem. Pharmacol. 37 (1988) 2607.
[21] A.E.C. Korst, J.B. Vermorken, W.J.F. van der Vijgh, J Chromatogr. B 693 (1997) 167.
[22] S.A.B.E. van Acker, D. van den Berg, M.N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, W.J.F. van der Vijgh, A. Bast, Free Radical. Biol. Med. 20 (1996) 331.