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TRACK 6

ADVANCES IN LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY

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SESSION CHAIR : DR. LUIGI SILVESTRO
3S-Pharmacological Consultation & Research GmbH, Germany

SESSION CO-CHAIR: DR. SIMONA RIZEA SAVU
3S-Pharmacological Consultation & Research GmbH, Germany

SESSION INTRODUCTION

TITLE: VALIDATION OF BIOANALYTICAL METHODS; INVESTIGATION OF BACK-CONVERSION IN INCURRED PHARMACOKINETIC SAMPLES
DR. LUIGI SILVESTRO, 3S-Pharmacological Consultation & Research GmbH, Germany

TITLE: ICP-MS COUPLING TO HPLC TO QUANTIFY UNKNOWN METABOLITE OF DRUGS IN BIOLOGICAL FLUIDS
DR. SIMONA RIZEA SAVU, 3S-Pharmacological Consultation & Research GmbH, Germany

TITLE: LC/MS APPLICATION IN THE DRUG DISCOVERY
DR. MAGDY MOHAMMED, Pharmaceutical and Drug Industries Research Division, National Research Center, Egypt

COFFEE BREAK & POSTER SESSION

TITLE: COMPARISON OF DIFFERENT MASS SPECTROMETRY IONIZATION TECHNIQUES TO ANALYZE DESOGESTREL IN PLASMA SAMPLES
DR. MARTIN MURER, 3S-Pharmacological Consultation & Research GmbH, Germany

TITLE: ANALYTICAL METHODS FOR DETERMINATION OF SOME SELECTED DRUGS IN PHARMACEUTICAL FORMULATIONS BY HPLC
DR. V. JAYATHIRTHA RAO, Organic Chemistry Division II, Indian Institute of Chemical Technology, Hyderabad, India

TITLE: DEVELOPMENT OF A DISPERSIVE MICRO-LIQUID LIQUID METHOD FOR THE EXTRACTION OF PYRETHROID PESTICIDES FROM ENVIRONMENTAL MATRICES FOR DETERMINATION BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
DR. SAEED S. ALBASEER, Institute of Science and Technology, Jawaharlal Nehru Technological University, India

TITLE: A VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF AMLODIPINE BESYLATE AND TELMISARTAN IN PHARMACEUTICAL FORMULATION
DR. ASHA B. THOMAS, Padm. Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pune, India

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Validation of Bioanalytical Methods; Investigation of Back-Conversion in Incurred Pharmacokinetic Samples

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Introduction: Lately growing attention has been dedicated to the occurrence of back-conversion, intended as the transformation of a drug metabolite to the parent compound during samples handling. Back-conversion brings to inaccurate quantitative determinations and EMA is imposing the validation of back-conversion in analytical methods for bioequivalence (Guideline on Bioequivalence 2010). While it is clear now that validation procedures shall check back-conversion, other methods than the use of biological samples spiked with metabolite standards, are unavailable. Unfortunately the metabolism of several well-known drugs is unclear and new molecules are coming: what to do? A validation approach based on incurred samples of subjects treated with studied drugs was developed; the results collected on 20 drugs will be presented.

Materials and methods: In all validations, HPLC-MS/MS and reversed phase or ion exchange columns separations were employed. These data compare the evaluation of back-conversion by classical procedures (spiked samples) with the findings gathered using incurred samples analyzed soon after sampling, then after different period/conditions of storage.

Results and conclusions: With one drug, only the incurred samples permitted to validate back-conversion and with another-one they allowed to perform a validation when metabolite standards were unavailable. In all other cases both approaches gave similar results; it never occurred that incurred samples results were contradictory with those based on spiked samples. In conclusion the incurred samples approach is very useful, if not the only one in some cases, to adequately validate analytical methods for back-conversion.

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ICP-MS Coupling to HPLC to Quantify Unknown Metabolite of Drugs in Biological Fluids

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Introduction: The application of HPLC-MS/MS in pharmacokinetic studies has been extremely helpful permitting to solve complex metabolism problems; it remains however a main problem to quantify the newly identified metabolites until a quantitative standard is available. Aiming to quantitate the concentrations of unknown metabolites in absence of adequate quantitative standards, ICP-MS has been combined to HPLC and, as an example, experimental results obtained on clopidogrel, a potent antiaggregant and antithrombotic drug characterized by an extensive metabolization, are here presented.

Materials and methods: This research was carried out using triple quadrupole MS/MS systems (API-4000 and API 5000 Applied Biosystems) and a quadrupolar ICP-MS (ELAN 6100 Perkin Elmer). HPLC separations were carried out (Agilent or Perkin ELmer HPLC pumps) on reversed phase or ion exchange columns eluted with different mobile phases in gradient or isocratic conditions. HPLC-MS and MS/MS analyses were performed in positive or negative ions mode in order to get the maximum of chemical structure information; in case of ICP-MS chromatographic traces with masses characteristic of sulfur were acquired. Plasma samples collected from subjects treated orally with clopidogrel as well samples obtained by “in vitro” metabolization of clopidogrel were analyzed and compared with a few synthetic clopidogrel metabolite standards.

Results and conclusions: Results obtained using different models of HPLC-ICP interfaces and mobile phase composition will be presented. Under adequate conditions of mobile phase composition and flow rate the application of ICP-MS to HPLC proved to be an effective tool to quantify known and unknown clopidogrel metabolites.

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LC/MS Application in the Drug Discovery

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Liquid chromatography (LC) combined with mass spectrometry (MS) creates an ideal analytical tool for the laboratory. The high-performance liquid chromatography (HPLC) has been the laboratory tool of choice for separating, analyzing, and purifying mixtures of organic compounds since the 1970s. An HPLC column can separate almost any mixture that can be dissolved. A mass spectrometer can ionize the separated peak solution and provide a molecular weight for each peak component. An LC/MS/MS system can fragment the parent ion into a distinctive fragmentation pattern and can separate the daughter ions for identification and quantitation. The characteristic fragmentation pattern from each parent ion can be identified by comparison to fragmentation patterns produced by standard computerized databases. There are currently three principal application areas in LC/MS, but the technique has much wider potential application and is in fact already being applied to a variety of fields. The first main area is compound discovery and identity confirmation in pharmaceutical manufacturing or drug discovery. The second application area, called *proteomics,* is protein structure determination by LC/MS. A growing subset of these studies is in the field of DNA/RNA structure studies; although the name is not in common use currently, an appropriate term would be *nucleomics*. The third area of application is in metabolite and trace contaminates studies. Our main interest here is the compound discovery; anyone doing organic synthesis or isolating compounds from biological sources has an interest, first, in establishing the activity of the compound, then its molecular weight, and finally, the compound's definitive structure. In this study we will focus step by step on the identification and characterization of new drugs from the medicinal plant *Solandra grandiflora* using LC/MS.

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Comparison Of Different Mass Spectrometry Ionization Techniques to Analyze Desogestrel in Plasma Samples

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Introduction: The development of HPLC-MS/MS has opened completely new horizons in analytical chemistry: polar and unstable compounds can be now easily analyzed with adequate sensitivity. Apolar compounds, once considered as best target for mass spectrometry, are now a critical class of compounds for HPLC-MS. In the present work desogestrel, a progestogen often used in therapy, has been selected as a model apolar compound to compare GC/MS and HPLC-MS/MS, with and without derivatization, in order to develop a highly sensitive quantitative method.

Materials and methods: In case of HPLC-MS/MS electrospray, atmospheric pressure chemical ionization, photoionization and experimental electron capture sources were tested. In case of GC/MS both electron impact as well as chemical ionization (positive and negative) were employed. The samples were analyzed as such or after derivatization with trimethylsilylimidazole (GC only), heptafluorobutyrylimidazole or pentafluoropropionylimidazole. The same SLE method was used for desogestrel isolation from plasma samples (0.6 ml aliquots) both for GC-MS and HPLC-MS/MS analyses.

Results and conclusions: In HPLC-MS/MS the best results (LLOQ 200 pg/ml desogestrel in plasma), without derivatization, were obtained with the photoionization source using a reversed phase separation. The derivatization with perfluorinated imidazoles gave adequate derivatives for electron capture ionization; critical technical aspects are still under evaluation due to the experimental source employed. With GC-MS a better sensitivity was achieved in case of the derivatives (LLOQ 50 pg/ml), using negative chemical ionization; a long analytical separation, with a 60 m DB-5 column, was however mandatory in order to eliminate the interference from endogenous compounds.

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Analytical Methods for Determination of some Selected Drugs in Pharmaceutical Formulations by HPLC

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Human health is most important for any country. Throughout the world spending percentage for keeping the good health of people is huge. Drug (medicine) discovery as well as its development is complex and highly competitive processes that are defined as the series of specialized events performed to satisfy quality criteria. In the development of the drugs (medicines), several steps are taken to assess their effectiveness on the particular disease with minimal side effects due to the main drug and no harmful effects due to the impurities present in the drug. It becomes very obvious to analyze the purity of the drug and impurities present in the drug. To understand these aspects, analysis of drugs in bulk and in formulations becomes essential. To analyze the drugs in formulations, one should have a proper method in which the drug is properly separated, identified and quantified. Time to time new methods need to be developed and validated to analyse these drug species in the bulk and formulation. To this end, High Performance Liquid Chromatography (HPLC) is the most important analytical technique in the pharmaceutical industry to test the products for both qualitative and quantitative analysis. HPLC methods are relatively standard and used to test the raw material, bulk drugs and formulations. Here we describe a simple, sensitive, reproducible and rapid HPLC methods developed and validated for some selected drugs: Ritonavir, Atomoxetine, Repaglinide, Valacyclovir and Glimipiride in pharmaceutical formulations.

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Development of a Dispersive Micro-Liquid Liquid Method for the Extraction of Pyrethroid Pesticides from Environmental Matrices for Determination by Reversed-Phase High Performance Liquid Chromatography

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The conventional multiresidue methods involve the consumption of large quantities of toxic organic solvents which necessitate further clean up steps before analysis. In addition, the use of large amounts of toxic solvents for sample preparation is not eco-friendly and difficult to justify. Miniaturization can serve well in this regard and is currently a major trend in analytical chemistry. In the present work we developed a method for the rapid trace analysis of residual pyrethroid pesticides in agricultural waters by high performance liquid chromatography with diode array detection using dispersive liquid–liquid microextraction (DLLME). To the best of authors' knowledge, this is the first application of DLLME for the extraction of pyrethroid pesticides from aqueous matrices. Several parameters of the extraction procedure such as type and volume of extraction solvent, type and volume of dispersive solvent and salt addition were evaluated to achieve the highest yield and to attain the lowest detection limits. The DLLME procedure optimized consists in the formation of a cloudy solution promoted by the fast addition to the sample (5 ml) of a mixture of carbon tetrachloride (extraction solvent, 55 µL) and acetone (dispersive solvent, 500 µL). The tiny droplets formed and dispersed among the aqueous sample solution are further joined and sedimented (25 µL) in the bottom of the conical test tube by centrifugation. Once extracted, the pesticides were directly injected and separated by RP-HPLC comprising a short column (Hiber- purospher star RP-18 end capped column (150 x 4.6 mm I.D., 5 µm particle size) and a PDA detector.

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A Validated Stability Indicating RP-HPLC Method for the Determination of Amlodipine Besylate and Telmisartan in Pharmaceutical Formulation

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A new Reversed Phase-High Performance Liquid Chomatographic method was developed and validated for the quantitation of Amlodipine besylate and Telmisartan in pharmaceutical formulations. Determination was performed on a HPLC Binary gradient system (Agilent Technologies, 1120 Compact LC HPLC) using a Hypersil-BDS (C_{-18}) column (5 µm, 250 mm 4.60 mm), a mobile phase containing 0.05 M potassium dihydrogen ortho phosphate: acetonitrile (60:40% v/v) in isocratic mode at a flow rate of 1.4 mL/min with UV detection at 237.0 nm. The retention time (t_R) for Amlodipine besylate and Telmisartan were 5.47min and 8.53 min respectively. The response was linear in the concentration range of 1-50 µg mL^{-1} for both Amlo and Tel with regression coefficient (r^2) 0.9987 and 0.999 respectively. The method was validated for precision, accuracy robustness and ruggedness as per ICH guidelines. The LOD and LOQ were found to be 0.03512 µg mL^{-1}, 0.10664 µgmL^{-1} and 0.04663 µg mL^{-1}, 0.14132 µg mL^{-1} for Amlodipine besylate and Telmisartan respectively. The mean recoveries of Amlodipine besylate and Telmisartan were found to be 99.78% and 100.02% respectively. The robustness of the developed RP-HPLC method evaluated by making deliberate variations in the method parameters such as change in flow rate [-0.1 level- 1.3 ml/min; 0.0 level- 1.4 ml/min; +0.1 level -1.50ml/min], ratio of aqueous: organic composition and pH of the mobile phase [+1:-1 level-(61:39/pH-6.41); 0:0 level -(60:40/pH-6.50);-1:+1 level -(59:41/ pH-6.47)] on the retention time, tailing factor and % content indicated that the developed method was unaffected by small changes in method parameters and is robust. The ruggedness of the developed method which was also evaluated by studying the effect of parameters like different analysts and chemicals and solvents (Qualigens- Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India and Universal Labs. Mumbai, India) employed exhibited low % R.S.D values for retention time, tailing factor and % content indicating that the developed method is rugged. The specificity of the method evaluated by forced degradation studies suggest that the method could effectively separate the drugs from its degradation products and can be used for stability-indicating analysis. Due to its simplicity, high precision and accuracy, the proposed RP-HPLC method can be used for the estimation of Amlodipine besylate and Telmisartan in pharmaceutical preparations.

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Carbon Isotope Ratio Mass Spectrometric Studies in Medicine

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The use of stable carbon isotopes in metabolic research on humans has expanded significantly since the early 1980s. This is due to a combination of factors such as the availability of increasing variety of labeled compounds, absence of health risk from radiation and more significantly due to the development of sophisticated instrumentation and greater availability of analytical facilities. Usage of ^{13}C labeled substrates in human studies has provided an array of information especially on the biochemistry, physiology and disease status. Some examples of this include the utilization of ^{13}C-octanoate in the Gastric Mobility Breath Test, ^{13}C-Methionine in the methionine Breath Test for measuring hepatic mitochondrial function, and ^{13}C-Phenylalanine in ^{13}C-Phenylalanine Breath Test. While simpler ^{13}C substrates such as sugars, amino acids and fatty acids are readily commercially available, proteins and carbohydrates generally are not. One can obtain these by labeling photosynthetic organisms with $^{13}CO2$ during photosynthesis and then isolating the compound of interest. One can also take advantage of the naturally enriched substrates for metabolic studies by a prudent selection of these material from plants following different photosynthetic pathways. Most of the human diet is derived from food items from plants following the C_3 photosynthetic pathway which display more negative carbon-13 signature than the plants that follow C_4 type of photosynthetic metabolism. Thus by a simple and imaginative manipulation of the diet by mixing material obtained from both C_3 and C_4 plants, one could obtain a significant physiological information which might lead to the synthesis of newer pharmacological compounds.

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Solid-Phase Extraction of Tramadol from Plasma and Urine Samples Using a Novel Water-Compatible Molecularly Imprinted Polymer

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In this study, a novel method is described for the determination of tramadol in biological fluids using molecularly imprinted solid-phase extraction (MISPE) as the sample clean-up technique combined with high-performance liquid chromatography (HPLC). The water-compatible molecularly imprinted polymers (MIPs) were prepared using methacrylic acid as functional monomer, ethylene glycol dimethacrylate as cross-linker, chloroform as porogen and tramadol as template molecule. The novel imprinted polymer was used as a solid-phase extraction (SPE) sorbent for the extraction of tramadol from human plasma and urine. Various parameters affecting the extraction efficiency of the polymer have been evaluated. The optimal conditions for the MIP cartridges were studied. The MIP selectivity was evaluated by checking several substances with similar molecular structures to that of tramadol. The limit of detection (LOD) and limit of quantification (LOQ) for tramadol in urine samples were 1.2 and 3.5 μg L^{-1}, respectively. These limits for tramadol in plasma samples were 3.0 and 8.5 μg L^{-1}, respectively. The recoveries for plasma and urine samples were higher than 91%.

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Trace Analysis of Drugs based on Novel Metal Complexes using Electrochemical Techniques

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Analysis of drugs are required not for regulatory purposes only but also due to their toxicity. Apart from this the new frontiers in personalized therapy also envisage the development of new tools for fast, easy-to-use, low cost, and point-of-care analysis of drugs efficacy on the patients. Recently we developed an electrochemical technique for selective and sensitive detection of drugs based on novel metal complexes. This technique is demonstrated for trace analysis of Cefotaxime (third generation cephalosporin drug) and Azidothymidine (AZT, Zidovudine, Retrovir, 3'-azido-3'-deoxythymidine) antiviral drugs. The basic principle behind our technique was to accumulate the drug specifically over the sensing electrode based on the interaction of the functional groups present on drugs with the metal complex coated over the sensing electrode. After the accumulation of drug anodic or cathodic voltammetry was used for the analysis. Our technique was found to be sensitive enough to detect the drugs at nM level with negligible interference.

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Investigation of the Detailed Molecular Structure of the Human Stratum Corneum Ceramides [NP] and [EOS] by APCI and Nano-ESI Mass Spectrometry

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The aim of this study was to characterize two ceramide subclasses, CER[NP] and CER[EOS], of human stratum corneum (SC) and to identify the chemical structures of their subspecies. High performance thin layer chromatography (HPTLC) and normal phase high performance liquid chromatography (NP-HPLC) were used for the separation of CER fractions of interest, whereas nanoelectrospray tandem mass spectrometry was applied to identify the chemical structures in detail. Thus, CER[EOS] fragmentation revealed that in addition to linoleic acid other esterified fatty acids occur in the ω-hydroxylated-position (part E). Of particular interest is the identification of a 17:2 fatty acid located in this part of the molecule.

Several subspecies of CER[NP], including subspecies with odd numbers of carbon atoms in both chains, the non-α-hydroxylated fatty acid moiety (part N) and the phytosphingosine (part P) were detected. Furthermore, 12% of CER[NP] subspecies with an odd number of carbon atoms in more than one chain for one molecule were detected.

Similar results were obtained for CER[EOS]. Both, the esterified fatty acid (part E) and the sphingosine base (part S) were found to contain odd-numbered chain lengths.

The combination of the analytical techniques presented allows complete new insights into the molecular structure of the SC ceramides. Now the techniques are used to identify differences in the detailed molecular structure of the ceramides in healthy and diseased skin.

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A Novel Spectrophotometric Approach for the Measurement of Submicromolar Concentrations of Nitrite and Nitrate in Biological Fluids

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Nitric oxide (NO) is an important mediator. Alteration in NO production has been implicated in a number of clinical conditions including renal failure. NO has a short half-life and therefore it is difficult to directly assess its concentrations body fluids. Hence, nitrite and/or nitrate levels are assessed as surrogate biomarkers of total NO production in the human body. A variety of analytical methods (i.e. spectrophotometric, spectroflourometric, HPLC-coupled to spectrophotometric- or spectroflourometric detectors, HPLC-MS and GC-MS have been developed for the measurement of NO metabolites. Of these, Griess-reaction is the most widely employed. The aims of present study were to develop: 1) an improved protocol for the preparation of serum nitrite and total nitrite (sum of nitrite + nitrate) by generating a genuine blank for every individual sample; 2) optimizing the condition for protein removal by $ZnSO_4$; and 3) exploring the effect of residual Cd, Cu and Zn on signal detection following the Griess reaction.

Recipient of first kidney [n=20] were recruited. Serum sample collection started collected at day one pre-transplantation and continued up to 2 months post-transplantation (i.e. 20 separate time points). Sample preparation involved nitrate reduction employing a mixture of Cd/Cu as catalyst, protein removal, performance of the Griess reaction and UV measurement at 545 nm.

It was found that the medians for nitrite and total nitrite levels in the candidates for renal transplantation were 0.6 μM and 1 μM, respectively. Three days after kidney transplantation, the corresponding levels dropped to 0.3 μM and 0.4 μM and remained fairly unchanged during the course of investigation.

In conclusion, this investigation has revealed that total nitrite concentration in patients with end stage renal failure is approximately 1 μM which is markedly lower than those data previously by other investigators using similar techniques. Our data also implies that final spectrophotmetric measurement is influenced by the protocol for protein removal as well as the presence of residual Cd and Cu. These findings may explain the large differences in reported values for above-mentioned NO metabolites in health and disease.