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RP-HPLC EVALUATION FOR SOME INSULIN INTERACTIONS

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ABSTRACT

In present study the RP-HPLC characteristics were used for the evaluation of insulin interactions. These characters, such as retention time (RT), area under the peak (AP), peak height (PH), and peak width (PW) were all examined. Free or uncontrollable cholesterol (UC) and fenugreek extract (FG) have been chosen to be the fat and food tested metabolites. It seems, as a general conclusion, that food fragment is considered to be preventive agent more than to be a treatment, meanwhile, it is a pH value dependent interaction, i.e. more acid or base in blood is less responded by food metabolites.

The most important achievement in present study was the capability of the technique to specifically determine the insulin interaction with UC. The new sharp analytical measurement is centered in a new peak of RT of more than 4 min for the protein only connected with the presence of UC. The new peak for the hormone is another one than its preceding original peak of about 2 min. The other mentioned HPLC characters sensitivity to these particular interactions was determined and separately discussed.

A modified technique based on this fast and simple assay may add a nutritional and medical significance to the field of cholesterolemia, hence insulin interaction becomes one of the most important molecular biology diagnostic test that constitute cholesterolemia cluster factors. These reactions were strongly recorded in both chromatographic and chromophoricl elements, especially AP, PW and PH. Moreover, the HPLC data has been supported by some spectrophotometric techniques.

INTRODUCTION

Insulin is known to be the key ward both in homeostatic and homeorhastic metabolic systems. This role of biological control is presumably attributed to its hormonal balance effect. Several known or unknown food or biological metabolites are seen to affect this extremely important role. The balanced diet is the only way to keep well-balanced body in nutritionally and healthfully status. Hopefully the picture that's emerging is that body's hormonal balance is like a symphony. Insulin is one of the loudest and most important instruments. When its metabolism goes normally, the other hormone action in most cases are frequently optimized *(Ahmed et al. 2005 a and Ahmed et al.2005 b)*.

Insulin structure and function were subjected to intensive studies concerning its metabolic functions. Variation in metabolic actions suggests that the initial binding response may give rise to a number of different mechanisms, which then result in the different final effects *(Pugeat, 1995; Bigot, 2003 and Griffin and Wildenthal, 1998)*. Furthermore, a particular type of insulin response may be prominent in one cell type while in other cells different mechanisms are important (Martin, 1987). A further difficulty in elucidating the mechanism of action arises from the very wide range of

different metabolic responses to the hormone, which is the major hormone promoting anabolic metabolism, particularly the rapid responses.

Non insulin dependent diabetes mellitus (NIDDM) that account for nearly 80% of all types is, in large part, due to insulin resistance, a state when the target cells no longer respond to ordinary level of circulating insulin *(Chance. et al. 1968).* Actually, a radical modification in diet designed to reduce insulin resistance involves increased phytoestrogen intake that decreases the bioavailability of sex hormones in women specific age cycle *(Berrino et al. 2001).* These results confirm weight loss induced by a low calorie diet which is effective in improving hyperinsulinemia *(Crave et al. 1995).* Insulin, for instance, has been shown to cooperate thyroxin hormone and decrease the levels of dehydroepiandrosterone (DHEA) in the blood *(Lavallee et al.1997).*

Physicochemical methods were used to study the insulin binding properties. The X-ray crystallographic analysis for the tertiary structure indicate that the A-chain portion of the molecule is the more exposed including the 6-11 disulfide bridge and possibly involved in hormonal activity. The B-chain is in internal portion of the molecule and noncovalent binding between B-chains is responsible for the formation of the insulin dimmer and higher polymer. The iodination of A-chain tyrosine up to one atom/mole has little effect on the biological activity of insulin, but increasing iodination causes progressive inactivation *(Chance et al. 1968).* On the other hand, sulfated insulin is sometimes used in resistant diabetics due to their reduced antigonicity and cross reactivity with circulating antibody *(Czech, 1980).*

The sensitivity of the protein for interaction is specific. For example, most amino acids bind insulin weak and nonspecific and did not affect the protein structural conformation no matter the legend concentration . Moreover, L-aspartic acid did not bind at all. In contrary, L-phenyl alanine reacts in two steps and affects the conformation structure of insulin. The characters of conformational changes here are depending upon the legend concentration *(Anzenbacher and Kalous, 1967).*

In general, most bioactive proteins (BP) upon binding with certain substances are loosing their activity. Some other factors interact these reactions in some cases. The techniques used to measure these reactions included titration curves, differential absorption spectra and measurement of optical rotation. These have indicated that all regions at low pH value, the heptaglobin molecule, for instance, undergo important structural changes. The altered structural protein was unable to bind hemoglobin and can not be determined on the basic of its increased proxidase activity *(Pavlicek and Kalous, 1967).*

There are signs that RPLC of proteins may have passed the zenith of the popularity. Overtaken by high efficiency, other LC supports which can deliver separation comparable to RP-HPLC in resolution, but carrying greatly reduced risks of denaturation and loss of biological activity. The very simplicity of RP-HPLC has made it easy to tailor conditions to suit successful separation of protein with a few restricted classes, e.g. growth factor, peptides hormones and immunmodulators, virus proteins, hemoglobin's and ribosomal protein *(Oliver, 1989).*

In present study the HPLC technique peak's characters were used to determine insulin interactions with some food and fat metabolites that may add an important cheap analytical tool in evaluating its extremely important biological actions.

MATERIALS AND METHODS

Chemicals: Insulin, Bovine insulin of Novo Denmrak regularly used of 20 IU/ml (42 µg/ml) prepared for direct injection was used. This was diluted one hundred times before incubation with free cholesterol (UC) or fenugreek extract (FG). The incubated solutions were phosphate buffer at pH values around the normal blood pH range (7.365).

The UC solution was cholesterol of analytical grade of Prolabo (386.4 MW) Needham Market Suffolk, England, which dissolved as 0.01 mg/ml in chloroform. Meanwhile, the FG has been prepared after boiling 10-g fenugreek seeds in 40 ml water for 10 min. before filtration. Commassi blue G250 reagent was prepared according to *Bradford (1976)*. All other used chemicals including phosphate salts were of pure laboratory analytical grade.

Insulin Interactions: The incubation of the hormone with the above-mentioned reagents has been all carried out at 37 °C. The legends under investigation, i.e. UC, FG and Commassi blue G250, were reacted for almost 5 min at a set of pH values of 6.64, 6.98, 7.38, 7.73 and 8.04 buffer of 0.1 N and 1/15 ionic strength.

The cholesterol binding procedure was adapted to that of *Ryan and Chopra (1976)* and modified by *Ahmed et al. (1989)*. To start the reaction, an amount of 0.1 ml of UC was added to the insulin solutions (4-ml), meanwhile, an aliquot of 0.05 ml of the other reactive agents were used.

Visible and UV spectra: All insulin preparation at given pH values were tested at 595nm according to *Bradford (1976)*. Each sample of fresh preparation was then scanned throughout the UV of WL from 190 to 400nm. The measurement has been carried out using Beckman UV spectrophotometer model DU 7400.

RP HPLC estimations: An aliquot of 20 microliter of each insulin sample was injected in an HPLC apparatus of Hewlett Packard series HPLC 1050, USA, using hypercil ODS C18 column. The gradient 20-50 acetonitril/water for almost 5 min runs in the presence of 0.1% TFA were carried out. The VUD detector at 210nm was used.

RESULTS AND DISCUSSION

Normaly, insulin RT makes a 2.737-min under the chromatographic condition was used. According to this specific protein separation technique, the pH value, as shown in Table (1), positive or negative shift depending on the acidic or basic blood range pH values, respectively. This Table shows also the effects of incubation with UC and either of C/F or F/C, or first incubation with UC or FG and vice versa, on that physical parameter of insulin. In some details, the pH value shift made nearly one second forward in slight acidic pH value to almost two seconds reward in relative weak alkaline solution. To put it more clearly, pH has hasten a sharp correlation with insulin

RT that moved from one second ahead to more than 1.5 back within a 0.66 acidic blood shift or base, respectively. These systematic changes have been pronounced in the same direction with the presence of free cholesterol (UC) and fenugreek (FG), particularly in the farest pH value used. Meanwhile, seemingly less affected by FG alone.

Table (1) : Changes (as +/- %) in retention time (RT) of insulin under pH value shift or incubation with cholesterol (UC) or/and fenugreek (FG).

pH shift	+/- sec	FG	UC	C/F	F/C
- 0.66	+ 0.96	+ 1.02	+ 1.08	+ 1.26	+ 0.90
- 0.40	+ 0.66	+ 0.78	+ 0.72	+ 0.60	+ 0.54
7.38	2.737	+ 0.06	- 0.30	- 0.06	- 0.36
+ 0.35	- 0.90	- 1.38	- 1.08	- 0.96	- 1.08
+ 0.66	- 1.44	- 1.32	- 1.62	- 0.66	- 1.56

Where: FG and UC insulin incubated with fenugreek and cholesterol; C/F and F/C with both of them cholesterol and fenugreek first, respectively.

Data in this Table may support the probability of insulin stability in FG administration. In contrast, UC gave an opposite effect in the total absence of FG. It seems that the food fragment here is considered to be preventive agent more than to be a treatment.

Area under the peak, as another HPLC character for insulin reaction, is tabulated in Table (2). It has been slightly changed except in case of incubation with UC. Taking the normal blood pH in consideration, FG is seen again to act as a protector for the hormone when first treated the protein. However, this shift area % may confirm the fact that a strong chemical reaction is established between the hormone and UC, which has been noticed earlier *(Ahmed et al.1999).* Data in normal blood pH value support a remarkable role for FG concerning insulin stability, which may be less effective with the blood pH value deviation. The pH value, however, determines the detection limit and depends on the RPC condition *(Oliver, 1989).* This Table shows also the effects of incubation with UC and either of C/F or F/C on those physiochemical properties of insulin. However, the sharpness of insulin peak is not practically influenced.

Table (2) : Changes (as +/- %) of insulin peak area (AP) response to UC and FG incubation in mAU sec/100.

pH shift	+/- sec	FG	UC	C/F	F/C
- 0.66	+3.7	+1.23	+7.4	-3.70	+0.74
- 0.40	-0.86	-2.35	-0.74	-7.40	-11.73
7.38	8.10	+0.99	-16.66	-10.62	-1.48
+ 0.35	+0.12	-0.63	-14.15	+1.20	-3.08
+ 0.66	-0.62	-3.09	-13.20	+5.18	+0.12

Where: FG and UC insulin incubated with fenugreek and cholesterol; C/F and F/C with both of them cholesterol and fenugreek first, respectively.

In Table (3), except in case of applying FG and UC at normal pH, a small boarding less sharper width for the protein peak are noticed. In another words, more PW shifting occurred at higher pH values. An exceptional range

was seen at lower pHs when UC was first incubated with insulin before the addition of FG. However, the sharpness of insulin peak is not practically influenced.

Table (3): Changes in width of insulin peak (PW) with UC and FG incubation in mAU sec /100

pH shift	+/- sec	FG	UC	C/F	F/C
- 0.66	3.42	3.30	3.36	3.66	3.42
- 0.40	3.36	3.30	3.36	3.60	3.36
7.38	3.42	3.36	3.36	3.48	3.66
+ 0.35	3.54	3.42	3.48	3.54	3.60
+ 0.66	3.60	3.42	3.48	3.54	3.84

Where: FG and UC insulin incubated with fenugreek and cholesterol; C/F and F/C with both of them cholesterol and fenugreek first, respectively.

The most remarkable sign that has been established for peak height is its reduction in the presence of UC. It is clear in Table (4) that what so-called insulin FGs protection is persisted at normal blood pH. It seems to be a pH dependent interaction; i.e. more acid or base shift in blood is less responded by food metabolites.

Table (4) : Changes (as +/- %) in height of insulin peak (PH) with UC and FG incubation in mAU sec /100.

pH shift	+/- mAu	FG	UC	C/F	F/C
- 0.66	+5.95	+1.69	-8.86	-9.70	+ 4.22
- 0.40	+ 1.27	+ 1.27	-5.91	-13.08	-2.53
7.38	2.73	+ 3.38	- 14.77	- 13.50	- 4.22
+ 0.35	- 3.38	-0.42	- 19.83	- 4.22	- 10.97
+ 0.66	- 5.91	- 3.80	- 19.93	- 12.58	- 10.97

Where: FG and UC insulin incubated with fenugreek and cholesterol; C/F and F/C with both of them cholesterol and fenugreek first, respectively.

As a matter of fact, insulin is most probably more sensitive at late ages in association to the blood chemical changes for several different reasons. The positive or negative response due to insulin chemical reaction that has been reflected by RP-HPLC characterization, therefore, is occurred according to the nature of food metabolite whether a stabilizer or inactivate agent.

The FG was reported in many cases to reduce blood sugar, urinary sugar excretion, TC, and TG, with no change in insulin levels *(Sharma et al. 1990)*. Moreover, in a controlled study of people with NIDDM, FG of 25g/d for 24 weeks significantly reduced blood glucose levels *(Sharma et al. 1996)*.This may be contributed to some constituents in natural food sources particularly metal ions. Gymnema Sylvester, for instance, may decrease the required daily dose of insulin *(Shanmugasundaram et al. 1990)* by containing these specific food constituents. Herb use may therefore be under the suppervision

of health care professional. Furthermore, people using insulin should avoid alcohol that interacts insulin action *(Threlkeld, 1997).*

Tobacco, in addition, decreases insulin reactivity and it compounds the health problems associated with diabetes *(Anderson et al. 2001).* In direct way, it has noticed that serum insulin level was significantly lower in oligofructose-fed rats both after eating and in the glucose tolerance test *(Kok and Delzenne, 1998).*

One of the most common protein stabilizer is the Chromium (Cr). Chromium is believed to enhance the effectiveness of insulin. This protein damage is the principle reason that diabetics have lower life expectancy than the normal. Cr allows insulin to work to its fullest extent. There is now scientific evidence that Cr picolinate can accelerate fat loss without compromising lean tissues *(Anderson,1997).* The presence of Cr and other metal ions such as Zn or Cu are most likely the main constituents behind the chromatographic change response of insulin.

Specific HPLC insulin/cholesterol peak (ICP):'

This unique peak being formed for insulin only in the presence of UC and comes two minutes later, almost 4.7 min, has different characteristics. Although it has more stable RT as shown in Table (5), all PA, PH and PW are very sensitive for the protein interaction.

Table (5) : Specific insulin/cholesterol peak under different conditions of icubation.

pH shift	RT	RTx	RTy	Area	Ax	Ay	Width	Wx	Wy	Height	Hx	Hy
-0.66	4.73	4.74	4.72	0.50	5.22	2.50	0.05	0.13	2.52	0.11	1.00	0.18
-0.40	4.71	4.72	4.72	1.88	2.00	3.60	0.09	0.20	0.54	3.30	0.40	0.46
7.38	4.71	4.72	4.72	2.45	4.50	1.00	0.09	0.11	0.34	2.33	0.70	0.07
+0.35	4.72	4.73	4.72	7.20	2.00	1.75	0.09	0.13	0.22	1.35	0.31	0.01
+0.66	4.72	4.72	4.72	4.35	5.00	2.75	0.09	0.11	0.26	0.81	0.86	0.65

Where: A peak area, W width and H height. X is C/G and Y is G/C.

However, using RF as HPLC parameter, less than 0.9 sec has been detected as longest variation of RT for this peak, which was seen to be pH dependant and FG as well. The FG seems to act as a correcting agent for what has happened in the presence of UC.

As a mechanism, RPC tend to trap any unfolded protein present in the unfolded form and depends much more on the overall amino acid composition, so structural feature also play an important though less well defined part in retention mechanism *(Oliver, 1989).* The insulin in that type of interaction might loss its proper folding structure as well as most of its proper functions. It is clear that the longer the peptide chain, the more hydrophobic side chains will be involved in transfer and be more steeply peptide retention is likely to depend on modifier concentration.The interaction of protein is responded by RP packing. The proteins in native state normally spends very little time in the absorbed state that affect the peak shape

Area under the peak is greatly responded as clear in Table (5). The unstable chromophor peak must be referred to an unstable chemical structure

that formed for the hormone. This should frequently existed according to the condition under which it forms.

Likewise, width of that protein's peak even with UC was controlled, but it becomes more triggered to the stationary phase when FG added assuming the presence of more polar solute. The most remarkable sign here is the two or even three times more response in peak width that occurred when FG's fragment conjugates the protein before UC. In consequences, peak height goes the reverse way in the presence of these legends. Note that there is no ICP peak in the appcence of UC at all.

The hormone binding capability was also tested using UV and Bradford spectra. As it shown in Table (6) that pH shifting for the protein can be detected using Bradford spectra, meanwhile, the influence of incubation with UC or FG first can be accurately responded. This might be useful in case of using the techniques together in order to draw more reasonable data about the protein interactions. Again, legend of specific roles such as food, fat and drug components must be of great help in this concern. In the front of these are multivalent ions. The presence of the metal ion in various studies has pronounced effects on protein stability. For example, the apoprotein is dramatically more susceptible to proteolysis than is the Zn-containing form *(Berg, 1990)*. The small angle X-ray scattering results suggest that the apoprotein is monomer whereas the Zn-containing protein is dimmer.

Table (6) : UV and Bradford spectra of the hormone binding capability

pH shift	BF	UC	210nm	C/F	F/C
- 0.66	0.120	0.030	1.100	2.500	2.150
- 0.40	0.110	0.060	1.100	2.760	2.300
7.38	0.100	0.080	1.000	2.650	2.150
+ 0.35	0.105	0.100	0.800	2.800	2.100
+ 0.66	0.110	0.110	0.800	3.000	2.000

Phenomenologically, it appears that metal-based domains are generally smaller than ones devoid of crosslinks. This might not be possible to gain enough free energy from favorable interactions to overcome the conformational entropy of the unfolded *(Trow et al. 2000)*. Thus, metal binding may be a very useful process for forming bumps and rides to extend from the surfaces of proteins that are well suited for interactions with other macromolecules and, in this case, shows the powerful of using LC analytical techniques in this area.

A new methodology for testing this relationship between insulin and cholesterol will appear soon. This technique can be used for measuring both of them separately.

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استخدام الكروماتوجرافى فائق السرعة في تقييم تفاعلات الأنسولين

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***قسم الإقتصاد المنزلي- كلية التربية النوعية بدمياط-جامعة المنصورة**

ثبت أن للأنسولين في لطريقة المستخدمة امتصاص عند دقيقتين ويتأثر بـرقم الحموضـة وكـذلك التحضين مع الكوليستيرول الحر ومستخلص الحلبة كما يتأثر ارتفاع قمة الامتصاص وعرضة والمساحة تحتة بتفاعلات الأنسولين مع الكوليستيرول.

وأهم ما ثبت في هذا البحث أن للأنسولين في وجود الكوليستيرول امتصاص جديد بعـد ٤ دقـائق بالإضافة إلى الإمتصاص

الأول بعد دقيقتين.وقد وجد أن تأثيرمستخلص الحلبة قد يرجع إلى وجود عنـصر الكروميـوم وان دورها واقي لهذا الهرمون.وتفاعلات الأنسولين أصبحت احدي أهم اختبارات بيولوجيا الجزيئات لتـشخيص حزمة العوامل الخاصة بالكوليستيرول وعلاقتة بأمراض القلب والشرايين.ويبدو أنة باستخدام هـذة الطريقـة التحليلية الجديدة يمكن الحصول علي معلومات مفيدة في مجال التغذية والطب.