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DETERMINATION OF 5-METHYLTETRAHYDROFOLIC ACID IN PLASMA AND SPINAL FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, USING ON-COLUMN CONCENTRATION AND ELECTROCHEMICAL DETECTION

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SUMMARY

Chromatographic conditions for the determination of 5-methyltetrahydrofolic acid in plasma and spinal fluid are described, involving simple pretreatment of the sample. Electrochemical detection was used. The linear range of the method is more than 10^3. Recovery from plasma and spinal fluid is 100%, and the detection limit of the method is $2 \cdot 10^{-9}$ *M*, sufficient for the detection of endogenous plasma and spinal fluid levels. The detection conditions are discussed. Endogenous concentrations of the compound in plasma and spinal fluid were determined and correlated with a folate bioassay. Plasma concentrations have been shown after the administration of leucovorin which is used in anticancer therapy.

INTRODUCTION

Folic acid analogues play an important role in several biochemical processes [1]. In man, 5-methyltetrahydrofolic acid (5-MeTHF) is the main analogue found to be present in plasma [2]. Its concentration in plasma is important for the determination of folate deficiency in patients [3]. Folate activity in serum is determined by microbiologic assay [4] or radiometric analysis using a labeled drug [5]. For the treatment of malignancies 5-MeTHF plays a role as the active

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metabolite of leucovorin which is used in the prevention of methotrexate toxicity [6—9]. Its concentration may be important to estimate the optimal antitumor effect [10,11]. In test samples 5-MeTHF has been separated from other folate analogues by high-performance liquid chromatography (HPLC) [12]. In plasma and spinal fluid the determination of 5-MeTHF is usually carried out by microbiological assay [13].

For the analysis of body fluids, a sensitive HPLC method is expected to be fast and selective and to have a wide linear dynamic range. Generally, bioassays are hampered by interference due to the presence of folates and antibiotics. As 5-MeTHF is very sensitive to oxidation [14], relatively large amounts of antioxidant must be added to the sample. Since the endogenous plasma concentrations lie between 10^{-9} *M* and 10^{-8} *M*, the determination of 5-MeTHF demands high efficiency of the chromatographic system. For this purpose a sensitive electrochemical detection was investigated. Among the electrochemical properties of folate analogues reduction has been reviewed [15]. However, oxidation at a glassy carbon electrode had not been previously reported. For easily oxidizable compounds this detection method is sensitive and selective.

A two-column concentration system [16] in combination with this detection method may have the following advantages: (1) the large ratio of antioxidant concentration, added to the sample medium, with respect to the small concentration of 5-MeTHF gives rise to a serious separation problem, but this is considerably diminished by the first separation step; (2) the possibility of injecting large sample volumes improves the lower detection limit; (3) extra sample clean-up of compounds interfering in the chromatogram, by elution from the concentration column, is included.

EXPERIMENTAL

Chromatographic equipment and columns

The liquid chromatograph has been described before [16]. Ion-exchange columns (Partisil SAX) were obtained from Whatman (Maidstone, Great Britain) and reversed-phase columns from Chrompack (Middelburg, The Netherlands). The particle size was 10 μm for both materials and the column dimensions were 25 cm × 4.6 mm I.D. and 4.6 cm × 3 mm I.D., respectively.

Detection system

An electrochemical detector with a large-surface electrode was used [17]. A glassy carbon electrode (finally polished with diamond powder) with a surface of 2.65 cm^2 was used. This material (grade GC-10, obtained from Tokai Carbon, Tokyo, Japan) has a porosity of 0.2—0.4%. Polyester spacers were used with a thickness of 87 μm and 25 μm. The polyester film (Melinex, type S) was obtained from ICI Holland (Rotterdam, The Netherlands). The electrode potential was held at +0.3 V vs. Ag/AgCl/0.001 *M* Cl^-. The detection system is commercially available from Kipp Analytica (Emmen, The Netherlands).

Before analyzing samples with concentrations just above the detection limit the electrode was allowed to equilibrate overnight at constant potential. Early plasma peaks in the chromatogram and foreign eluent, coming form the void volume of the concentration column, were

vented in order to avoid possible electrode "poisoning". For simultaneous measurement of other folate analogues (e.g. during drug treatment) a UV detector (Schoeffel Instruments, Westwood, N.J., U.S.A., Model 770) was placed in series upstream from this detector.

For the measurement of the coulometric yield in the detection cell an infusion pump was used (Infors, Basle, Switzerland, Type 5003).

Number of electrons, involved in the oxidation reaction

The number of electrons which are transferred per molecule in the oxidation reaction was calculated by Faraday's law [17] at a coulometric yield of 1.0 and was found to be 1.45.

Reagents and standards

All reagents were of analytical grade. Demineralised water was used for the preparation of all solutions, except for the concentration eluent. For the concentration eluent a solution of 0.015 *M* citrate in 0.015 *M* phosphate buffer (pH 4.95) was used. Sodium azide, 0.5—1 mg/l, was used in order to prevent the growing of micro-organisms. The analytical eluent consisted of 0.05 *M* sodium phosphate buffer (pH 4.95)—methanol (4:1, v/v) and contained 0.001 *M* sodium chloride for the electrochemical detection. For the deproteinization of samples a solution containing 10% (w/w) of trichloroacetic acid (TCA) in 0.1 *N* HCl was used. A solution of 1% of L-(+)ascorbic acid was freshly prepared each day. 5-MeTHF was obtained from Sigma (St. Louis, Mo., U.S.A.). The samples were standardized by means of the formula $\epsilon = A \cdot W/Q$ where ϵ = molar extinction, A = peak area, W = eluent flow-rate and Q = amount of compound. From Gupta and Huennekens [18] a value for ϵ of $31.7 \cdot 10^3$ $AU \cdot mol^{-1} \cdot l \cdot cm^{-1}$ is obtained for pH 7. This value was corrected by us for the difference in pH. By this calculation the purity of the commercially availably samples was found to be only about 60%.

Sample treatment

Sample treatment consisted of the following two steps: protection against oxidation and a deproteinization/centrifugation step.

To 0.9 ml plasma or spinal fluid, 0.1 ml of a solution containing 1% ascorbic acid was added. The mixture was allowed to equilibrate for 3 min. During vigorous mixing 1 ml TCA solution [10% (w/w) in 0.1 *N* HCl] was added. After centrifugation at 2000 *g* for 5 min 1 ml of the clear supernatant could be injected.

In order to prevent oxidation by dissolved oxygen during sample pretreatment an antioxidant is necessary. For this purpose the following compounds were tested: sodium sulfite, dithioglycol (1,2-ethanedithiol), 2-mercaptoethanol, cysteine·HCl and ascorbic acid. For a one-column separation on either a reversed-phase (RP-8, Merck, Darmstadt, G.F.R.) or an ion-exchange (Partisil SAX) column sodium sulfite gave the best peak. However, the addition of sulfite was found to be insufficient for stabilizing 5-MeTHF. The other agents, except ascorbic acid, contained impurities which were detected by the electrochemical detector. Ascorbic acid was therefore the best choice for stabilization. Because the recovery from plasma was found to decrease when using lower

amounts of ascorbic acid, only a freshly prepared solution was used. For storage fresh plasma samples were frozen immediately and stored at −20°; when thawed ascorbic acid was added immediately. The deproteinizated samples were injected within 30 min. After two hours at ambient temperature the concentration of 5-MeTHF in this mixture was found to decrease by about 5%, but could be less by keeping the samples in ice.

Quantitation

To determine the concentration of 5-MeTHF, its peak height in the chromatogram is measured. Homogenized plasma containing 5-MeTHF was divided into samples of 1 ml. Each day, the concentration of 5-MeTHF was measured in two samples in order to correct for changes in peak height.

RESULTS

Chromatographic procedure

A reversed-phase column (RP-8, Merck) was tested for a combined concentration and first separation step, followed by an extra separation on an ion exchanger (Partisil SAX) as described earlier for the analysis of methotrexate [16]. Column dimensions and the composition of the analytical eluent were similar. A concentration eluent volume of 7 ml was chosen, which was enough to produce a good sample clean-up during concentration, while still small enough not to cause elution from the concentration column.

Detection conditions

Although folate analogues show high UV absorption at wavelengths between 280 and 300 nm, UV detection did not appear to be useful for the detection of endogenous plasma levels of 5-MeTHF. This is demonstrated in Fig. 1 where the same separation is shown with UV detection and with electrochemical detection, indicating the increased sensitivity of the latter.

In order to investigate the detection signal as a function of eluent flow-rate in a static way the oxidation current resulting from a solution containing 5-MeTHF, flowing directly through the detector, was measured.

By means of a precise infusion pump the flow-rate was increased stepwise. 5-MeTHF was previously purified by elution on the two-column system. A plot of measured current against flow-rate is given in Fig. 2. At the lowest flow-rates a coulometric yield of 1.0 is reached. This yield is the fraction of all the molecules that react at the electrode [17]. The actual yield was calculated for various flow-rates of the 5-MeTHF containing eluent (dashed line). A flow-rate of 120 ml/h was used for the determination of 5-MeTHF. A coulometric yield of 12% can be determined from Fig. 2.

Detection limit, linear dynamic range, recovery

The method has a detection limit of $2 \cdot 10^{-9}$ *M* (0.9 ng/ml) for both plasma and spinal fluid. The standard deviation in peak height is 7.5% at $2 \cdot 10^{-9}$ *M* ($n = 7$) and 3.5% at concentrations higher than $3 \cdot 10^{-9}$ *M*. The upper limit of the linear dynamic range was measured to be at least 10^{-5} *M*. The linear dynamic range is more than 10^3. In the static measurement the linear range was lower

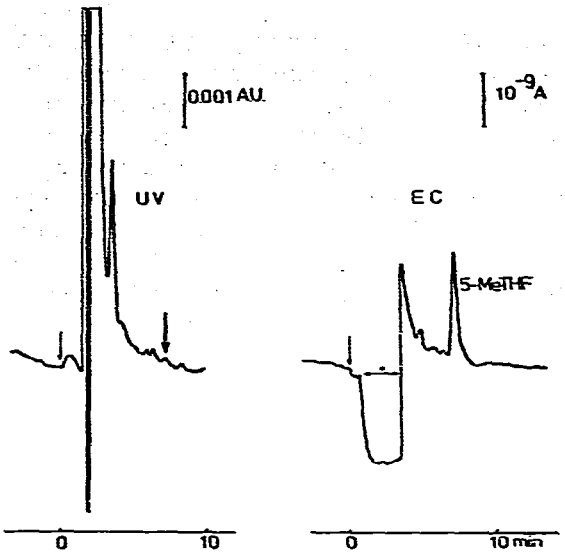

Fig. 1. Chromatogram of a plasma sample for the two-column system using UV detection at a wavelength of 290 nm and electrochemical (EC) detection at a working electrode potential of + 0.3 V. Injection volume, 1 ml; concentration of 5-MeTHF $5 \cdot 10^{-9}$ *M*; asterisk indicates venting just upstream from EC detector.

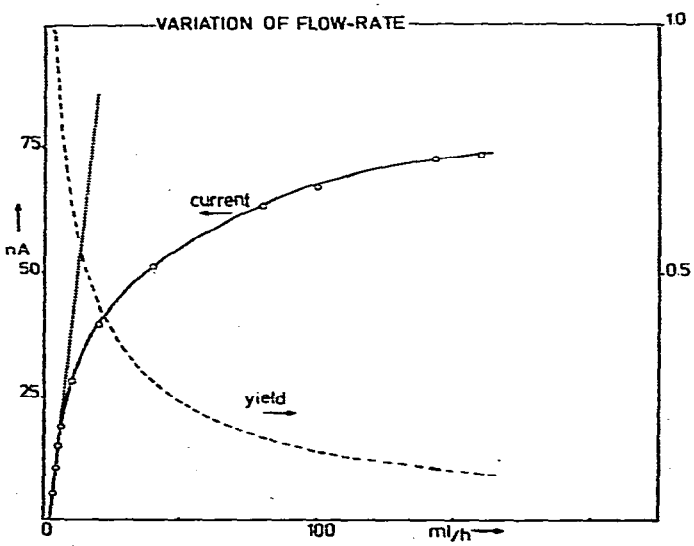

Fig. 2. Static measurement of oxidation current against flow-rate for a solution containing $1.3 \cdot 10^{-7}$ *M* 5-MeTHF (solid line). Working electrode potential + 0.3 V, liquid film thickness 87 μm. The current vs. flow-rate at a coulometric yield of 1.0 is represented by the dotted line; the coulometric yield vs. flow-rate by a dashed line.

and can be explained by a higher accumulation of reaction products which have to be resorbed from the electrode surface. Under the conditions described under Sample treatment the recoveries from plasma and spinal fluid were found to be 100% over the measured concentration range.

Correlation with total-folate assay

In clinical analysis for the determination of folate deficiency in patients the measurement of the concentration of 5-MeTHF is often based on the growth of *Lactobacillus casei* [4]. The concentrations of patient samples, obtained by this method, are plotted against concentrations obtained by the HPLC method for both plasma (n = 83) and spinal fluid (n = 49) (Fig. 3). Statistical data are given in the legend to Fig. 3. The diffuse pattern can be explained by the interference of the other compounds present in the sample effecting bacteria growth. The systematic deviation from the line connecting equal values is probably due to standardization of the bioassay using solutions of folic acid instead of 5-MeTHF [4].

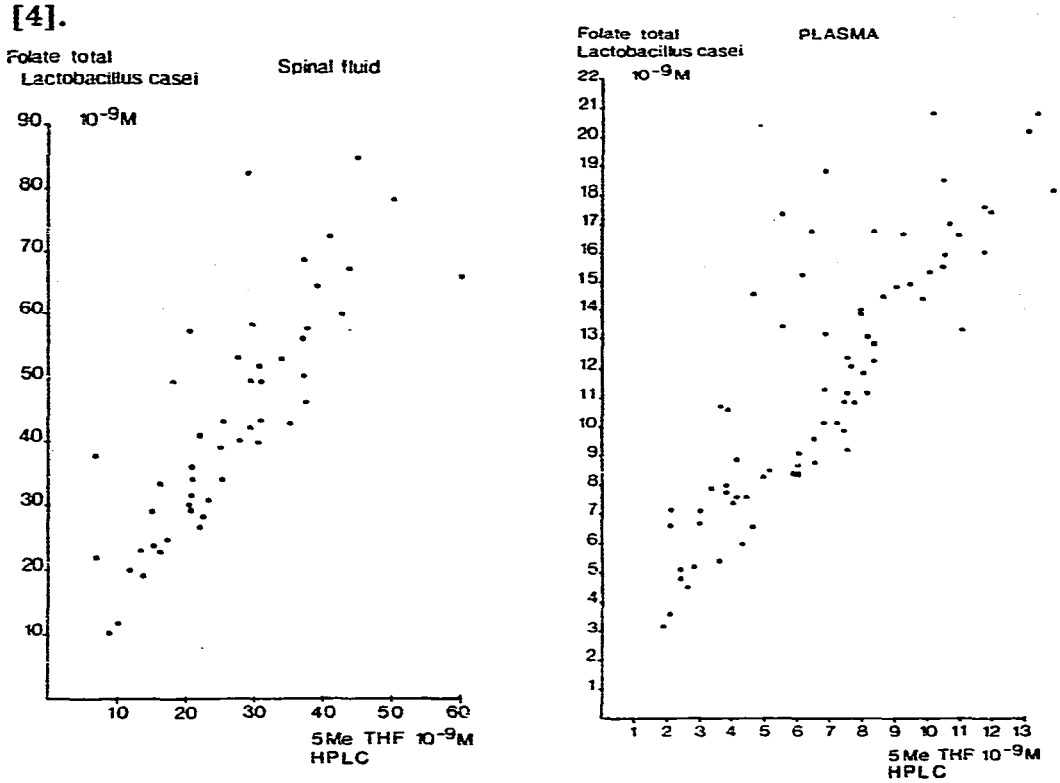

Fig. 3. Correlation of concentrations of 5-MeTHF with total folate measurements using the growth of *Lactobacillus casei* for plasma (n = 83) and spinal fluid (n = 49) samples; computer generated regression lines are represented by $y = 3.45 \cdot 10^{-9}\ M + 1.31 \cdot 10^{-9}\ M \cdot x$ and $y = 8.95 \cdot 10^{-9}\ M + 1.30 \cdot 10^{-9}\ M \cdot x$, respectively, where y = ordinate and x = abscissa; regression coefficient for both cases = 0.83.

Measurement during administration of folates

When folic acid, leucovorin (Ca-folinate) and methotrexate were present in the plasma they showed a sensitivity of less than 0.1% of that of 5-MeTHF under the described conditions. Relatively high concentrations of these other folates can be determined simultaneously by UV detection. The analytical column did not show a good separation of these compounds, and by altering the pH this selectivity could not be substantially improved.

5-MeTHF concentrations can be measured simultaneously with leucovorin or folic acid concentrations. The combination of folic acid and 5-MeTHF in rat plasma is shown in Fig. 4.

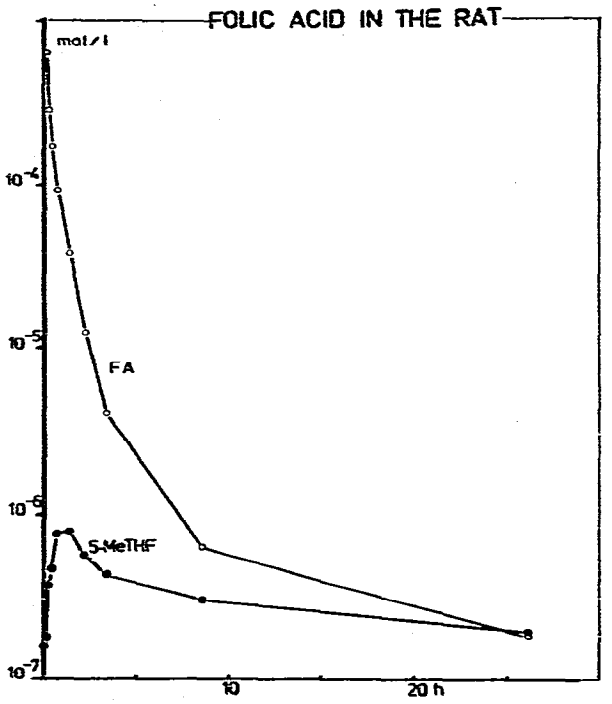

Fig. 4. 5-MeTHF plasma concentrations (EC detection at + 0.3 V) after intraperitoneal administration of folic acid (FA) (UV detection at 280 nm) in the rat (dose 90 mg/kg).

Plasma concentrations in man

Plasma concentrations of 5-MeTHF were measured after oral administration of 15 mg leucovorin (Ca-folinate) in two volunteers. The plasma concentration vs. time profile is shown in Fig. 5, indicating a rapid conversion of leucovorin to 5-MeTHF. The latter compound was shown to have a half-life of 3.5 h in the plasma of these volunteers.

Measurement in urine samples

After addition of ascorbic acid to a human urine sample the concentration of 5-MeTHF was found to increase by about 70% in 8 h. This phenomenon can be explained by the stimulated chemical generation from other folates present in urine or the breakdown of endogenous folates to 5-MeTHF. When no ascorbic acid was added the concentration fell rapidly. Because of these fluctuations determination of 5-MeTHF in urine was less reliable.

DISCUSSION

One-column system

When deproteinized plasma was injected directly into a reversed-phase column or an ion-exchange column a large, unretained, tailing peak was seen from

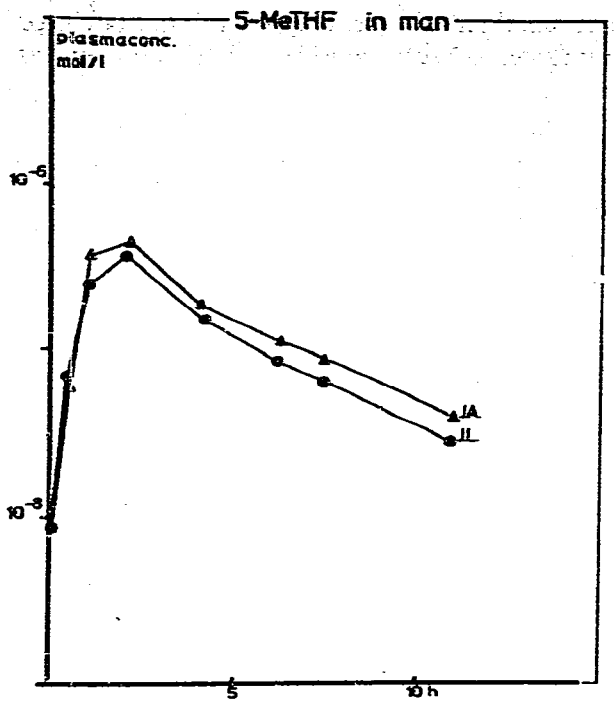

Fig. 5. 5-MeTHF plasma concentration vs. time curves after oral intake of 15 mg leucovorin in two healthy volunteers, 26 (J.A., ▲) and 27 (J.L., ●) years of age.

both the ascorbic acid and the TCA. TCA could be extracted just before injection by diethyl ether, but the concentration of 5-MeTHF in the aqueous layer also decreased considerably. This can be explained by the formation of ion-pairs which are extracted in the organic layer and/or by destruction of the compound.

Concentration eluent volume

Tap water was also investigated as the concentration eluent used for the methotrexate determination [16]. In Fig. 6 the peak height is presented as a function of the concentration eluent volume. It can be concluded that tap water is not useful here, due to elution of 5-MeTHF from the concentration column. After the addition of citrate the retention was considerably increased. However, the growth of micro-organisms in this medium was a serious problem. The addition of 2-hydroxy-1-isopropyl-4-methylbenzene (thymol) had a favourable effect in retarding this growth but appeared to decrease the retention of 5-MeTHF during concentration. This was not the case after the addition of 0.5—1 mg/l sodium azide, which was found to be the most useful for this purpose. In Fig. 6 an upper limit of 12 ml is seen for the concentration eluent.

Choice of electrode potential

The peak height as a function of electrode potential is shown in Fig. 7, indicating a half-wave potential of + 0.3 V (vs. Ag/AgCl/0.001 *M* Cl^-). When the potential increases, a higher sensitivity can be expected, but a lower selectivity

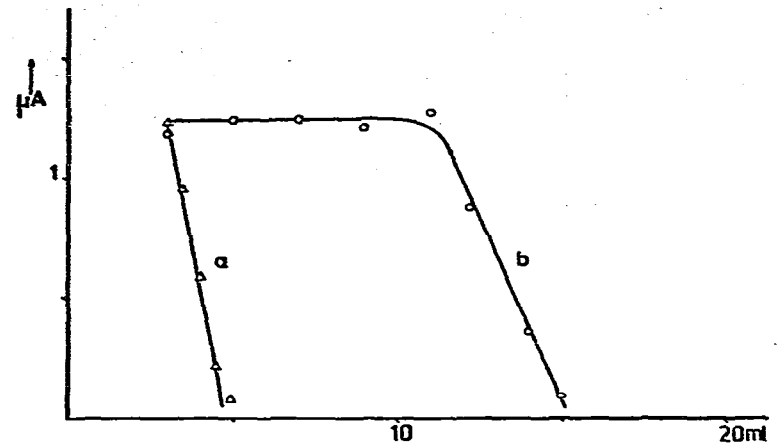

Fig. 6. Effect of citrate on retention during the concentration step. The peak height versus the volume of the concentration eluent for two compositions of concentration eluent is given: (a) tap water; (b) a solution of 0.015 *M* sodium citrate in 0.015 *M* phosphate buffer (pH 4.9), containing 0.5—1 mg/l sodium azide.

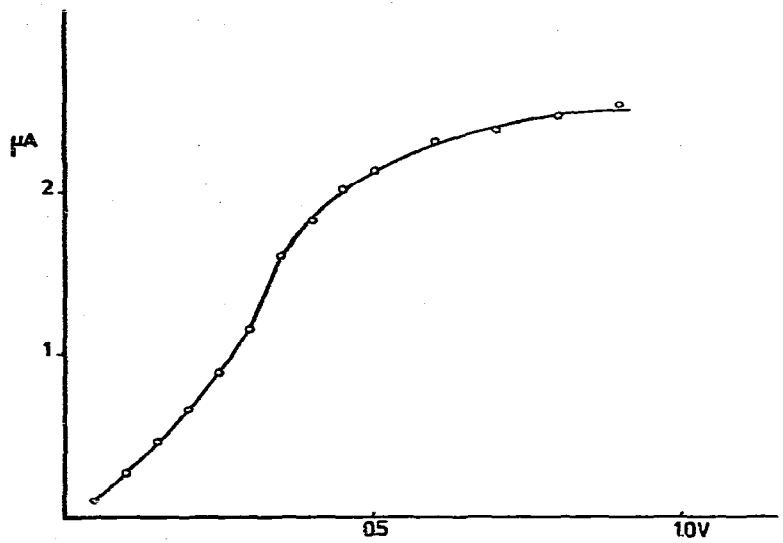

Fig. 7. Maximal peak height in μA vs. working electrode potential (vs. Ag/AgCl/0.001 *M* Cl^-) after injection of $1.8 \cdot 10^{-9}$ mole 5-MeTHF.

towards other oxidizable plasma or spinal fluid components occurs. The difference in the measured chromatogram found by an increase in potential from + 0.3 to + 0.5 V is demonstrated in Fig. 8. A potential of + 0.3 V appeared to be optimal with respect to sensitivity and selectivity.

Quantitation — peak height versus peak area

Whether peak height or peak area is taken as a measure for 5-MeTHF depends on the electrochemical conditions. In voltammetric detection the signal is proportional to the concentration in the detection cell. In order to reduce the decrease in concentration near the electrode surface only a small electrochemical

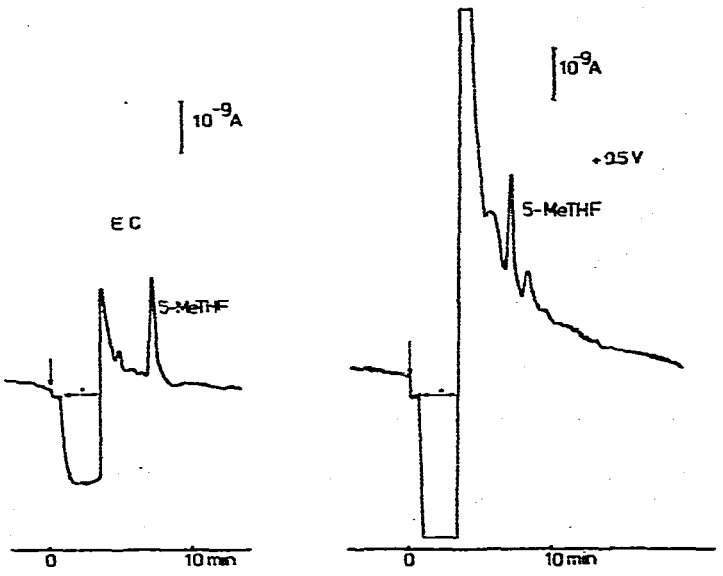

Fig. 8. Effect of electrode potential on detection selectivity for a plasma sample containing 5-MeTHF ($5 \cdot 10^{-9}$ *M*). This figure should be compared with Fig. 1; the asterisks indicate venting period. Chromatograms shown at + 0.3 V and + 0.5 V.

conversion is allowed. In this case the peak height is used for quantitation. However, when the electrochemical reaction or adsorption/desorption processes of reagents and products are rate-limiting an increase in sensitivity is not expected by facilitating transport. From Fig. 2 it can be concluded that under the present chromatographic conditions the voltammetric character dominates. So the peak height should be taken for quantitation. In agreement with these findings dynamic measurement showed more variation of peak area than of peak height when the flow was varied.

CONCLUSION

The method described is useful as a rapid, sensitive and specific determination of 5-MeTHF in plasma and spinal fluid. The selectivity for 5-MeTHF related to other folates in patient samples was obtained by carefully selected detection conditions rather than by separation properties of the analytical column.

In general, this phenomenon should be studied more extensively when considering optimal detection conditions in electrochemical detection. In the determination of methotrexate and 7-hydroxymethotrexate [11,16] interference of 5-MeTHF is excluded by elution during the concentration step. The application of columns with a smaller inner diameter should be investigated for analogous analytical problems. By using micropacked columns with inherent lower eluent flow-rates the detection limit would be decreased by an increase in detector sensitivity.

The behaviour of 5-MeTHF in the body after administration of leucovorin during anticancer therapy is under investigation.

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