Research Networking Programmes

Short Visit Grant ☒ or Exchange Visit Grant ☐

(please tick the relevant box)

Scientific Report

Scientific report (one single document in WORD or PDF file) should be submitted online within one month of the event. It should not exceed eight A4 pages.

***Proposal Title**:* The learning of GC-MS and conditions of sample-preparation process for GC-MS for the investigation of different endocrine diseases

Application Reference N°: 5670

1) Purpose of the visit

The aim of the visit was the investigation of 9 samples of urine by GC-MS methods with studying of preliminary sample-preparation step and comparing of these results with HPLC-UV data for the same samples, received in Russia.

The objects of research were urine samples of donors, having following disorders: 1 adrenocarcinomas, 1 virile syndrome, 3 Cushing's syndrome, 3 hyperaldosteronism and 1 urine sample of health donor. Previously all samples of urine were prepared by liquid-liquid extraction and investigated by HPLC-UV method (Agilent system) in Russia. The HPLC-UV method allows to measure 4 diagnostically important corticosteroids: free cortisol (FF), free cortisone (FE), 18-hydroxy-corticosterone (18-OH-B), 6-hydroxy-cortisol (6-OH-F) and the relations FE/FF and 6-OH-F/FF which are important for diagnostics too. However, this methods doesn't allow to determine diagnostically important metabolites of steroids.

The application of HPLC-UV method gave the following results. 1.It was observed that donor having adrenocarcinomas has characteristic chromatographic profile: extremely high levels of free cortisol and cortisone, 18-hydroxy-corticosterone, the ratios FE/FF and 6-OH-F/FF abnormally shift towards to free cortisol. 2.Inspite of the fact that donor has the virile syndrom diagnosis, the quantities of steroids and their relations have normal levels. 3. The samples of urine of donors having Cushing's syndrom showed the high levels of free cortisol, 6-hydroxy-cortisol and 18-hydroxy-corticosterone (the quantity of free cortisone is within the normal range), the ratio FE/FF abnormally shifts towards to free cortisol and chromatographic profiles have additional unidentified peaks.

4. The chromatogramms of urine samples of donors having the hyperaldosteronism diagnosis showed the slightly increased levels of free cortisol and cortisone, 18-hydroxy-corticosterone and additional unidentified peaks were registrated.

Thus, it is necessarily to use an additional criterion - urinary steroid profiling by high resolution gas chromatography–mass spectrometry (GC-MS) provides qualitative and semi-quantitative data on steroid and their metabolites excretion. This information provides a composite picture of the quantitatively major biosynthetic and catabolic pathways.

2) Description of the work carried out during the visit

This project allowed to learn the GC-MS method and conditions of sample-preparation process.

Before the determination of steroids and their metabolites it was nesessary to make a sample peparation, including steps:

1. The solid-phase extraction (SPE) of urine (24h collection) samples. Steroid metabolites are excreted in the urine mainly as glucuronide and sulphate conjugates with smaller amounts as free compounds. These are extracted together from urine using Sep-Pak® cartridges and eluted together using methanol.
2. The hydrolysis of conjugated steroids with following SPE step. Steroid conjugates were hydrolysed using a lyophilised enzyme preparation from the digestive juice of the edible snail, Helix pomatia. This also contains β-glucuronidase, which cleaves glucuronides, and steroid sulphatase which cleaves 3-sulphates but does not cleave 17- and 21- sulphates as efficiently. The steroid solution was buffered to pH 4.6, which is the optimum pH for the enzyme activities. After a 3-day incubation at 37degC, the freed steroids were extracted using Sep-Pak cartridges again.
3. The addition of internal standards. Internal standards are required for the GC analysis to identify steroids of interest by their position relative to the standards and for quantification. Three internal standards are used: 17-alfa-androstane-3-alfa,17-alfa-diol, stigmasterol and cholesterol butyrate. The first one elutes before the naturally occurring steroids; cholesterol butyrate elutes after them. Stigmasterol is included because it is relatively difficult to silylate and its peak height, in comparison with the other standards, provides a check on the completeness of the derivative. They are dispensed in a mixture containing 100 microgramms/ml of each. The quantification by GC-MS uses the 17-alfa-androstane-3-alfa,17-alfa-diol.
4. The derivatization and washing of determined steroids. The formation of a chemical derivative by methyl oxime-trimethylsilyl ether (MO-TMS) renders the steroid more stable, more volatile and greatly reduces ionic interactions, all of which are advantageous for GC-MS analysis. All ketone groups (except at position C11) reacted readily using incubation at 60degC for 90 minutes with formation of oxime-derivates. After addition of TMS reagent the samples were additionally incubated at 100degC for overnight.
5. Preparation samples for the analysis. The aliquote of cyclohexane was added to all incubated samples and washed by water twice. Thus, the organic solution was transferred into vials and was ready for test by GC-MS method.

The next step of investigation was the GC-MS analysis of received samples. The analysis was carried out on The Perkin Elmer Clarus 500 GC-MS system. Gas chromatography was carried out on capillary column Agilent (VF-1ms (OV-1), 30m x 0.32mm id x 0.45mm od, film thickness 0.1 micrometers), other conditions are following:

3uL injection volume, carrier gas helium, flow 1.80ml/min; split flow 45ml/min, splitless injection, with valve open for 3 min. GC programme: 50 degC 1 min, 20 deg/min until 175 deg, 2.5 deg/min until 270 deg; hold 2min. MS conditions (Perkin Elmer Clarus system): multiplier voltage 470 V; transfer line temp 270 degC, source temp 220 degC, data acquisition 16-40 min. Mass 100-900 scan time 0.4 sec, interscan delay, 0.1 sec.

The GC is fitted with a split-splitless injection port which is normally used in the splitless mode. This means that at the point of injection, the gas flows only down the column, so that vaporised sample is transferred only onto the column. After 3min, the split valve is opened to clear any residual sample out of the injector. The injection incorporates a cold trap: injection takes place with the column at 50degC. At this temperature, the steroid derivatives are immobile, so that the sample is concentrated as a narrow band at the front of the column. The temperature is rapidly raised to 180C, at which point the injected material becomes mobile, and then increased at a slower rate during the analysis until 270degC is reached.

Controlled temperature increase (temperature programming) is essential for steroid profiling because of the wide polarity range and complexity of the mixture of steroid metabolites found in urine. Peak widths remain relatively constant over the duration of the analytical run.

As a result, the steroids were identified by comparing retention times and their mass spectral characteristics with pure standards and were quantified using internal standards.

3) Description of the main results obtained

Gas chromatography method is fast with a good resolution. Combined with a mass spectrometer detection is highly sensitive, specific, has very good linearity and data can be obtained for all steroids. The quantitative results were obtained for following steroids: androsterone (A), aetiocholanolone (Ae), DHA, 11oxoAe, 11A, 11Ae, 16-alfa-DHA-I, pregnanediol (P2), pregnanetriol (P3), androstenetriol, tetrahydrocortisone (THE), tetrahydro-11-dehydrocorticosterone (THA), tetrahydrocorticosterone (THB), alloTHB, tetrahydrocortisol (THF), alloTHF, alfa-Cortolone, betaCortol+ betaCortolone. The analysis of urine sample of relatively health donor showed the normal levels of steroids metabolites with characteristic chromatographic profile.

Concerning the urine sample of donor having adrenocarcinomas, it was observed that levels of all steroids are extremely increased, excepting THA which quantity is lower than normal level. Excretion of cortisol metabolites was about twice normal. The most important markers are the increase of DHA and 5-pregnene-3beta,17alfa,20alfa-triol levels. All that confirms the diagnosis and agrees with HPLC-UV results.

The chromatogramm of the donor with virile syndrome has increased levels of aetiocholanolone, DHA, pregnantriol, androstentriol and cortisone metabolites. The main markers of 21-hydroxylase deficiency are tetrahydro-11deoxycortisol (THS) and 11-oxo-pregnantriol, but the last steroid had the low quantity in our case. Anyway the GC-MS method shows abnormal steroid profile which help us to predict the presence of desease.

The most important feature of Cushing's syndrome is the high excretion of both C19 and C21 metabolites of cortisol. It was observed that urine of donors having this desease has high levels of androsterone, aetiocholanolone, androstentriol, THE, THF, alloTHF, alfa-cortol and the sum of bCortol+ bCortolone. This results agrees with HPLC-UV experiments.

The chromatogramms of urine samples of donors having the hyperaldosteronism diagnosis showed the increased levels of alloTHF and 17-hydroxy-progesterone. The quantity of androgen metabolites was different and depended on the day of menstrual cycle.

Thus, the main results of the visit:

1. The method of sample-preparation of urine, including the hydrolysis and derivatisation steps, was studied;
2. The GC-MS method was used for the investigation of prepared samples. It allowed to receive useful information about quantity of steroids and their metabolites in samples. The comparison between results of the same samples received in our and London's Steroid Laboratory was made. Moreover, these experiments allowed to have an experience in the field of mass-spectrometry. All that is very useful for introduction GC-MS method in practice and diagnostics of different diseases, particularly tumors in Russia.

4) Future collaboration with host institution (if applicable)

It is planned to continue the collaboration together with Prof. Norman F. Taylor, Steroids Laboratory of King's College Hospital and Science Investigation Laboratory of Chromatography of the North-Western State Medical University named after I.I. Mechnikov. On the base of gained experience we plane to make sample-preparation and further GC-MS analysis of urine samples of patients having different endocrine diseases in Russia. The results will be discussed with Pr. Taylor and interesting samples might be useful for development of the data base of endocrine deseases, including tumors.

5) **Projected publications / articles resulting or to result from the grant *(ESF must be acknowledged in publications resulting from the grantee's work in relation with the grant)***

Together with Prof. Norman F. Taylor it is planned to have a publication related with results of the research including HPLC-UV and GC-MS data (ESF will be acknowledged in publications).

6) Other comments (if any)