



Pharmaceutisch Weekblad

Scientific edition

Supplement N

Supplement to Pharmaceutisch Weekblad Scientific edition,
volume 11, number 6, 15 December 1989

Abstracts of papers

Pharmaceutical and Biomedical Analysis Meeting

Organized by the Working Group Pharmaceutical and Biomedical
Analysis (Werkgroep Farmaceutische en Biomedische Analyse)

Oss (the Netherlands), 17 November 1989

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R. Both-Miedema and E.H.J.M. Jansen

Several high performance liquid chromatographic (HPLC) methods have been reported about the determination of amino acids with pre-column derivatization. In our hands the derivatization method using dabsyl chloride gave the best results. The present report describes the derivatization procedure and HPLC analysis of dabsyl-amino acids for a number of applications in our Institute.

The derivatization procedure with dabsyl chloride was performed for 10 min at 70 C. Gradient reversed phase HPLC was used for the separation of the dabsyl-derivatives, usually with a mixture of 100 mM NaAc (pH 6.5), methanol and acetonitrile. For each application the gradient was optimized to obtain the best HPLC separation.

The dabsyl derivatives shown a number of advantages relative to other derivatives, like:

- A very good stability of the dabsyl-derivatives for at least one week.
- A good reproducibility of 0.5 to 4.2 %.
- A good limit of detection of about 3 pmol or 300 pg (S/N = 10).
- A complete HPLC separation for all 20 amino acids.
- Specific detection at a wavelength of 436 nm.

In our Institute, the here described pre-column derivatization with dabsylchloride was applied to a variety of investigations in which amino acids are involved, like total amino acid analysis of protein hydrolysates for several applications, monitoring of the consumption of amino acids in animal cell cultures for the production of biologicals, determination of glycine in preparations of anti-thymocyte globulins, the ratio between tryptophan and four other hydrophobic amino acids in the study of the pre-menstrual syndrome and the determination of thyronine residues in the thyroid for toxicological investigations. In conclusion, the pre-column derivatization of amino acids with dabsyl chloride appears to be a good and practical method with a number of distinct advantages.

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G. de Groot, R. Koops.

Histamine is an essential protein in several processes in man. In food products such as fish, histamine is formed during storage. Consumption of food with high concentrations of histamine can cause serious intoxications. In our Institute, a study was started in human volunteers to assess a 'no-effect' level of histamine. One of the subjects was the pharmacokinetics of histamine after ingestion of histamine-containing food. For this purpose a method was developed for the determination of histamine and its main metabolite 1-methylhistamine in human serum. Development was directed to automation of both sample preparation and derivatization.

Isolation of the analytes from serum was by solid-phase extraction (SPE) on a 100 mg C-18 cartridge (J.T. Baker). Retention of the analytes (primary amines) on the sorbent was based on secondary interactions of the amine groups of the analytes with ionized silanol groups of the sorbent. Since these are very strong interactions, a selective sample clean-up on the cartridge was obtained. Derivatization of the compounds with o-phthalaldehyde (OPA) was necessary prior to HPLC analysis to give products with good chromatographic and fluorescent properties. This reaction was on-line carried out in a stainless-steel reaction coil (length 1 m, 0.75 mm I.D.) through which the OPA reagent was pumped. The reaction products were directly transported to a short precolumn (30 x 4 mm filled with 5 µm Nucleosil C-18) where they were retained and thereby isolated from the alkaline (pH 9.2) OPA reagent. Subsequently, only a small "heart cut" of the precolumn eluate was transported to the analytical column (200 x 3.0 mm filled with 5 µm Hypersil ODS). Detection was by fluorescence detection (excitation wavelength 328 nm, emitted light detected above 417 nm). The fluid transport between the reaction coil, the precolumn and the analytical column was automated with a column switching system (MUST).

Further subjects of investigation are automation of the SPE and the determination of the compounds in urine.

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SAMPLE PRETREATMENT OF BODY FLUIDS BY ISOTACHOPHORESIS PRIOR TO LIQUID CHROMATOGRAPHIC ANALYSIS

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R.W. Sparidans and F.M. Everaerts

Among the sample pretreatment techniques for HPLC-analysis in bio- and clinical chemistry, isotachopheresis (ITP) is of growing interest. Compared to the liquid-liquid (LL) and solid-phase (SP) isolation procedures, which are frequently used in these fields of application, ITP offers a high selectivity as a sample pretreatment step for ionogenic components in complex samples prior to HPLC analysis.

In this study a number of model components like codeine, morphine, theophylline, phenylacetic acid and homovanilic acid were investigated with respect to the recoveries from blank and spiked serum and urine samples. This included also samples with a relatively high ionic strength comparable to i.e. a 3 M hydrochloric acid solution which is often applied for breaking protein-drug bindings in body fluids. Recoveries of 90% with standard deviations of 4% for the ITP pretreatment method were observed.

An additional advantage of this sample pretreatment technique is the considerably extended lifetime and chromatographic stability of the HPLC-columns due to the strong clean-up effects of the ITP-technique.

1. H.A. Claessens et al., *Chromatographia*, 26 (1988) 351.

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FULLY AUTOMATED SAMPLE PREPARATION AND HPLC ANALYSIS OF DESMETHYLDIAZEPAM IN SERUM.

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In our Institute, the abstinence behaviour of benzodiazepines after chronic use is extensively studied in rats. Desmethyldiazepam is one of the test substances. One of the subjects of the study is the pharmacokinetics of the benzodiazepines after chronic oral administration and after bolus injection. As a consequence, large series of samples are currently analyzed in these studies.

The method for the determination of benzodiazepines we used for several years, included isolation of the analytes from serum by solid-phase extraction (SPE) followed by clean-up of the SPE extract. The present study describes automation of these steps. In the final procedure, the SPE was automated with the ASPEC (Automatic Sample Preparation with Extraction Columns; Gilson), a pipetting and injection robot with three degrees of freedom. We programmed the ASPEC to precondition a 100 mg 'High-Hydrophobic' C-18 SPE cartridge (J.T. Baker), to carry out the SPE of the sample and the internal standard prazepam, to dilute the sample eluate and to inject the extract in twofold into an HPLC with automated column-switching system, which provided clean-up and separation of the analytes.

Clean-up of the SPE extract was on a short precolumn (10 x 3 mm filled with 5 µm Hypersil ODS). Only a small 'heart-cut' of the precolumn eluate was transported to the analytical column (150 x 4.6 mm filled with 5 µm Hypersil ODS). The SPE of a sample was carried out simultaneously with the clean-up and chromatography of the previous sample. UV detection was at 226 nm.

An important part of the study was the development of a Quality Assurance System which recorded the extraction and clean-up recovery and the performance of the precolumn and the analytical column.

The overall isolation and clean-up recovery was about 100 %. The limit of detection was 15 µg/l using 150 µl of serum. The precision was typically 3-5 % at 1500 µg/l. Repeatability (RSD) of reference samples over one month was 11.9 % at 170 µg/l (N=22) and 7.9 % at 1500 µg/l (N=24). The application range was 15-5000 µg/l.

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The cytostatic agent daunorubicin is effective against leukemia, but cardiotoxicity is an important side effect, common to all anthracyclines. Since anthracycline metabolites are thought to contribute to the observed cardiotoxicity, a quantitative method for the determination of all metabolites in plasma as well as in tissues is needed as a basis to further investigate the correlation between toxicity and the amount of each metabolite formed.

Using Sep-pak C_{18} cartridges we were able to extract daunorubicin and its five metabolites including the aglycones with recoveries ranging from 50-90%. Fluorescence detection following HPLC separation permitted detection limits ($S/N = 2$) as low as 0.2 to 0.9 nM in plasma and 0.8 to $3 \cdot 10^{-11}$ mol/g in tissue (depending on the specific metabolite), which compare favorably with literature data. The method showed linearity from 1-250 nM in plasma and 0.04-4.0 nmol/g in tissue ($r \geq 0.998$). Repeatability was determined at spiking levels of 10 and 100 nM in plasma and of 0.1 and 1.0 nmol/g in tissue, resulting in within-day coefficients of variation ranging from 2-7% for plasma and 3-12% for tissue (depending on the metabolite). Between-day variation for tissue is comparable (5-10%), whereas plasma values are slightly higher (3-12%). Because of large inter-compound differences in fluorescence quantum yield and recoveries, separate calibration curves have to be used for each anthracycline to calculate their concentrations in the samples.

In conclusion, this method offers the opportunity to determine daunorubicin and all metabolites with high sensitivity in one run, using a uniform method for plasma and tissue.

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ION-EXCHANGE LIQUID CHROMATOGRAPHIC ANALYSIS OF PHOSPHONATES IN PHARMACEUTICAL PREPARATIONS.
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Phosphonates form a class of compounds all characterized by the presence of one or more $O=P(OH)_2$ -groups. Several representatives of this class have been introduced in the clinic recently. Trisodium phosphonoformate (Foscarnet) is an antiviral drug with in vitro activity against e.g. cytomegalovirus. Bisphosphonates (e.g. 3-amino-1-hydroxypropane-1,1-bisphosphonate, APD) are used in the treatment of diseases involving calcium metabolism, e.g. Paget's disease. Sensitive and selective analysis of phosphonates in quality control has been a problem in the past because of inadequacies in both the chromatographic separation and the detection of these compounds.

This paper reports a uniform approach for the analysis of phosphonates currently of pharmacotherapeutic interest using ion-exchange chromatography with conductivity detection. Chromatographic separation is performed on a Waters IC-PAR anion-exchange column using low millimolar concentrations of nitric acid as the mobile phase. Detection occurs by conductivity measurement. Sample preparation involves dissolution of the sample in distilled water, followed, if necessary, by filtration through an 0.22 μ m filter prior to injection. Retention of the phosphonates can be controlled by varying conditions such as the ionic strength and/or the pH of the eluents. The detection system provides sufficient sensitivity for the determination of these phosphonates in pharmaceutical preparations. Estimated detection limit for APD is 0.2 μ g absolute amount. The chromatographic system provides good selectivity towards possible impurities and decomposition products such as phosphate ions. In conclusion ion-exchange chromatography with conductivity detection is a simple, fast, sensitive and selective method to determine phosphonates in pharmaceutical preparations.

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Thyroglobulin is the major storage for the thyroid hormones tri- and tetraiodothyronine (T3 and T4) and their precursors mono- and diiodotyrosine in the thyroidal follicle. In studying disturbances in thyroid hormone biosynthesis by toxic compounds it is essential to establish changes in the amount of iodinated tyrosine and thyronine residues in thyroglobulin. In order to determine these residues by high performance liquid chromatography (HPLC) the efficiency of various digestive procedures for the breakdown of thyroglobulin was investigated.

The thyroglobulin was treated with pronase, trypsin, chymotrypsin, aminopeptidase-M (AP-M), carboxypeptidase-A, -P and -Y in several combinations and sequences. Three HPLC methods were used to monitor the extent of proteolysis: a slightly modified reversed phase method (1) with stepwise elution for the determination of tyrosine and thyronine residues, a methanol-water-0.1% trifluoroacetic acid gradient for the determination of peptide residues and a reversed phase system for the analysis of amino acids and peptides after dansyl derivatization.

The treatment of thyroglobulin with trypsin and chymotrypsin alone resulted in a very low content of free hormones, precursors, tyrosine and tryptophan and a high content of peptide residues. Treatment with chymotrypsin (which splits at the C-terminal site of aromatic amino acids) followed by various carboxypeptidases resulted in a higher level of the free compounds as could be expected theoretically. The best result, however, was obtained with the combination of pronase and AP-M or the combination of trypsin, pronase and AP-M. The former combination gives also the expected relationship between T3 and T4 (2).

1. P.R. Kootstra et al., J. Chromatogr., 458 (1988) 175.

2. S.C. Werner, S.H. Ingbar, The Thyroid. Harper&Row, New York 1978.

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THERMOSPRAY LC/MS AND LC/MS/MS IN THE ANALYSIS OF LABILE COMPOUNDS IN COMPLEX BIOLOGICAL MATRICES

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Solving bioanalytical problems puts high demands on the applicability of thermospray LC/MS. Key parameters to an appropriate selectivity and sensitivity are the choice of the ionization mode, and the optimization of the many interdependent experimental parameters, such as the repeller potential, the vaporizer temperature, the solvent composition and the flow-rate. Rapid optimization strategies for these parameters have been developed as a result of our systematic studies.

Most of our applications are concerned with the identification of unknowns in complex (biological) matrices. In general, thermospray mass spectrometry is a soft ionization technique, supplying minor fragmentation. However, various ways are available to obtain useful structural information, such as repeller-induced and collision-induced fragmentation and thermal degradation.

In this paper two bioanalytical examples, demonstrating the various thermospray ionization modes and the various MS/MS scan modes, are discussed.

Considering the growing importance of the determination and identification of (desulfo-)glucosinolates in extracts of plant materials, a rapid screening method for desulfo-glucosinolates, based on thermospray LC/MS/MS, has been developed. Because all desulfo-glucosinolates show a loss of the sugaring (162 amu) by collision-induced dissociation in the collision quadrupole, a constant neutral loss scan of 162 amu can be applied for screening and identification of desulfo-glucosinolates in sprout extracts¹.

After administration of heptabarbital an unknown peak was observed in a rat urine LC(UV)-chromatogram, which was expected to be a heptabarbital metabolite. Metabolites of heptabarbital are expected to show similar collision-induced dissociation in MS/MS mode, because of structure similarities. By combining parent and daughter scans in thermospray LC/MS/MS, this unknown peak has been identified as a heptabarbital metabolite. The structure of the metabolite has been confirmed with GC/MS and high resolution electron impact, using a solids-probe as well².

1. In cooperation with J. Vuik and R.H. de Vos. TNO-CIVO Institutes, Zeist.

2. In cooperation with A. Stijnen and M. Danhof, Division of Pharmacology, Center for Bio-Pharmaceutical Sciences, Leiden.

P.S.L. Janssen, R.L.A.E. Hamelincx, P.A.T.A. Melgers.

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A specific and rapid HPLC method for the simultaneous assay of debrisoquine, an antihypertensive agent, and its most important metabolite 4-hydroxydebrisoquine in urine, was developed.

The sample preparation was done by direct injection of the urine samples after a fivefold dilution with water.

Separation was performed on a 100 x 4.6 mm Microsphere 5 µm CN glass column using a mobile phase containing phosphate buffer, acetonitrile and triethylamine (10 : 90 : 0.05). The pH of the mobile phase was 3.5.

Detection was by spectrofluorescence measurement at the excitation wavelength of 220 nm and the emission wavelength of 562 nm.

The lower limits of quantitation were 0.25 mg.L⁻¹ and the detection limits were about 0.1 mg.L⁻¹ using 200 µL of urine sample. For both compounds the linearity of the method was significant (p < 0.05) over the calibration range of 0.25 to 20.0 mg.L⁻¹. In general, correlation coefficients of 0.9990 or higher were found.

The good reproducibility of the method was shown by the intra- and inter-day reproducibility. For debrisoquine coefficients of variation were observed of less than 3.5 % and 3.6 % for the intra- and inter-day reproducibility respectively. The 4-hydroxydebrisoquine showed similar results namely coefficients of variation of less than 3.5 % and 4.5 % respectively. The accuracy of the method, expressed as the deviation of the observed concentration from the nominal concentration, was less than 3.5 % for both compounds.

The method is selective, sensitive and very accurate and it showed to be very useful in the phenotyping of individuals with respect to aromatic hydroxylation.

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Amino acid analysis (AAA), i.e. the simultaneous separation and quantification of the 20 peptide/protein building blocks, is a basic tool in biomolecular analysis. From the pioneering work of Moore and Stein in the 1950's until now, many different methods for performing the technique on dedicated analyzers have been proposed. With the advent of HPLC and, consequently, the diversity of possible chromatographic conditions, a more flexible performance of AAA has come within reach. As a result a variety of HPLC methods for the determination of amino acids, each having its own specific pros and cons, is available now.

In our laboratory various peptides and proteins, widely varying in structure and in sample characteristics and thus demanding a specific approach, are investigated. Important parameters for performing AAA of these preparations are the available sample quantity, the matrix composition (presence of salts, detergents, pharmaceutical fillers, etc. in the sample), whether or not information on secondary amino acids is required and the aimed reproducibility. Based on these aspects and regarding the characteristics of four established procedures^{1,2}, a decision scheme has been set up to select the best HPLC-AAA method for each particular sample preparation.

1 P.S.L. Janssen, et. al. Chromatographia, 22 (1986) 345.

2 P.S.L. Janssen, et. al. Chromatographia, 22 (1986) 351.

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AN EXPERT SYSTEM FOR THE SELECTION OF INITIAL HPLC CONDITIONS FOR THE ANALYSIS OF BASIC PHARMACEUTICALS; VALIDATION AND EVALUATION

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Objective. Validation and evaluation of an expert system developed for the selection of initial HPLC conditions.

Content. An outline of the project "Expert Systems for Chemical Analysis" has been given¹.

Four domains were selected, which together cover the entire field of method development in HPLC.

The first step is to obtain a chromatogram within a correct retention range ($3 \leq k' \leq 10$). A prototype expert system was built which especially gives advice on the mobile phase composition and the pH for the analysis of basic compounds. The mobile phase composition as proposed is a summation of the polarity contributions of so-called structural fragments. These fragments are obtained by dividing the structure of the molecule to be analysed into small units (phenyl, CH₂, OH etc.). The polarity of some of these fragments is not affected by the pH, while the polarity of others depends strongly on the pH, viz. N-containing groups.

In the validation and evaluation process, the advice of the expert system on real samples received for analysis has been checked. On the basis of these results, existing rules are adapted and new rules are added. Also rules have been implemented to guide the operator in case the first proposal results in a peak (or peaks) which is (are) not in the correct range of capacity factors.

Conclusions. For the analysis of Organon compounds, the consultation of the expert system resulted in 75 % correct proposals.

1 D. Goulder et al. Chromatographia, 26 (1988) 237.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ENANTIOSEPARATION OF AMINO ACIDS

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During the synthesis of peptides, racemization is of major concern as a high degree of optical purity is necessary for adequate pharmaceutical and clinical studies. In this respect a reliable method for the determination of the L/D configuration of amino acids is essential. In general the physical properties of the L and D enantiomeric pairs are identical, so they cannot be separated directly on a (conventional) achiral reserved-phase HPLC column. Therefore an automatic pre-column derivatization procedure was developed in which the L/D amino acids are converted into diastereoisomeric pairs. O-phthalaldehyde (OPA) in combination with the homo-chiral thiol N-acetyl-L-cysteine (L-NAC) react in alkaline medium with the enantiomeric amino acids, forming (highly fluorescent) isoindoles, the diastereoisomeric product pairs. Subsequently the various derivatized L/D amino acids are separated on-line by reversed phase HPLC on a C₁₈ column, applying a ternary gradient, and detected by fluorimetry.

The developed technique is a good starting point for the ready and sensitive determination of the L/D configuration of amino acids. By this procedure two-fold quantitative information can be obtained:

- optical purity of the amino acids used as starting material for peptide synthesis;
- optical purity of the composing amino acids, after hydrolysis of the synthesized peptide (racemization check).

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Adequate analytical support to the complicated multi-step synthesis, isolation and purification procedures is of utmost importance in modern peptide chemistry. Moreover the end product, i.e. the purified peptide, should meet high quality requirements. As to these aspects the information obtained from reversed-phase high performance liquid chromatography (RP-HPLC) and from high performance capillary electrophoresis (HPCE) was evaluated. RP-HPLC is most valuable due to its versatility and high separation power. Its main separation mechanism is hydrophobic interaction. HPCE is a new promising technique in peptide analysis. The capillary zone electrophoresis (CZE) mode is a most valuable addition to RP-HPLC as it is based on quite another separation mechanism, i.e. electrophoretic mobility. Capillary isotachopheresis (CITP) gives useful information on the nature and the quantity of non-peptidic components in peptide preparations. The combined use of the three techniques, RP-HPLC, CZE and CITP, results in complementary information on the quantitative composition of synthetic and natural peptide preparations¹.

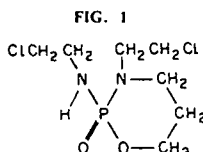
1 P.S.L. Janssen, et. al. J. Chromatogr., 470 (1989) 171.

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BIOANALYSIS OF IFOSFAMIDE BY CAPILLARY GAS CHROMATOGRAPHY

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Ifosfamide (ifo, fig 1) is an oxazaphosphorine with therapeutic superiority over cyclophosphamide (cy), in many experimental animal tumours. Our ongoing study of the pharmacology of oxazaphosphorine requires development of bioanalyses of parent compounds and metabolites. Ifo can be determined by HPLC or GC, both applying packed column technology. We exploited capillary column technology in the gas chromatographic analysis of Ifo. Short methylsilicone columns (5 nm, i.d. 0.53; filmthickness 2.65 um, Hewlett Packard) proved to be capable of complete elution of ifosfamide without thermal degradation at temperatures between 250 C (injection) and 230 C (column temperature). The limit of detection was in the low picomol range with FID (50 pMol at 35/1 split/splitless mode). In contrast to cy, ifo is not subjected to intraalkylation during GC (1,2). A single liquid-liquid extraction enabled selective and sensitive determination of ifo in biological fluids. Without derivatization, the limit of determination is 1 ug/ml (signal to noise ratio 3:1, FID) which is sufficient for routine analysis of ifo.



1. E.A. de Bruijn, PA Leclercq, UR Tjaden, HRC & CC 9 (1986) 89.
2. E.A. de Bruijn et al., Biomed Environm. Mass Spectrom 14 (1987), 643.

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Vitamin D₃ is metabolized to 25-hydroxyvitamin D₃ [25-(OH)D₃] which in turn is transformed into the biologically active metabolite 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], playing a major role in the calcium (Ca) and phosphate (P) metabolism. The normal plasma concentrations of 25-(OH)D₃ and 1,25-(OH)₂D₃ are 30-70 nmol/L and 60-160 pmol/L, respectively. Because production and clearance are regulated by the Ca/P and vitamin D status, it is interesting to study the pharmacokinetics of 25-(OH)D₃ and 1,25-(OH)₂D₃ under various conditions. To avoid disturbance of the physiological pharmacokinetics, a tracer dose (< 10% body pool) has to be administered. Use of non-radioactive (¹³C) labelled metabolite is preferred for the intended human pharmacokinetic studies. For such studies very sensitive GC/MS methods have to be developed.

On column (CP-Sil-5 CB, 10 m, 0.2 mm) detection limits with EI (70 eV) and PCI (NH₃ reagent gas) were about 10 pg for a) the trimethylsilyl (TMS) and heptafluorobutyric acid (HFBA) derivatives of 1,25-(OH)₂D₃ and b) the TMS derivative of 25-(OH)D₃. In contrast to the detection limit for 1,25-(OH)₂D₃, the detection limit for 25-(OH)D₃ is sufficiently low for the intended pharmacokinetic studies. The electron-capturing (EC) HFBA and pentafluorophenyl dimethylsilyl derivatives of 1,25-(OH)₂D₃ were not suited for NCI due to abundant loss of the negatively charged EC-group.

It can be concluded, that the detection limit for 25-(OH)D₃ is sufficiently low but that alternative procedures have to be developed for 1,25-(OH)₂D₃ to obtain derivatives suited for EC-NCI permitting quantitation at the sub-pg level.

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COMPARISON OF HPLC METHODS FOR THE ANALYSIS OF BASIC DRUGS

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Objective. To study the HPLC analysis of basic compounds on the basis of present knowledge.

Content. Problems which are often encountered for the analysis of basic compounds are severe peak tailing and low separation efficiency. Although the main reasons for these problems are well-known, universal solutions are not found. Even more, the chromatographer gets confused of the variety of suggestions given by the specialists and of the various stationary phases brought onto the market.

A systematic study has been carried out, in which 32 test compounds were selected varying in:

- (i) pKa value (range 1-10),
- (ii) polarity (retention index 400-1600),
- (iii) number of N-atoms (range 1-5) and
- (iv) structural type of N-atom.

The following parameters were studied:

- (i) pH of the mobile phase,
- (ii) type of modifier,
- (iii) use of an additive in the mobile phase to suppress peak tailing and
- (iv) type of stationary phase.

Conclusion. Some new types of stationary phases clearly improve peak shapes of basic substances. However, the use of additives to suppress peak tailing is still often necessary.

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DETECTION OF CYTOCHROME P-450 ISOENZYMES WITH LUMINOL-MEDIATED CHEMILUMINESCENCE

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For the purification of cytochrome P-450 isoenzymes from rat liver microsomes several chromatographic techniques have been used, like low pressure chromatography, affinity chromatography and high performance liquid chromatography. The control of a purification step can be done by a combination of enzymatic activity determination or spectrophotometric analysis and electrophoresis.

Here we introduce a new, simple, sensitive and fast way for the detection of cytochrome P-450. This method is based on the haem catalyzed oxidation reaction of luminol, which is a well-known phenomenon in the chemiluminescence immunoassay field. Since the cytochrome P-450 isoenzymes contain a haem group, we have tried to use it as a catalyst to produce a chemiluminescence signal in a luminol-mediated reaction. The system described in concept by Akhrem (1) was optimized and consists of luminol and cumene hydroperoxide in borate buffer pH 10.1 (2).

The optimized system has the following advantages and characteristics. A high intensive chemiluminescence signal is produced with a maximum signal between 1 and 2 min. The limit of detection has been increased substantially compared with conventional methods of analysis. The limit of detection is about 0.01 μ l microsomes which gives a signal of ten times the blank value. In addition, the time of analysis has been decreased dramatically to 10-30 sec per sample, depending on the kind of luminometer used.

Thus far this method has been applied in our laboratory for a fast monitoring of cytochrome P-450 isoenzyme patterns after purification steps and for a newly developed assay method for the total content of cytochrome P-450 in microsomes.

1. A.A. Akhrem et al., Biomed. Biochem. Acta, 44 (1985) 1591.
2. E.H.J.M. Jansen et al., Anal. Chim. Acta, in press (1989).

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ANALYSIS OF JOSAMYCIN BY HPLC

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In 1970 it was shown that josamycin, a macrolide antibiotic, produced by the actinomycete *Streptomyces narbonensis* var. *josamyceticus*, was identical¹ to leucomycin A₃.

Chromatographic analysis of commercial samples was carried out under the following conditions:

Column: Hypersil C18 5 μ m (Shandon)

Temperature: 30 °C

Mobile phase: acetonitrile - 0.2 M tetrabutylammonium hydrogen sulphate - 0.2 M phosphoric acid - water (25:3:5:67)

Flow rate: 1.0 ml/min

Detection: UV absorption at 232 nm

Sample: 1 mg/ml in acetonitrile - water (30:70)

The proposed method shows a good selectivity towards the different components in the analysed samples. Different C18 reversed phase materials can be used. The mobile phase does not contain corrosive substances and ensures a prolonged column life time.

A major component content between 80 and 90 % was found.

The following additional components were identified, in order of decreasing amount: leucomycin A₄, leucomycin A₁, leucomycin A₇, leucomycin A₆ and platenomycin A₁.

The samples also contained small amounts of degraded or rearranged products such as demycarosyljosamycin and isojosamycin, a product formed in acidic media through allylrearrangement.

- I S. Omura et al., J. Antibiot., 23 (1970) 511-513.

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ANALYSIS OF BIOGENIC AMINES

S.V. Rose, J.P. Crombeen, L.G.D. Lammerts van Bueren

HPLC analysis of biogenic amines is of growing importance. The main problem with such compounds is their elution as asymmetric peaks under RP conditions. In some cases the required detection sensitivity is an additional problem because of the absence of native UV-absorbance/ fluorescence. Several techniques were evaluated:

Pre-column derivatization of the amino function with FLEC (fluorenyl ethyl chloroformate) prevents tailing and improves detectability. Excess FLEC is hydrolysed to a fluorescent compound which may disturb analysis. To avoid extraction procedures, a second reaction with 1-amino-adamantane is introduced to shift the interference to the back of the chromatogram.

Ion pairing is a suitable alternative, reducing tailing and increasing retention. Separation of catechol-amines is performed using lauryl sulphate. Reproducibility of the separation is influenced by several parameters: eluent composition, pH, etc. It was demonstrated that the influence of mobile phase pH can be lessened by using a phosphate buffer, instead of an ammonium acetate buffer.

Strong cation exchange proves to be a useful alternative for the separation of catecholamines because of the reduction of parameters as compared to ion pairing techniques.

Mixed phase HPLC is a suitable alternative to ion pairing for the analysis of choline and acetylcholine. To meet the required detection level, these compounds are selectively converted in a post-column Immobilized Enzyme Reactor (IMER) to betaine and hydrogen peroxide. The latter can be detected electrochemically. Ion pairing gives limited reproducibility and the ion pairing agent gradually inactivates the enzymes in the IMER. The use of a mixed phase column results in fast analysis and good peak shapes. The detection limit is 50 fmol/component.

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THE INFLUENCE OF DIFFERENT ANTIOXIDANTS ON THE STABILITY OF TRETINOIN IN A CREAM

H. Rosing, F. Elferink, F.J. van de Vaart

The Formulary of the Dutch Pharmacists (FNA) contains a formulation for Cremor tretinoïni 0,05%, which is used in the treatment of acne vulgaris. Tretinoïni or all-trans retinoïc acid is easily oxidizable, thermally instable and isomerizes fast when exposed to radiation. Previous investigations at our laboratory showed a rapid degradation of tretinoïni in the cream. Therefore we studied the influence of α -tocopherol (0.1% w/w), citric acid (0.01% w/w), a combination of both and butylated hydroxytoluene (0.04% w/w) on the stability of tretinoïni in the cream at 4°, 21° and 31°C.

Tretinoïni was determined by HPLC. The chromatographic system consisted of a Lichrosorb RP18 10 μ m column (250 x 4.6 mm i.d.), a mobile phase of methanol-water-glacial acetic acid (175 + 25 + 1, v/v) at a flow rate of 2.0 ml/min and UV-detection at 351 nm.

Our study showed that the degradation of tretinoïni was increased by α -tocopherol at 31°C in comparison with the cream without any antioxidant! The pro-oxidant effect of α -tocopherol has been described and is concentration dependent (1) The increase of degradation is probably due to the oxidation of α -tocopherol to unstable hydroperoxidic products (2). These unstable hydroperoxides might generate new free-radical chains which may accelerate the degradation of tretinoïni. So α -tocopherol is not effective in preventing the oxidation of tretinoïni in the cream.

The stability tests revealed that butylated hydroxytoluene was the most suitable antioxidant. After 2 years at room temperature more than 90% of the tretinoïni was still present in Cremor tretinoïni, stabilised with 0.04% butylated hydroxytoluene.

- (1) M. Rieger, Cosmetics & Toiletries, 102 (1987) 83.

- (2) D.J. Carlsson, et al. J. Am. Oil. Chem. Soc., 53 (1976) 656.

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Organic anions, accumulated in blood sera of patients with chronic renal failure^{1,2}, were separated by closed-system capillary zone electrophoresis (CZE)^{3,4}, in a pH 6 carrier electrolyte system. Hippuric acid (HA), p-hydroxyhippuric acid (PHHA), and uric acid (UA) were tentatively identified by co-elution of standards with ultrafiltered serum. Repeatabilities of migration times and peak areas of HA, PHHA, and UA in serum samples were around 0.7% and 6% in CZE respectively. Accuracy and precision of the CZE-method were comparable with those of an HPLC gradient elution method. The analysis time of HA with CZE (8 min), is much smaller than with HPLC (90 min). Capillary zone electrophoresis is very suitable for the selective and rapid analysis of (UV-absorbing) anionic constituents in ultrafiltered uremic serum, without initial sample pretreatment. The observed "solvent effect", due to the high concentration of a major constituent in the sample, may prove to be useful for enhancing the detectability of minor components in CZE-analysis.

Specific clinical-biochemical or metabolic studies require the accurate measurement of the serotonin (5-hydroxytryptamine, 5-HT) concentration in platelet rich (PRP) and platelet poor plasma (PPP). We developed a rapid, simple and sensitive HPLC method with electrochemical detection for the assay of 5-HT in PRP and PPP without pre-purification of the plasma sample. We also studied the effects of centrifugation speed and brake effects after the end of the centrifuge run on the concentration 5-HT and the number of platelets.

The [5-HT] and the number of platelets (N) decrease with increased centrifugal speed. With a centrifugal speed above 500 g the [5-HT] and N are stable. At low speed (160 g) brake at the end of the centrifuge run has a negative effect of N and [5-HT]. At high speed (900 g) the difference between brake and no-brake are neglectable.

PRP (160 g., 15 min., 4°C., no-brake) or PPP (900 g., 15 min., 4°C., brake) is diluted with a 0.001 M Na₂HPO₄ buffer pH=2.8 and an internal standard (IS) is added. The sample is sonicated, centrifuged and 1 ml supernatant is injected in a coupled column HPLC system. The sample is passed through a pre-column, which is packed with a strong cation-exchanger (polar phase aromatic sulfonic acid silane bonded to silicagel), where the basic compounds are adsorbed. By a column switching method, the basic compounds are desorbed and eluted through a reversed phase C-18 analytical column, by passing a 0.05 M (NH₄)₂HPO₄ buffer pH=5 with 0.08% EDTA and 0.2% Triethylamine. After desorption of the basic compounds, the cation-exchanger must be regenerated. This can be done during the remaining analysis time. The total analysis time of 1 ml plasma is 12 minutes, the detection limit is 0.6 nmol 5-HT/L plasma, the recovery in plasma is almost quantitative (with good reproducibility). Reference values for 5-HT concentrations in healthy adults are 40 nmol/L for PPP and 1037 ng/E9 platelets for PRP (n=14).

- 1 A.C. Schoots, J.B. Dijkstra, S.M.G. Ringoir, R. Vanholder, C.A. Cramers. Clin. Chem., 34 (1988) 1022.
- 2 A.C. Schoots, P.M.J.M. De Vries, R. Thiemann et al. Clin. Chim. Acta, 185 (1989) 91.
- 3 F.E.P. Mikkers, F.M. Everaerts, T.P.E.M. Verheggen. J. Chromatogr., 169 (1979) 1.
- 4 T.P.E.M. Verheggen, A.C. Schoots, F.M. Everaerts. J. Chromatogr. in press.

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DEVELOPMENT OF A CONVENIENT HPLC SYSTEM WITH CHEMILUMINESCENCE DETECTION TO ANALYSE ABEI LABELLED COMPOUNDS.

O.M. Steijger¹, E. Hoksbergen¹, R.A. Baumann¹, J.J.M. Holthuis² and U.A.Th. Brinkman³.

BIOANALYSIS OF SURAMIN

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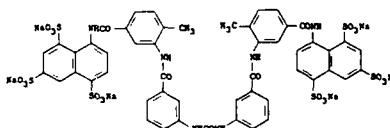
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Suramin is a polysulphonated naphthylurea (fig 1) that has been used as antitrypanosomal agent since the early 1920's. Its anti AIDS and anticancer activities have been demonstrated recently. A non-toxic, active 300 ug/ml steady-state level is indicated to exploit suramin activity optimally; therefore frequent monitoring is required.

In recent years, there has been increasing interest in chemiluminescence detection in HPLC, because chemiluminescence detection is a very sensitive method. One of the chemiluminescence methods is based on the reaction of luminol (3-aminophthalic hydrazide) with hydrogen peroxide in the presence of a catalyst in alkaline solution. We used an isoluminol analogue, ABEI (N-(4-aminobutyl)-N-ethylisoluminol) to label carboxylic acids and amines. The disadvantages of a conventional HPLC system is that three pumps are necessary, one for the mobile phase and two post-column for hydrogen peroxide and the catalyst respectively. To simplify the HPLC system we have utilized an on-line electrochemical flow cell in front of the detector to generate the oxidant in stead of delivering the hydrogen peroxide by a pump. The electrochemical generation of the oxidant gives, beside the advantage of omitting one pump, a better performance then the post-column addition of hydrogen peroxide. This HPLC system offers the opportunity to analyse drugs in biological matrices by a sensitive and improved method.

A liquid chromatographic method for suramin has been developed based on the use of tetrabutylammonium bromide (TBABr) as an ion-pairing agent with UV detection. Sample pretreatment includes deproteination by an organic solvent, and addition of TBABr to increase the recovery of the almost complex protein bound suramin. The minimum detectable concentration in plasma amounts to about 0.1 ug/ml which allows to monitor patients treated with the drug. Recovery of suramin from plasma was calculated to be 95.9%; the within-assay precision amounts to 104.5% ± 2.4% at 50.5 ug/ml and for 202 ug/ml 95.5 ± 4.2%. Protein binding at 500 ug/ml was 0.11% which is in agreement with previous data. It is concluded that the present assay allows frequent, rapid, selective and sensitive monitoring of suramin in body fluids of patients under suramin treatment.

FIG. 1



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DETERMINATION OF CHLORAMPHENICOL IN PIG TISSUES, MILK AND EGGS USING A MONOCLONAL ANTIBODY-MEDIATED CLEAN-UP PROCEDURE

C. van de Water and N. Haagsma

An earlier developed method (1) for a specific antibody-mediated clean-up and concentration procedure for the HPLC-analysis of chloramphenicol (CAP) in pig tissues is modified with respect to the preparation of the immunoaffinity (IAC) columns and elution procedure. The IAC-procedure is also made suitable for the analysis of CAP in milk and eggs (2).

Aqueous extracts of pig tissues, defatted milk and centrifuged egg homogenates were directly applied on IAC-columns which were prepared by coupling monoclonal antibodies against CAP to a carbonyldiimidazole-activated support. Elution of CAP can be performed by methanol or by a glycine/NaCl solution. The latter solution offers much better perspectives for re-use of the columns. In subsequent HPLC-analysis no matrix interferences were observed. Good recoveries were obtained at 1-100 µg kg⁻¹ spiking levels.

1. C. van de Water and N. Haagsma. *J. Chromatog.* 411 (1987), 415-421.

2. C. van de Water, D. Tebbal and N. Haagsma. *J. Chromatog.* 478 (1989), 205-215.

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SENSITIVE CHEMILUMINESCENCE DETECTION OF XANTHINE OXIDASE AS LABEL IN IMMUNOASSAYS

R.H. van den Berg and E.H.J.M. Jansen.

A new class of chemiluminescence labels is described (1,2) which combines the advantages of an enzyme label and a radioisotope. The system consists of the enzyme label xanthine oxidase which produces hydrogenperoxide and superoxide anionradicals with hypoxanthine as substrate. The signal reagent contains in addition an Fe(II)-EDTA complex and luminol. The metal complex decomposes hydrogenperoxide into very reactive hydroxyl radicals (Fenton reaction) which react with luminol to produce chemiluminescence. The Fe(III)- is oxidized back to the Fe(II)-complex by the superoxide radical according to the second leg of the Haber-Weiss reaction.

One of the most striking features of this enzyme enhanced label system is the long-term production of a constant level of light. The half-life time of the chemiluminescent signal is about 30 hrs. In addition a very low detection limit was obtained of 3 attomol xanthine oxidase per tube. The enzyme preparation of xanthine oxidase (EC 1.2.3.2) can be stored at 4 C for at least 6 months without loss of enzyme activity. Both the stability of the enzyme preparation and the signal reagent is very good. Because of the long-term kinetic signal various measuring devices can be used (2), like a tube-luminometer, a microtitreplate luminometer, a sensitive instant photographic film, or even a liquid scintillation counter.

Other oxidases were tested also in this reaction in order to develop a sensitive assay for these enzymes for use in toxicological experiments. Besides xanthine oxidase also a number of other oxidases can be detected with great sensitivity in this system, like glycolate oxidase, sulphite oxidase, glucose oxidase, aminoacid oxidase, allantoinase, uricase, alcohol oxidase, lactate oxidase and choline oxidase.

1. A. Baret, V. Fert, *J. Biolum. Chemilum.*, 4 (1989) 149.

2. E.H.J.M. Jansen et al., *J. Biolum. Chemilum.*, 4 (1989) 129.

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DETERMINATION OF PHENYLALANINE METABOLITES IN RELATION TO MATERNAL PHENYLKETONURIA

W.A. van den Ham, E.H.J.M. Jansen and A.H. Piersma,

Phenylketonuria (PKU) is an accumulation of phenylalanine (PHE) and its metabolites in blood and urine which is caused by a deficiency of the hepatic enzyme phenylalanine dehydroxylase. At our Institute the embryotoxicity of PHE and its metabolites is investigated in rat post-implantation embryo culture.

To monitor the changes in metabolite concentrations in culture we have developed a quantitative determination using isocratic reversed phase high performance liquid chromatography (HPLC). The mobile phase was optimized with respect to the percentage of organic modifier and the concentration of sodium acetate in order to separate seven components: tyrosine, phenylalanine, tryptophan and the phenylalanine-metabolites phenyllactic acid, o-hydroxyphenylacetic acid, phenylacetic acid and phenylpyruvic acid. A complete baseline separation was obtained with the following conditions: a column (4.6x150 mm) with Hypersil-ODS (5µm) eluted with acetate-buffer (0.035 M, pH 5.5) : methanol = 98.5 : 1.5, v/v. The optimal detection wavelength was determined as 210 nm after spectroscopic investigation of the various metabolites.

Before HPLC analysis the samples were deproteinized. Five methods were tested: precipitation with zincsulfate/bariumhydroxide, perchloric acid, sulfosalicylic acid and methanol and by heat denaturation. The best method was the addition of diluted acetic acid until pH 5.0-5.5 followed by boiling for several minutes. After centrifugation the supernatant could be used directly for HPLC injection.

The linearity was tested for both the low (0.20 to 2.0 µg/ml) and high concentrations (10 to 150 µg/ml) of the several components. Correlation coefficients obtained were between 0.9997 and 1.0000.

The method was successfully applied for the analysis of PHE and metabolites for embryo culture media and the determination of PHE in human reference plasma.

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MULTI-RESIDUE ANALYSIS TO DETECT THE ABUSE OF β-AGONISTS IN SLAUGHTER ANIMALS

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J.C.M. Farla, H.J. van Rossum

The use of β-agonists is one of the latest inputs into animal production. The effect of these compounds is a clear shift from fat to protein deposition in e.g. veal calves and cattle¹. An example of a very effective adrenergic β-agonist is clenbuterol. However also relatively old human drugs like salbutamol and terbutaline as well as newly developed agents like cimaterol show these effects.

At our Institute we developed a multi-residue method in which multi stands for both multi matrix and multi residue. The primary extraction of course depends on the matrix, e.g. muscle, liver, urine or animal feed. Sample clean-up and detection are identical for all samples. For sample clean-up we developed a immunoaffinity matrix by immobilizing antibodies against clenbuterol on a solid carrier². The individual β-agonists are subsequently detected and identified as TMS-derivatives by gaschromatography-mass spectroscopy. Special attention is given to the identification criteria. If these cannot be fulfilled using electron-impact ionization alternative derivatization or ionization techniques have to be used.

With the procedure described it is possible to detect and identify β-agonist in the low or sub ppb range.

1 H. Timmerman, In Beta-agonists and their effects on animal growth and carcass quality: Elsevier, London, 1987, 13.

2 L.A. van Ginkel et al. *J. Chromatogr.*, 489 (1989) 11.

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R. van Gijn and J.H. Beijnen

The anti-arrhythmic drug tocinide is used in the treatment of ventricular arrhythmias. Adverse effects are common during tocinide therapy. The frequency increases at plasma concentrations > 10 mg/L. We report a simple gas chromatographic (GC) method using nitrogen-phosphorous detection (NPD) for tocinide after acylation with valeric acid anhydride. Sample pretreatment: 500 µL plasma + 500 µL internal standard solution (methaqualon) + 500 µL 5N NaOH + 5 mL diethylether. After extraction, the organic phase is evaporated at 60 °C in the presence of 10 µL of valeric acid anhydride. The residue is dissolved in methanol and injected into the GC. Column: OV 1-10% on Chromosorb 80-100 mesh, 6 feet; flow: helium 45 mL/min, hydrogen 3 mL/min, air 100 mL/min. Column temperature: 210 °C. Recovery: >95%. Detection limit: 10 ng/mL. Spiked plasma samples, containing 10 mg tocinide/L are stable at 4 °C and -25 °C for at least seven days. The presented assay is suitable for routine therapeutic drug monitoring.

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O. van Tellingen, J.H. Beijnen, H.R. van der Woude and W.J. Nooven.

A High Performance Liquid chromatographic procedure, including sample pretreatment, is presented for the analysis of the L-isoleucine-ethyl ester of de-acetylvinblastine. To 1 ml plasma, 10 µl (10 mg/l) vinblastine is added as internal standard. This sample is prediluted with 5 ml 0.5 M phosphate buffer pH=3. Next 5 ml chloroform is added and the tubes are shaken vigorously for 10 minutes. After centrifugation for 10 minutes (3000 g, 4°C) the aqueous layer is discarded. The organic layer is transferred to a clean tube and evaporated under nitrogen (37°C). The residue is dissolved in 100 µl acetonitrile by sonification for 10 minutes. An aliquot of 50 µl is injected in the HPLC system. Separation of the compounds is achieved on a glass column (200 x 3 mm) packed with Spherisorb-Si (5 µm) and eluted with a mixture comprised of 10 mM citrate buffer (pH=3) and acetonitrile (20/80). Fluorimetric detection (exc. 270 nm/ em. 345 nm long pass) is used giving a detection limit of 1 µg/l in plasma. The assay is linear from the detection limit upto at least 10 mg/l with good reproducibility. Typical variation coefficient are less than 7 percent.

RAPID DETERMINATION OF SURAMIN IN PLASMA BY USING ION-PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

R. van Gijn, J.J.M. de Clippeleir, S. Horenblas, W.J.M. Underberg and J.H. Beijnen

Suramin is a drug used for the treatment of parasitic diseases. The drug blocks receptors for tumor growth factors and clinical studies are now in progress with patients suffering from advanced prostatic cancer. Accurate drug level monitoring during suramin therapy is of paramount importance in view of its toxicity and therapeutic efficacy. We report a simple, rapid, selective assay for the bio-analysis of suramin applicable for routine analysis. The method is a modified version of that from Teirlynck et al. (1). Sample pretreatment: 100 µL plasma + 100 µL 5 mM tetrabutylammonium phosphate (TBAP) + 200 µL acetonitrile. After vortex and centrifugation the clear supernatant is injected onto the HPLC column. HPLC: column: Bondapak C18/Corasil; eluent: 460 mL methanol + 50 mL acetonitrile + 490 mL 50 mM phosphate buffer pH 6.5 containing 5 mM TBAP; flow: 0.8 mL/min; UV detection: 313 nm; injection 25 µL. Recovery: > 80%; detection limit: 5 µg/mL; linear upto 1000 µg/mL. The assay can be used for routine drug analysis.

(1) Teirlynck O, et al. J Pharm Biomed Anal 7 (1989) 123.

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DETERMINATION OF AMPICILLIN IN PLASMA, MUSCLE TISSUE, KIDNEY AND LIVER WITH HPLC.

K. van Twillert, P.W.M. Zeiljans, M. Olling, H.C.J.M. Derks.

For the estimation of the bioavailability of intramuscular ampicillin preparations a sensitive assay in plasma, muscle tissue, kidney and liver was developed. After digestion of the tissue with collagenase and protease for 20 hours, the supernatant or plasma was treated with mercuric chloride. The 2-hydroxy-3-phenylpyrazin formed was extracted with ethyl acetate. The residue was dissolved in the mobile phase and chromatographed on an HPLC. The HPLC conditions were: column: 150 x 4.6 mm with Nucleosil ODS 5 µm, temperature 50 °C; mobile phase: 50% methanol in water; detection: fluorescence detector, excitation 325 nm, emission 420 nm. The method was linear from 0.01 to 10 mg/l or kg for all matrices ($r^2 = 0.999$). The reproducibility was in: plasma 7.4% to 14%; kidney 4.4%; liver 10%; muscle tissue 14%. The minimal detectable amount was 2.5 ng, and the lower limit of detection was 10 ng/ml plasma and 20 ng/g kidney, liver and muscle tissue. The enzymatic digestion of tissue is carried out easily and can be accomplished over night. The recovery, as compared with ampicillin standards in water, was larger than 100%. This is probably due to the fact that the reaction to convert ampicillin to the end product was more complete in plasma and tissue homogenates than in water. The large variation in the reproducibility in plasma, mainly caused by between day variation, could be explained by the same cause. The described method is suitable for use in pharmacokinetic studies when standards are used that are added to the relevant matrices instead of water.

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IDENTIFICATION OF TETRACYCLINES BY THIN-LAYER CHROMATOGRAPHY ON SILICA GEL

Weng Naidong, Th. Cachet, E. Roets and J. Hoogmartens.

The tetracycline antibiotics form a large family of therapeutically useful compounds. A considerable number of publications on the identification of tetracyclines by planar chromatography have appeared. The European Pharmacopoeia first prescribed the use of kieselguhr, in the second edition cellulose was used as the stationary phase.

Difficulties with the separations are mainly attributed to the formation of complexes with metallic ions and to the regulation of the moisture content of the stationary phase, which is very critical.

The procedure presented here permits the identification of chlor-tetracycline, demeclocycline, doxycycline, metacycline, minocycline, oxytetracycline and tetracycline by thin-layer chromatography on silica gel layers, previously sprayed with a 10 % m/v solution of sodium edetate adjusted to pH 7.0, pH 8.0 or pH 9.0 depending upon the tetracycline to be identified and using dichloromethane-methanol-water (59:35:6) as the mobile phase.

The method is applicable to laboratory-made layers and precoated plates of various origin. No special precautions regarding the moisture content have to be taken.

1 European Pharmacopoeia first edition 1973.

2 European Pharmacopoeia second edition 1980.

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The introduction of mixture experimental design in the optimization of liquid extraction of drugs from biological matrices

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The scope of chromatographic assay method optimization has always been lying towards the optimization of the chromatographic part. The sample pretreatment has often been neglected and optimized with trial and error methods, although it is an important step in assay methods. In the literature, scarce attention has been paid to this part of assay methods.

Often, solutes to be analysed in a chromatographic system have to be extracted from all kinds of matrices, because direct injection of samples into a HPLC-system cannot be performed. This often causes column deterioration. Like chromatography, extraction has to be carried out as selectively and as quantitatively as possible. Chemists exclusively want to extract the solute(s) to be determined and with high recovery: the higher the recovery, the more sensitive an assay method is. Especially in pharmaceutical analysis, where concentrations of analytes decrease nowadays, sensitivity and selectivity become major goals. More over, the less disturbing contaminants are being extracted, the easier the chromatographic procedure will be.

We introduce mixture experimental designs for the optimization of the liquid extraction of sulphamethoxazole and sulphamethizole from plasma, with the recovery as the response factor to be optimized and the fractions of the different mixture components in the extraction solvent as the control variables.

As mixture components we use methylene chloride (dipole interactions), chloroform ("proton donor") and *t*-butylmethylether ("proton acceptor").

Different types of regression models were calculated for the recovery of sulfamethoxazole and sulfamethizole:

Linear : Recovery = $\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3$
Quadratic : Recovery = $\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$
Special Cubic : Recovery = $\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3$

This systematic approach for the optimization of liquid extraction gives good results for the extraction of sulphamethoxazole and sulphamethizole from plasma. An adequate model for the description of recovery is the Quadratic Model. The calculated models predict high recoveries in the optimum.

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DETERMINATION OF PANCURONIUM AND ITS HYDROLYSIS PRODUCTS IN PAVULON® BY QUANTITATIVE THIN LAYER CHROMATOGRAPHY

G. Windhorst.

Pancuronium bromide, a bis-quaternary amino steroid, is successfully used as a long acting neuromuscular blocking agent.

Thin Layer Chromatographic methods have been developed for the quantitative determination of pancuronium and its hydrolysis products in an injectable formulation.

Application of sample and reference solutions is done band-wise using a Linomat IV application apparatus.

Separations are performed on HPTLC silica gel 60 plates¹ using solutions of lithium perchlorate or sodium iodide^{2,3} in water/propanol-2/acetonitrile mixtures as the mobile phases.

The detection is carried out by an iodine derivatization. Chromatograms are quantitatively evaluated by densitometry. Performance characteristics have been established. The precision, accuracy and linearity of the method are excellent.

1 G. Szepesi et al. *J. Chromatogr.*, 328 (1985), 279.

2 R.A. de Zeeuw et al. *Anal. Lett.*, 9 (1976), 831.

3 R. Giebelmann. *Pharmazie* 40 (1985), 178.

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