In Vivo Distribution of Hydrocortisone Over Whole Blood: A Novel Method for the Extraction of Erythrocytes

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SUMMARY

Ten mg hydrocortisone (HC) was administered intravenously to a healthy volunteer after a dexamethasone suppression test and HC concentrations were determined from 1-270 min in plasma, plasma water and on erythrocytes. HC was extracted from erythrocyte concentrates with high efficiency by HC-poor plasma or by human or bovine albumin solutions. Determination of HC in the plasma of the volunteer mainly gave insight about the concentration time course of HC bound to plasma proteins. One minute after HC injection the amount associated with erythrocytes was about half the amount bound to plasma proteins. Decrease of HC in plasma, and hence on plasma proteins, was monophasic from 30-270 min with a half-life of 116 min. Decrease of HC associated with erythrocytes and HC free in plasma water was biphasic from 30-270 min and initially HC diminished about five times faster from these compartments than from plasma proteins. At the end of the observation period half-lives on plasma proteins, erythrocytes and in plasma water were similar, i.e., @ 120 min.

It is concluded from these as well as from previous in vitro experiments that erythrocytes gain importance as HC carriers at increasing HC blood concentrations. Once charged, erythrocytes yield HC much more readily than do plasma proteins. This "last come first go" phenomenon of association of HC with erythrocytes is known also to exist for certain drugs. It indicates erythrocytes as important transporters of non-freely water soluble compounds.

Key words: Hydrocortisone - Erythrocyte extraction - Erythrocyte binding - Protein binding - Free fraction

INTRODUCTION

Blood is a mobile tissue supplying all other tissues and mainly consisting of erythrocytes in a matrix of plasma. It is propelled from larger vessels into capillaries where it is kept apart from the other tissues by a 0.5 micron thick deformable and sometimes discontinuous membrane. By passing the membrane body constituents dissolved in the fluids of pericapillary tissues can be taken up into blood and vice versa. Compounds poorly soluble in plasma water are also transported in blood by association to plasma proteins and erythrocytes. From in vitro experiments it has been demonstrated that partition ratios of the body constituent hydrocortisone (HC) and of the drugs valproate (VPA) and phenytoin (DPH) over the blood compart-

ments plasma water, plasma proteins and erythrocytes depend on concentration (1-3). At higher concentrations plasma proteins approach a state of saturation whereupon erythrocytes in turn become of more importance as transport medium. On the other hand it could be shown that erythrocytes charged with the mentioned compounds release their cargo much more readily to blank plasma water than do plasma proteins. This phenomenon of concentration-dependent association with and release from erythrocytes was described as a "last come first go" principle and was operating for both drugs within their therapeutic ranges of blood concentrations (1, 2) and for HC in the physiologic range (3).

In vivo a corresponding phenomenon has been
demonstrated, as with declining blood concentrations in a dose interval, erythrocytes lost both drugs much faster than plasma proteins, whereas the half-life of the drugs in plasma water had an intermediate value (1, 2). This indicated that the pool of drug linked to erythrocytes is more mobile than the one bound to plasma proteins.

The study reported here had two main objectives, i.e., first to establish whether the half-lives of hydrocortisone on erythrocytes, in plasma water and on plasma proteins in a dose interval would increase in a similar order as that observed for the two drugs. Second to this question of kinetics a new approach to the determination of HC on erythrocytes in blood was investigated. As plasma proteins bind HC much stronger than erythrocytes, it should be possible to “extract” erythrocyte concentrates with HC-poor plasma. As most analytical procedures in clinical chemistry are based on plasma samples, such an extraction technique would facilitate the determination of compounds associated with erythrocytes.

MATERIALS AND METHODS

(Radioactive) compounds, solutions and reagents

Ten mg HC for injection was prepared as a sterile solution in 1 ml ethanol 98% in the hospital pharmacy. Labelled HC (1,2-H$_3$), spec. act. 50 Ci/mMol and a purity of 97.8% was purchased from Amersham and used as such.

Albumin solutions used for extraction: 680 mg human albumin fraction 5 (Sigma A-1653) or 680 mg dried, demineralized bovin albumin (Poviet Prod., Amsterdam), 60 mg NaCl and 22 mg NaHCO$_3$ were dissolved in 10 ml water; VPA-C$_{14}$, labelled at both C-atoms adjacent to the a-group, spec. act. 23 mCi/mMol was obtained from Sanofi-Labaz, Brussels; DPH-C$_{14}$, spec. act. 58 mCi/mMol, from Amersham.

FETI ADVANCE reagents were obtained from Syva (Merck, Darmstadt). RIA reagents: the antibody was obtained from Prof. Dr. J. Thyssen, Dept. of Nucl. Med., State Univ. of Utrecht. Scintillation solution, Packard Scintillator 299.

Apparatus

Hematocrit capillaries, length 75 mmm, i.d. 1.3-1.4 mm, volume $0.67 \mu l$ per mm, Schott, W. Germany; for plasma ultrafiltration: YMT filters, Amicon, Oosterhout, The Netherlands; liquid scintillation counter, Packard Tricarb 4640; Biofuge A hematocrit centrifuge, Hereaus, W. Germany.

Between procedures whole blood, plasma and plasma ultrafiltrate were stored at 4°C. All procedures were performed at room temperature.

In vivo experiments

Acquisition of HC-poor plasma

A male volunteer, 53 yr, 72 kg, received 1 mg dexamethasone orally at 11.00 p.m. Blood was collected the next morning at 10.00 a.m. and the plasma to be used for extraction of erythrocytes was checked on HC depletion using the FETI ADVANCE method.

Concentration-time profile of HC in fractions of blood

In the same volunteer a dexamethasone suppression test was performed as before. The next morning starting at 10.00 a.m. 5 ml blood samples were drawn from a heparinized catheter in the V. cubiti at times (min) 0, 1, 4, 6, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 150, 180, 210, 240, 270 and collected in EDTA-K$_3$ coated containers. Between times 0 and 1, 10 mg HC was injected i.v. into the V. cubiti contralateral to the catheter. The 5 ml blood samples were immediately divided into a portion of 1.5 ml and in one of about 3.5 ml. The first portion was used for analysis of non-radioactive HC in blood fractions using FETI and RIA methods; 2.5 ml of the second portion were added to test tubes containing an amount of @ 0.4 $\mu$Ci HC-H$_3$ dissolved in 50 $\mu$l HC-free plasma, agitated and allowed to stand minimally for 1 h at room temperature. In these samples the partition of HC over blood fractions was checked by measurement of radioactivity.

Measurements in blood using FETI ADVANCE and RIA

Erythrocyte concentrates

Immediately after sampling six hematocrit capillaries were filled with blood and centrifuged. The hematocrit was determined and the parts containing plasma or erythrocytes separated from each other by cutting the capillary well below and above the buffy coat using a glass knife. The parts containing erythrocytes were cut in two, and after measuring the lengths of the fragments with a vernier, transported six to six into two narrow test tubes containing 200 $\mu$l of HC-poor plasma. The six fragments contained about 40 $\mu$l erythrocyte concentrate. The erythrocyte contents of the fragments were centrifuged (5 min at 3000 rpm) into the HC-poor plasma, agitated and allowed to stand for one night at 4°C. The samples were shaken afterwards by a vortex.
mixer in case not all capillary fragments had lost their contents. After this extraction the hematocrit of the erythrocyte plasma mixture was determined, the plasma separated by centrifugation and 120 μl transported to a test tube for HC measurement with RIA.

**Plasma**

After filling of the six capillaries the remaining blood was centrifuged and 120 μl of plasma transferred to test tubes for HC measurement with the routinely used FETI ADVANCE method (4).

**Plasma water**

About 350 μl of the remaining plasma was used for centrifugation through YMT filters yielding about 100 μl of plasma water. During centrifugation the temperature rose from 22 to 25°C (3). Because in plasma water HC concentrations appeared to be too low for measurement with FETI, these samples were analyzed with RIA. FETI and RIA methods were compared using plasma samples.

**Procedures with hydrocortisone-H\textsubscript{3} enriched blood**

**Erythrocyte concentrates**

About 40 μl of the remaining plasma was used for centrifugation through YMT filters yielding about 100 μl of plasma water. During centrifugation the temperature rose from 22 to 25°C (3). Because in plasma water HC concentrations appeared to be too low for measurement with FETI, these samples were analyzed with RIA. FETI and RIA methods were compared using plasma samples.

**Plasma and plasma water**

These portions were prepared as described above. Duplo volumes of 50 μl were counted for radioactivity.

**Determination of radioactivity**

Either 50 μl plasma used for extraction or 50 μl plasma or plasma water or the capillary fragments which contained the extracted erythrocytes were transferred to glass counting vials and 1 ml of 0.1 N NaOH was added. To erythrocytes and plasma water 50 μl blank plasma and to plasma and plasma water 10 μl of blank erythrocytes were added to make all samples equal in color quenching. With a vortex mixer the erythrocytes were shaken out of the capillary fragments. To decolorize 0.5 ml 30% H\textsubscript{2}O\textsubscript{2} was added after 0.5 ml 2-propanol to prevent foaming; the samples were left uncapped overnight. Thereafter the samples were heated for 2 h in a waterbath at 45°C. After cooling 10 ml scintillator 299 was added. Counting was done with luminescence correction.

**Extraction of erythrocytes with albumin solutions**

Tracer amounts of radioactive HC, DPH and VPA as such or supplemented with non-radioactive substances were added separately to portions of 1 ml of whole human blood obtained after a dexamethasone suppression test, resulting in amounts of 18.2 or 200 ng HC, 0.27 or 10 μg DPH and 1.8 or 100 μg VPA per ml. After 30 min equilibration at room temperature the procedure as described above was followed except that 200 μl human or bovine albumin solution instead of HC-poor plasma was used for extraction of erythrocyte concentrates.

**Calculations**

In the calculations it was taken into consideration that erythrocyte concentrates contain 2% entrapped plasma (1) and hence that only 98% of the volume consists of erythrocytes. Furthermore, in the used batch of hematocrit capillaries 1 mm corresponded with a volume of 0.67 μl. (The equations used for calculating the results are available from the authors upon request).

For curve fitting from 30-270 min optimum fit was obtained by using the equation $Ae^{-x_1t}$ to plasma concentrations and the equation $Ae^{-x_1t} + Be^{-x_2t}$ to plasma water and erythrocyte data, in which A and B are intercept parameters, $x_1$ is the distribution rate constant and $x_1$ and $x_2$ are the elimination rate constants, and t is time in minutes.

**RESULTS**

**Agreement between FETI ADVANCE and RIA measurements**

A plot of plasma HC concentrations obtained with FETI ADVANCE on the abscissa against the results from the same samples obtained with RIA on the ordinate showed a linear relation: $y = 1.046 x - 0.06$ with a correlation coefficient of 0.994 (n = 18). Hence, both methods used for measurement of stable HC generated nearly equivalent results.

**Extraction performance**

Figure 1 shows on the ordinate the performance in extracting erythrocyte concentrates with HC-poor plasma expressed as percentage extracted from the total amount of radioactivity originally present on erythrocytes. On the abscissa the concentration of HC in the plasma of the blood samples is shown. In general, extraction efficiency then more than 90%. A slight
dependence of extraction performance on HC content in blood is probable.

**Concentration-time course and half lives of HC in blood fractions**

Figure 2 shows the concentration of HC in plasma as determined with FETI ADVANCE and in plasma water and erythrocytes as measured with RIA. As can be inferred from the lacking data at later time points, concentrations in erythrocytes and plasma water decreased to blank values or to the detection limit at 20 min resp. 150 min. The figure also indicates the amount of HC bound to plasma proteins, calculated by subtracting plasma water concentrations from plasma concentrations. It illustrates that measuring plasma concentrations of HC only gives an insight in the course of its concentration on proteins.

After the partition ratios of radioactive HC over the fractions of whole blood were established, it could be calculated from the plasma concentration how much of the substance should be present in the erythrocyte and plasma water fractions. The calculated values are depicted together with the concentrations measured with RIA in Figure 3.

Evidently, in plasma water the predicted and observed concentrations show the same course and match fairly. This is also the case for the HC concentrations which are present on erythrocytes from 1-20 min after injection.

By comparing Figures 2 and 3 it appears that from 30 min onward values in plasma follow a straight line in the semilog plot, whereas the values of HC in the plasma water fraction, shown both as those calculated from the partition of radioactivity over the fractions as well as those obtained when measuring stable HC, do **not**. The same is true for the elimination from the erythrocyte fraction. The plasma data of Figure 2 from 30-270 min fit to a straight line, corresponding with only one half-life of 116 min. Curve fitting of the plasma water and erythrocyte data of Figure 3 based on radioactive measurements resulted in a biexponential curve corresponding to two half-lives. Table 1 shows these elimination half-lives with standard deviations.

From the table it appears that elimination from the erythrocyte and plasma water fraction proceeds almost 5 times faster in the early 30-270 min period than from the plasma fraction, whereas for the lower concentrations at later time points the half-lives in the three blood
**In vivo distribution of hydrocortisone**

**FIG. 2.** Log concentration time curve of hydrocortisone in plasma, plasma water, on plasma proteins and erythrocytes in a human volunteer after a bolus injection of mg hydrocortisone. + hydrocortisone in plasma as measured with FETI ADVANCE; ○ hydrocortisone bound to plasma proteins; □ hydrocortisone associated with erythrocytes as measured with RIA; △ hydrocortisone in plasma water as measured with RIA.

**FIG. 3.** Log concentration time curves of hydrocortisone in plasma water and on erythrocytes as measured with RIA compared with curves calculated from the partition of radioactivity. ❀ open symbols: hydrocortisone associated with erythrocytes as measured with RIA; □ closed symbols: predicted amount of hydrocortisone associated with erythrocytes from radioactive measurements; △ open symbols: hydrocortisone in plasma water as measured with RIA; △ closed symbols: predicted amount of hydrocortisone in plasma water from radioactive measurements.
TABLE 1. Hydrocortisone half-lives with S.D. in plasma, plasma water and erythrocytes 30-270 min after injection

<table>
<thead>
<tr>
<th>Blood fraction</th>
<th>Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>116.2 ± 3.1</td>
</tr>
<tr>
<td>Plasma water</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>124 ± 18</td>
</tr>
<tr>
<td>Plasma water</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>130 ± 40</td>
</tr>
</tbody>
</table>

fractions are much the same. There is no difference in elimination rates from plasma water or erythrocytes.

DISCUSSION

Hydrocortisone-poor plasma appears to perform very well as an extraction medium for erythrocytes and correction for HC left on erythrocytes will generally not be necessary. However, plasma which meets the requirement of non-coagulation of erythrocytes and which at the same time is free of HC (as is the case following a dexamethasone suppression test) will not always be readily available. As more practical alternatives the extraction performances of human and bovine albumin solutions were also investigated. From these experiments summarized in Table 2 it can be inferred that @ 40 μl of erythrocyte concentrate charged with HC, VPA or DPH can be extracted with very high efficiency with 200 μl of albumin solution.

An albumin solution is a much more convenient matrix in further quantitative analysis than erythrocytes. In view of the growing interest in the role of erythrocytes as carriers with a distinct function in transport (5-7) this simplified analysis may facilitate further studies in this field.

In the erythrocyte extract measured with RIA an unexpected high value of 76 ng per ml HC was measured at time zero (Fig. 2). At 20 min and afterwards concentrations leveled off to this value. To study this unexpected phenomenon the determination of HC in erythrocytes was repeated in two volunteers following a dexamethasone suppression test. HC-poor human plasma and solutions of human and bovine albumin were used as extractants. Human plasma drawn after dexamethasone suppression as well as human albumin solutions still appeared to contain a slight quantity of @ 7 ng HC or a similarly reacting substance per ml by RIA determination. Consequently, this concentration would be the lower limit of the amounts detectable in erythrocytes when using these extraction agents. Bovine albumin solutions, however, have the advantage that they are free of HC. Using this medium for extraction no HC was found in erythrocyte concentrates obtained after dexamethasone suppression. This makes bovine albumin solution the extraction medium of choice for HC in such cases. The high HC concentration at zero time in the first experiment was, however, not reproduced.

In Figure 3 the concentrations in plasma water as

TABLE 2. Extraction of erythrocytes with albumin solutions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in blood (μg/ml)</th>
<th>Percentage of radioactivity extracted from erythrocytes (measurements in duplo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human albumin</td>
<td>Bovine albumin</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.018</td>
<td>83.5 82.8</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>94.7 93.9</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>0.27</td>
<td>92.1 92.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>92.3 92.7</td>
</tr>
<tr>
<td>Valproate</td>
<td>1.8</td>
<td>94.8 93.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>97.3 97.6</td>
</tr>
</tbody>
</table>
In vivo distribution of hydrocortisone

stable HC are steadily below those found by radioactive measurements. Obviously the 2.2% impurities present in HC-H₂ are not or are less firmly bound to plasma proteins and thus mainly collected in the ultrafiltrate fraction (8). A systematic correction for this contamination to make the curve obtained with radioactive measurements overlapping the other one was not undertaken because of the uncertainty of the exact partition ratios of the contaminants over the three compartments.

The second objective of the study was to investigate whether the three compartments of whole blood would show distinct HC half-lives. As could be predicted from in vitro measurements on HC distribution over these compartments (3), and as expected on the basis of experiences with the drugs VPA and DPH (1, 2), data in Figures 2 and 3 and Table 1 indicate that from 30-120 min the protein fraction discharged HC relatively more slowly than the erythrocyte and plasma water fractions. Only towards the end of the period from about 120-270 min, when the two last mentioned fractions were relatively empty of HC, did their half-lives fall in step with that of the protein fraction. However, not only from 30-120 min, but also in the first 30 min erythrocyte and plasma water fractions lose HC more abundantly than does the protein fraction. From Figure 2 it can be inferred that one minute after injection the concentration in plasma was 1050 ng per ml, in plasma water 186 ng and on erythrocytes 566 ng. With a hematocrit of 0.39 and a blood volume of 6 liters this means that one minute after injection 5.2 mg (52% of the dose) was still in the circulation. From these 5.2 mg, 13% was in plasma water, 26% associated with erythrocytes and 61% bound to proteins. After 20 min the HC plasma concentration was 261 ng/ml, plasma water concentration 16 ng/ml, whereas erythrocytes contained 70 ng per ml. At this moment a hematocrit value of 0.37 was measured and hence in total blood 1.1 mg HC (11% of the dose) was still present, corresponding with a disappearance from the circulation of 4.1 mg in 19 min. This decrease stems for about 14% from a lowering of the plasma water concentration, for 28% from a lower concentration on erythrocytes and 56% from a lower concentration on plasma proteins, giving a ratio of the participation of these fractions of 1:2:4. However, the concentration ratios between the 3 blood fractions at 20 min in the same order are 1:2:15, indicating that not all fractions deliver HC proportional to concentration, the proteins also being in this early period slowest in yielding their HC contents.

At body temperature less HC might be bound to plasma proteins and consequently the concentration in plasma water would be more elevated than at room temperatures. We did not study methodically to what extent the association of HC with erythrocytes is dependent on temperature. However, in vitro partition of 200 ng HC spiked with HC-C₁₄ over 1 ml of blood yielded a concentration ratio of erythrocytes to plasma water of 1.98 ± 0.02 (n = 3) when processed in a cold room (all procedures at 7°C), whereas this ratio was 2.14 ± 0.02 (n = 3) when the same sample was processed at room temperature. Extrapolating these ratios to that in vivo at 37°C it is plausible that the transport of HC on erythrocytes might even be more important then already deduced from measurements in samples processed at room temperature.

The role that erythrocytes appear to have in the transport of substances not freely soluble in plasma water will be of special interest when drawing conclusions concerning distribution after bolus injections, for instance the A. carotid and subsequent distribution over brain tissue. In those cases the protein binding of the substance injected into a plug of blood will be saturated and hence in the early phase, much of the injected compounds is transported on erythrocytes which are more eager to discharged their cargo than proteins. This means that the fraction of substance available for rapid diffusion into tissues will be much larger than estimated if the concentration in plasma water only.

In conclusion, the results of this study suggest once more a "last come first go" type of association, in this case of hydrocortisone with erythrocytes in vivo, indicating erythrocyte bound hydrocortisone to be a more mobile store in the blood pool than protein bound hydrocortisone. This will be especially important in cases of HC substitution therapy or chemotherapy in which more elevated blood concentrations are obtained. Secondly, hydrocortisone-poor plasma and albumin solutions proved to be good extractants for erythrocyte-associated hydrocortisone.

ACKNOWLEDGEMENTS

The study was supported by the Netherland Organization for Applied Health Research (Grant TNO - GO - CLEO - A54) and by the "Christelijke Vereniging voor Verpleging van Lijders aan Epilepsie". Gratitude is expressed to Dr. P. Vermeij, head of the Pharmaceutical Dept. of the Leiden University Hospital for supplying a sterile hydrocortisone solution for injection, to Dr. D.N. Velis for medical supervision of blood sampling and to Ms. P.A. Plesman for secretarial help.
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