

# Blood Volume Determination Through New Generation 130/0,4 Hydroxyethyl-Starch: A Propaedeutic, *In-Vitro* Study

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## Abstract

**Background:** The importance of knowing the blood volume, in critically ill patients, collides with the difficulty to have its direct measure through a safe and economic method. Hydroxyethyl starch (HES) was introduced by Tschakowsky as a useful marker for the dilution method, calculating the HES concentration (HESC) in a solution by inducing an in-vitro hydrolysis of starch molecules into glucose monomers and dosing the consequent increase of the solution glucose level ( $\Delta$  GLUCOSE).

**Objective:** This study develops a simple and cheap laboratory technique which uses a new generation 6% 130/0,4 Hydroxyethyl starch as a possible "dilution marker" for the measurement of patient's blood volume maintaining Tschakowsky's study protocol. The aim is to refocus attention on an interesting method that could lead the way to a number of possibilities in critical area.

**Method:** We designed a two-phase in-vitro experiment. Firstly, we found out the suitable treatment duration to ensure a complete hydrolysis of starch molecules. Secondly, we aimed to the achievement of a univocal constant of proportionality (K) between  $\Delta$  GLUCOSE and HES concentration. HESC will be expressed as HESV/PV ( $\mu$ l/mL) where HESV represents the HES volume and PV the plasma volume. Plasma volumes were calculated as  $BV^*(1-Ht)$ .

**Results:** K was planned by means of a linear regression analysis between HESV/PV and  $\Delta$  GLUCOSE on 133 validated samples collected from 30 healthy volunteers. The obtained hematocrit values ranged between 39.9 and 48 (mean  $\pm$  CI 95%=42,62  $\pm$  2,93). This corresponded to HESC ranging from 0,033 to 0,038 HES (mL)/PV (mL) (mean  $\pm$  CI 95%=0,035  $\pm$  0,002). While hydrolysis times increase, glucose values tended to augment until they reached stable plateau. During the second phase we handled a total of 720 specimens. Hematocrit of collected samples ranged from 33,9 to 49 (mean  $\pm$  CI 95%=41,3  $\pm$  1,21). HESC ranged between 0,015 and 0,089 mL HES/mL PV (mean  $\pm$  CI 95%=0,037  $\pm$  0,003). The regression analysis showed that HESC equals 0,592 times  $\Delta$  GLUCOSE ( $R^2=0,947$ ).

**Conclusion:** This study might be the first step in reintroducing starches into the clinical management of critical patients, not just as therapeutic agents for volume resuscitation but even as useful markers in the diagnosis of hemodynamic derangements, improving fluid and blood therapy strategies.

**Keywords:** Dilution marker; Blood volume; Voluven; Glucose

## Introduction

The determination of patient's blood volume (BV) with a direct, safe and economical method still represents a great challenge of both clinical and scientific interest.

It's often important for clinicians, especially in critical area, to obtain measures (and not only estimations derived from several calculations) of some relevant parameters or physical dimensions. Currently, the only useful approach to directly measure the blood volume is represented by the so-called "dilution method". This technique is based on the assumption that, if a known amount of an easily measurable "marker substance" is added to an unknown volume, it is possible to calculate the latter's real value, according to the measured marker's concentration. The principle is well explained by the formula:

$$C=Q/V$$

(C=Indicator Concentration, Q=Indicator Quantity, V=Volume)

Despite this simple assumption, during the years none of the proposed markers was found to be suitable for clinical settings, because of their cost, potential toxicity or dosing complexity.

At the beginning of the XXI century Tschakowsky [1,2] introduced the use of Hydroxyethyl starch (HES) as a safe and economic marker for the dilution method.

HES is derived from a highly branched glucose polymer (amylopectin) obtained from either waxy maize or potato starch and is commonly used as a colloidal plasma expander during fluid resuscitation. More precisely, it is generated by nucleophilic substitution of amylopectin to ethylene oxide in the presence of an alkaline catalyst.

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Since direct HES dosages turn out to be expensive and almost unreliable, Tschaikowsky et al. [1,2] proposed a simple and indirect procedure to overcome the problem: they demonstrated it was possible to calculate the HES concentration ( $HES_c$ ) in a solution by inducing an in-vitro hydrolysis of starch molecules into glucose monomers and dosing the consequent increase of the solution glucose level ( $\Delta$  GLUCOSE).

Two conditions are necessary to make this attempt achievable: firstly, the hydrolysis of HES molecules must be complete (or at least, it has to progress at a constant degree). Secondly, a substance-specific constant of proportionality (K) is required to bind an observed  $\Delta$  GLUCOSE to the corresponding HES concentration.

Starting from these premises,  $HES_c$  is obtainable through the formula:

$$HES_c = K * \Delta \text{ GLUCOSE}$$

( $HES_c$ =HES Concentration;  $\Delta$  GLUCOSE=Augment in glucose concentration due to HES hydrolysis; K=Constant of Proportionality)

Since HES remains a long time into the vessels, it would be possible to take the reported concepts back to the in-vivo context: it seems possible to calculate an individual intravascular plasma volume (PV) through an intravenous injection of a known amount of HES. The corresponding blood volume (BV) equals the PV multiplied for the inverse of hematocrit (1-Ht).

During their studies, Tschaikowsky et al. refined the laboratory procedure of hydrolysis, calculated the K of proportionality and tested in vivo the feasibility of this method with extremely promising results.

Nevertheless, the HES they used (10%; 200/0,5) is hardly available today, since it has been discontinued.

The objective of this study is to develop a simple and cheap laboratory technique which uses a new generation Hydroxyethyl starch (6% 130/0,4) as a dilution marker. This could lead the way to routine bedside BV determination, which is a great opportunity to easily assess a fundamental parameter for a correct evaluation of the intravascular compartment and its variations in several conditions (e.g., in case of sepsis, hemorrhage, perioperative fluid management...) helping to ensure a proper management.

Unfortunately, due to an E.M.A. note concerning HES administration in critically ill patients occurred during the planning phase of this study (see discussion for further details) it was not possible to test our method during an in-vivo experiment.

## Materials and Method

In order to make a third generation HES feasible for blood volume measurements; we designed and realized a two-phase in-vitro experiment. The entire process lasted from September 2014 to February 2015.

For all measurements we used VOLUVEN® (Fresenius Kabi, Bad Homburg, Germany), a 6% solution of waxy-maze-derived HES (molecular weight,  $130000 \pm 20,000$ , degree of substitution=0.38-0.45; molar substitution=0.4; C2/C6 ratio=9) in isotonic saline.

Since HES are usually commercialized as solutions, the amounts of VOLUVEN used are expressed from this point on in mL (1 mL=0,06 g) or in  $\mu$ L. Consequently, HES concentrations are indicated as  $\mu$ L/mL.

The objective of phase 1 was to find out the suitable treatment

duration to ensure a complete (or at least constant) hydrolysis of starch molecules.

For this purpose we collected through precision pipettes 5 samples, each containing 3 mL of blood from a health donor in anticoagulated test tubes (Vacutainer K3-EDTA 7.5%, 0.072 mL).

0,06mL of 6% VOLUVEN® was added to each sample. Obtained solutions were subsequently centrifugated for 10 min at 3500 rpm in order to separate the plasma.

0,6 mL of plasma of each sample were collected through precision pipettes and treated according to Tschaikowsky findings: each sample was mixed with 0,15 mL of concentrated hydrochloric acid before being incubated in boiling water (100°C). Samples were hermetically sealed into specific test tubes for the purpose. Incubation was necessary in order to catalyze the hydrolysis of HES.

Each sample was incubated for a different time interval (namely 5 min, 10 min, 15 min, 20 min, 25 min).

When time was up, samples were recovered and opened. 0,55 mL of Tris buffer 3,33 M were subsequently added to samples with the aim of stopping the hydrolysis by neutralization of the acid. The reaching of a neutral pH was checked with a precision pH meter (pH  $7 \pm 0,5$ ).

Obtained samples were finally centrifuged for 2 minutes at 14000 G. Glucose levels were measured on supernatants in duplicate using the Roche Cobas (Roche Diagnostics).

Obtained values were reported on a glycemia/time graphic which showed a progressive increase of glycemia until the achievement of a fixed plateau.

As performing twice each processing and each measurement, charts were based on the means of two coupled values.

Overall, the experiment was repeated for 5 different healthy donors with the achievement of 5 glycemia/time curves.

The shortest incubation periods required for the attainment of steady plateaus were investigated and considered as the minimum treatment duration suitable to allow a complete (or constant) hydrolysis of HES molecules.

The second phase of the experiment was aimed to the achievement of a univocal constant of proportionality (K) between  $\Delta$  GLUCOSE and  $HES_c$ .

To reach this scope, we collected through precision pipettes 7 samples of blood (each containing 6 mL) from 30 healthy volunteers. Samples were collected into anticoagulated test tubes (K3-EDTA 0.072 mL+3 mL blood).

One of the samples was exclusively used in order to obtain a reliable determination of Ht. This was necessary in order to calculate the volumes in which HES would have been diluted (see below).

The remaining blood was treated as follows:

We added an increasing amount of 6% VOLUVEN® (namely 0,00 mL; 0,06 mL; 0,12 mL; 0,18 mL; 0,24 mL; 0,30 mL) to different blood specimens. Samples were therefore treated according to Tschaikowsky: 0,6 mL of plasma were obtained from each sample and treated by means of the addition of 0,15 mL of concentrated hydrochloric acid. Incubation in boiling water (100°C) followed. We obviously incubated acidified plasma as long as the first phase of this experiment pointed out.

Every measurement conducted from this point on was doubly checked: from each of the 6 differently diluted solutions we extracted 4 samples; 2 of these were incubated for 15 min, and the others for 20 min. After that, neutralization of HCl was obtained by means of a TRIS buffer solution and confirmed through a precision pH meter (the detailed procedure was identical to the one carried out during the first phase of the experiment). The mean glucose levels between the tubes boiled for 15 min. and those boiled for 20 min. were obtained and compared each other (see discussion for further details): if the difference was less than +/- 3 mg/dL, a stable plateau was reached thereby validating the measurement. In reverse, if the difference was higher than +/- 3 mg/dL, data were discarded.

Once the measurement was validated, the mean between the means was assumed as the glucose value corresponding to a certain HES<sub>c</sub>.

Tubes that became unsealed during the procedure were discarded. In such a case remaining tubes were considered for the analysis. In case of violation of both the 15' and 20' treated tubes the analysis would have been suspended. This scenario anyway never happened during the study.

As reported above HES are usually commercialized as solutions. Their concentration will therefore be expressed as HES<sub>v</sub>/PV (μl/mL) where HES<sub>v</sub> represents the HES volume and PV the plasma volume.

Plasma volumes were calculated as BV \* (1-Ht). Since samples were diluted by the amount of VOLUVEN® we added, respective HES<sub>v</sub> were summed up with the calculated plasma volumes in order to obtain the actual dilution volume we used for the analysis.

K was calculated by means of a linear regression analysis between HES<sub>v</sub>/PV and Δ GLUCOSE.

$$K = \Delta \text{GLUCOSE} / (1000 * \text{HES}_v / \text{PV})$$

(K=Constant of proportionality; HES<sub>v</sub>=HES volume; PV=Plasma volume)

For convenience, HES<sub>v</sub>/PV values were multiplied by a factor of 1000.

## Results

As regard to phase 1, the obtained hematocrit values ranged between 39.9 and 48 (mean ± CI 95% = 42,62 ± 2,93). This corresponded to HES<sub>c</sub> ranging from 0,033 to 0,038 HES (mL)/PV (mL) (mean ± CI 95% = 0,035 ± 0,002).

As easily predictable, while hydrolysis times increase, glucose values tended to augment until they reached stable plateau.

After 15 minutes of hydrolysis, plateaus were always achieved as indicated by the stability of glucose concentration when extending the boiling time up to 30 minutes (data are shown in Figure 1 and Table 1).

Notably, once plateaus were reached glucose values didn't trend to decrease by extending the hydrolysis times. Accordingly, to our premises, we can assume a time of 15 minutes as the minimum duration to obtain a complete (or constant) hydrolysis of HES molecules. This assumption allowed us to set up the second phase of this study.

We therefore decided to treat samples as explained in the previous section of this manuscript during the last phase of the experiment.

The coefficient of proportionality between Δ GLUCOSE and HES<sub>c</sub> was calculated through a linear regression analysis on 133 validated samples collected from 30 healthy volunteers.

From each donor we obtained 5 values of Δ GLUCOSE corresponding to 5 different HES<sub>c</sub>. 17 measurements were discarded since they were not validated as described above.

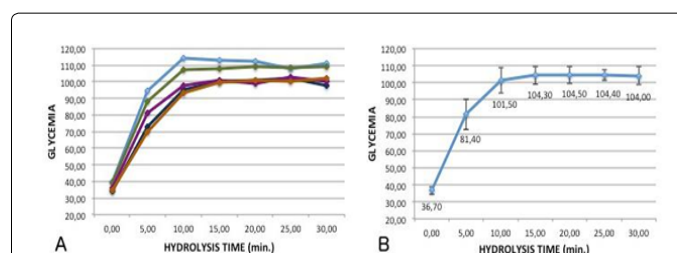
During the second phase of the study we handled a total of 720 specimens since each glucose determination required the treatment of 4 specimens. 42 specimens became unsealed during the catalyst phase of hydrolysis. As pointed out in the previous section, those specimens were excluded from the statistical analysis. Since the simultaneous opening of two coupled specimens never happened during the study, no data were lost due to tubes unsealing.

Hematocrit of collected samples ranged from 33,9 to 49 (mean ± CI 95%=41,3 ± 1,21). HES<sub>c</sub> ranged between 0,015 and 0,089 mL HES/mL PV (mean ± CI 95%=0,037 ± 0,003).

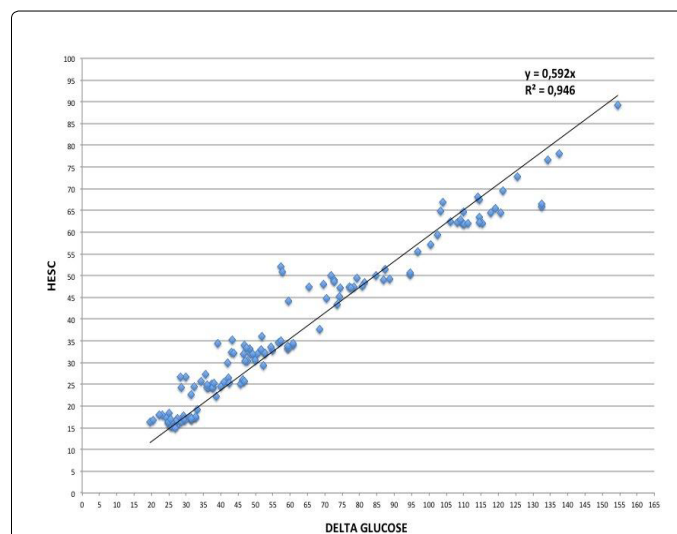
The regression analysis showed that is possible to calculate a K of proportionality: in this study HES<sub>c</sub> equals 0,592 times Δ GLUCOSE, with an high R<sup>2</sup> value (0,947). Data are shown in Figure 2.

## Discussion

Since we used a new and different marker in comparison with the one used in Tschaikowsky's study, with different chemical properties, we had to recreate the in-vitro setting of the experiment in order to identify the behavior of the new substance.



**Figure 1:** A: glycemic curves obtained by treating samples of plasma containing fixed concentrations of HES for different times. Each curve represents the analysis of a different sample. B: mean glucose values obtained by treating HES containing blood samples for different periods. A stable and long-lasting plateau was reached after 15 minutes of hydrolysis. Error bars represent 95% confidence intervals.



**Figure 2:** Regression analysis between Δ GLUCOSE and HES<sub>c</sub>. Δ GLUCOSE is expressed in mg/dL while HES<sub>c</sub> is expressed in mL/mL \* 1000.

HYDROLYSIS TIME (min)	0 min.	5 min.	10 min.	15 min.	20 min.	25 min.	30 min.
<b>GLUCOSE VALUE OF BLOOD 1 (mg/dl) (TREND)</b>	40,00	94,5 (+55)	114 (+19,5)	113 (-1)	112,5 (-0,5)	108 (-4,5)	111 (+3)
GLUCOSE VALUE OF BLOOD 2 (mg/dl) (TREND)	34,00	73 (+39)	95,5 (+22,5)	100 (+0,5)	101 (+1)	102,5 (+1,5)	97,5 (-5)
<b>GLUCOSE VALUE OF BLOOD 3 (mg/dl) (TREND)</b>	39,00	88 (+49)	107 (+19)	108 (+1)	109 (+1)	108,5 (-0,5)	109,5 (+1)
GLUCOSE VALUE OF BLOOD 4 (mg/dl) (TREND)	36,00	81,5 (+45,5)	97,5 (+16)	101 (+ 3,5)	99 (-2)	103 (+ 4)	100 (-3)
<b>GLUCOSE VALUE OF BLOOD 5 (mg/dl) (TREND)</b>	34,50	70 (+ 35,5)	93,5 (+ 23,5)	99,5 (+2)	101 (+1,5)	100 (-1)	102 (+2)
MEANS OF GLYCEMIC VALUES $\pm$ 95% CI (MEANS OF TRENDS $\pm$ 95% CI)	36,7 $\pm$ 2,35	81,4 $\pm$ 8,93 (+44,7 $\pm$ 6,7)	101,5 $\pm$ 7,63 (+20,1 $\pm$ 2,6)	104,3 $\pm$ 5,22 (+1,2 $\pm$ 2,45)	104,5 $\pm$ 5,16 (+0,2 $\pm$ 1,26)	104,4 $\pm$ 3,24 (-0,1 $\pm$ 2,76)	104 $\pm$ 5,22 (-0,4 $\pm$ 3,01)

Table 1: Results of phase one.

In particular, we analyzed the time needed to obtain a complete hydrolysis and the proportionality constant binding delta glucose to HES concentration.

About the first point, it is important to notice that a complete hydrolysis is not necessary to our purpose. In fact, it is enough to establish a constant hydrolysis rate of HES in order to obtain a univocal proportionality function between delta glucose and HES concentration.

During our study we maintained Tschaikowsky's study protocol in toto, thereby modifying the processing time of samples, in order to adapt the procedure to the new substance.

It is possible that the same VOLUVEN hydrolysis rate could be obtained earlier by increasing the reagents concentrations/HES<sub>v</sub> ratio, with evident advantages, both clinical and logistic. Further studies are desirable in this way.

During our tests, plateaus were reached between 10 and 15 min of hydrolysis (against the 7 min proposed by Tschaikowsky [1,2]), and they didn't decrease even up to 30 min of treatment. This fact rules out a possible hydrolysis effect by hydrochloric acid on glucose molecules.

Every measure we realized was checked for the reaching of a constant hydrolysis rate of HES (see results for further details) before being included in the statistical analysis.

This procedure notably limits the influence of possible procedural and/or analytic errors.

It emerges from the "materials and methods" section that during the second phase of the experiment we treated a HES-free sample as if it contained HES. This was mandatory in order to obtain the basal glycemic level of a subject.

Indeed, during HES hydrolysis, reagents were added to samples, which resulted inevitably diluted. Furthermore, the same substances which react with HES could react with glucose molecules, thereby causing an underestimation or even an annullment or a negativization of  $\Delta$  GLUCOSE.

Due to the high R<sup>2</sup> value we obtained from the statistical analysis and to the aforementioned "validation method" we applied we are very confident with the results we drew.

It is noteworthy that during this experiment samples were processed and analyzed by a single person and that measurements usually required less than one hour to be completed. Furthermore they were inexpensive. This make HES really suitable for the clinical context making it possible to open the door to routine bedside BV determinations.

Intravenous fluids administration represents one of the most common therapies during the acute phase of resuscitation.

The main indications are represented by severe hypovolemia, septic and/or inflammatory conditions, and all hemodynamic alterations that could be improved with a fluid load.

During the last decade a large debate took place for a consensus in the quality and quantity of the fluid therapy.

The publication of some important studies about the side effects of synthetic colloids [3-5], and a modern review of the traditional Starling's equilibrium [6-8], have both drawn the attention on the possible side effects deriving from an inaccurate volemic replacement strategy.

Furthermore it has been demonstrated that excessive fluid administrations (both of colloids and crystalloids) may produce tissue edema and severe electrolyte derangement. This could lead to higher morbidity, longer hospital stay and even increased mortality [9-13].

Fluid infusions must be also considered as any other pharmacological therapy: they can obviously improve outcomes when accurately provided, but they can cause potentially dangerous side effects as well [14-18].

Despite this, no clear guidelines exist concerning the choice of type, quantity and timing of fluid administrations [19-21].

The current modality of administering or removing fluids is centered on macro-haemodynamic and stroke volume. The preeminence of cardiac output in the assessment and treatment of hypoperfusion, in particular, can be confusing and lead to excessive volume administration. Not always the "volume responder" needs a volume expansion. If perfusion is adequate, further fluid administrations could be unnecessary, even if a volume challenge rises the cardiac output.

That is why the true circulatory defect, which requires correction, should be regarded as an inadequate tissue perfusion [22].

Furthermore, therapies like inotropes and vasoactive drugs (amines) may integrate support or substitute fluid resuscitation by augmenting venous return, cardiac contractility and preload [23]. These should be early considered during the management of unstable patients.

To do this, the vascular blood content (volemia) should be evaluated in a precise, objective and quantifiable manner. This level of evaluation is anyway still lacking.

For all these reason, the possibility to quantify a patient's blood

volume (BV) with a direct, safe and economical method still represents a great challenge, of both clinical and scientific interest.

Taking into account the recent literature review regarding hydroxyethyl starch toxicity, one may argue that the method we describe could be harmful for the patient [24,25].

This observation seems to be particularly rational considering that HES can harm the renal function of critical patients. As a matter of fact it has been observed that, after intravenous infusions, starches are taken by several tissues (including kidney, skin and bone marrow), and are stored into intracytoplasmic vacuoles. In kidney tubular cells, this accumulation is described as associated to osmotic nephrosis-like lesions and to an increased need for renal replacement therapy, especially in septic patients with a reduced glomerular filtration.

Accumulation of HES occurs early after infusions, lasts long and is dose related. Cumulative doses seem to be relevant rather than daily doses [26].

Although electronic microscopy proved the occurrence of HES deposition at 0,4-0,8 g/Kg, the clinical significance of this finding is still to be proven. In fact, even though a shared threshold for HES administration is still lacking, there is no evidence of association between cumulative starches doses inferior to 14 mL/Kg (10% HES 250/0,45; corresponding to 1,4 g/Kg) and increased risk of RRT [27,28].

Furthermore no studies have ever found an association between HES infusion and mortality.

Since we used administration of 100 mL or less of a 6% 130/0,4 HES (6 g) it appears safe to perform up to 15 BV measurements to an average 70 Kg weighted individual.

Despite that, some studies identified an increased risk of CRRT, dose related with starch administrations. For that and for the commonly used high doses of starches, an E.M.A. note occurred in October 2013. This notably limited the frame for Hydroxyethyl starch application.

In particular, it is reported that HES can be no longer used as plasma substitutes in critically ill patients (ICU average patients). This ruled out the possibility for us to test our method into an *in-vivo* context.

Anyway, since HES still has some clinical indications (e.g., acute hemorrhage occurring in a non-critical patient) we advocate for studies which compare blood volume measurements carried out through standard techniques and 6% 130/0,4 HES.

## Conclusion

The quantification of a patient's blood volume (BV) still represents a great challenge of both clinical and scientific interest. To date, all the validated techniques are unsafe (since they involve radioactive tracers) too much expensive in terms of resources ad time or too elaborate for clinical routine. This study aims to settle the bases for an affordable and feasible bedside tool for estimate the patients' volemia.

Nevertheless exploratory clinical trials aiming to reproduce, confirm and validate these results through *in vivo* measurements would be greatly valuable. Specifically, taking into account HES potential side-effects, attention should be addressed to find out the lowest suitable dose of HES to be administered for BV measurement.

Starting from the result of these studies, it might be justified to reintroduce starches into the clinical management of critical patients, not just as therapeutic agents for volume resuscitation but even as useful markers in the diagnosis of hemodynamic derangements.

## Competing Interest

The authors declare that they have no competing interests and that they didn't receive founding or technical support from Fresenius Kabi.

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