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SIMULTÁNNÍ STANOVENÍ KOVOVÝCH KATIONTŮ A ORGANICKÝCH ANIONTŮ V LÉČIVÝCH PŘÍPRAVCÍCH VYUŽITÍM REVERZNÍ KAPALINOVÉ CHROMATOGRAFIE A ELS DETEKCE

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SIMULTANEOUS ION-PAIRING REVERSED PHASE LIQUID CHROMATOGRAPHIC DETERMINATION OF METALS AND ORGANIC ACTIVE SUBSTANCES IN PHARMACEUTICAL FORMULATIONS USING EVAPORATIVE LIGHT SCATTERING DETECTION

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ABBREVIATIONS LIST

AES	Atomic Emission Spectrometry
ASC	Ascorbate
ASP	Aspartate
CCC	Countercurrent Chromatography
CIE	Capillary Ion Electrophoresis
CIT	Citrate
CZE	Capillary Zone Electrophoresis
DNA	Deoxyribonucleoic Acid
ELSD	Evaporative Light Scattering Detector
FAAS	Flame Atomic Absorption Spectrometry
FIA	Flow Injection Analysis
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
NFPA	Nonafluoropentanoic acid
PFPA	Pentafluoropropionic acid
RID	Refractive Index Detector
RNA	Ribonucleoic Acid
RT	Retention Time
SFC	Supercritical Fluid Chromatography
SIA	Sequential Injection Analysis

TFA	Trifluoracetic acid
UV-VIS	Ultraviolet-Visible

1. ITRODUCTION

Magnesium and potassium play an essential role in human health. Their compounds are described in pharmacopoeias [1, 2, 3] and are provided in pharmaceutical or food supplement formulations (mainly tablets, effervescent tablets or suspension) in relatively high amounts. In many cases these metals are provided in pharmaceuticals in form of salts of organic anions, for example acetate, aspartate, citrate, gluconate, pidolate, salicylate, stearate, etc.

Ascorbic acid (vitamin C) is one of the most important vitamines in human diet. It is often contained in pharmaceutical formulations (mainly in tablets and effervescent tablets) together with metals as magnesium, iron, selen or zinc. It is supporting their pharmacological effect, stabilising the formulation or supplying the source of vitamin C in the diet.

These compounds are succesfully used in the case of deficiency syndromes and other diseases. However, overdosage of these supplementary formulations can in some cases result in serious damage of organism. Therefore a reliable method of determination individual effective substances in the formulations is required.

Evaporative Light Scattering Detection (ELSD) has been recently used in many chromatographic applications as a quasi-universal detector, especially in case of absence of chromophoric groups in the analytes molecules [4]. The operation principle of ELSD mainly consists of three successive processes: (a) nebulization of the chromatographic effluent, (b) evaporartion of the mobile phase, and (c) detection of the non-volatile residual particles, by means of the measurement of the scattered light. Despite the wide use of ELSD in organic analysis (drugs, natural products, polymers) [4], very few inorganic analytes have been determined using LC-ELSD methods [sulfate (as counter-ion of aminoglycoside antibiotics [5]), sodium carbonate (in a drug substance [6])].

In this paper HPLC-ELSD methods have been developed and validated for the simultaneous determination of metal cations (potassium, magnesium) and organic anions (aspartate, ascorbate) of pharmaceutical use. These methods were applied on pharmaceutical

formulations. Using reversed phase column and volatile acids as mobile phase the separation of the aforementioned ions was successful and the detection by ELSD was achieved at the $\mu g\ ml^{-1}$ concentration level.

2. THEORETICAL PART

2.1. Magnesium, Potassium, Ascorbic Acid and Aspartic Acid

2.1.1. Magnesium

Magnesium is an essential mineral for human nutrition mainly found in foods like cereals, nuts, cacao, meat, milk and vegetables. Magnesium has several important functions. It is involved in energy metabolism, acting as a metal activator or co-factor for enzymes requiring adenosine triphosphate (ATP), in replication of DNA and in the synthesis of RNA and proteins; it appears to be essential for all phosphate transferring systems. Together with calcium, magnesium is involved in muscle contraction and blood clotting [7, 8]. Its deficiency occurs, in general as complications of other diseases like alcoholism, diabetes, and kidney failure and in some post-operative periods. Magnesium deficiency can be treated by oral or parental administration of some magnesium salts (magnesium supplement tablets). Oversupply in severe cases lead to coma and death [7].

Pharmaceutically is magnesium used in form of acetate, aspartate, carbonate, chloride, citrate, gluconate, glycerophosphate, hydroxide, oxide, phosphate, pidolate, salicylate, stearate, sulfate, trisilicate, etc. [3].

2.1.2. Potassium

Biological active-transport systems involving ions, in particular K^+, have important functions in the organism, which are essential for regulation of many intracellular activities. These systems are related in the transmission of information by the nervous system and in the excitation and relaxation cycle of muscle tissue [9, 10]. Besides, some enzymes require K^+ as a cofactor for the maximum catalytic activity, while metabolically supported gradients of K^+ and Na^+ across the cell membrane are involved in the maintenance of the membrane potential caused by an abrupt increase in the permeability of the membrane when it is stimulated [10, 11]. During chronic K^+ deficiency alterations in skeletal muscle function have

been described [12]. The changes may be associated with abnormalities of muscle membrane permeability and cellular function.

Pharmaceutically is potassium used in form of acetate, aspartate, bromide, carbonate, citrate, chloride, hydroxide, iodide,nitrate, phosphate, sobate, sulfate, etc.[3].

2.1.3. ***Ascorbic Acid***

Ascorbic acid, commonly known as Vitamin C, is one of the most important water-soluble vitamins in the human diet. It's important for forming connective tissue, bone, teeth, blood vessel walls, for unction of some enzymes, for metabolism of some substances (for example cholesterol) and for assimilation of iron and amino [13]. A diet deficient in Vitamin C may cause a person to develop scurvy. Vitamin C lowers the incidence of and mortality from two of the most prevalent diseases, cardiovascular disease and cancer [14]. Plants and some animals make their own Vitamin C, human body is not able to. For this reason, humans need to seek it from other sources. Vitamin C is present naturally in a wide range of foods, particularly fruits and vegetables. But ascorbic acid has limited stability and may be lost from foods during storage, preparation and cooking. In some foods, it is purposely added to attract consumers and to act as an antioxidant (e.g., to wines [15]) to prolong the shelf-life of the commercial product. Pharmaceutical preparations have ascorbic acid to add a supplementary source of Vitamin C in human diets [13].

Pharmaceutically it is used in form of ascorbic acid [3].

2.1.4. ***Aspartic Acid***

Aspartic acid is a non-esential aminoacid. It has a function in metabolism of urea. Its salt is aspartate, which is also involved in neurotransmission [16].

Pharmaceutically it is used in form of magnesium, potassium and arginin aspartate and aspartic acid [3].

2.2. Determination of Magnesium, Potassium, Ascorbate and Aspartate

2.2.1. Determination of Magnesium

According to the Czech Pharmacopoeia, magnesium in pharmaceuticals is determined by complexometric titrations with disodium edetate at alkalic buffer [3].

Several articles were published describing altenative analytical methods of determination of magnesium in pharmaceutical preparations. These include flame atomic absorption spectroscopy [7], sequential injection analysis [17] and recently a multi-commutation-based flow system for multi-element analysis [18].

A variety of other instrumental methods is used for magnesium analysis in non-pharmaceutical samples as beverages, food or body fluids. These include UV-Vis spectrophotometry [19], ion chromatography with a piezoelectric detector [20], inductively coupled plasma atomic emission spectrometry [21], inductively coupled plasma mass spectrometry [22] and ion selective electrode [23]. Other methods used for magnesium analysis are based on flow based procedures, eg continuous on-line feedback based flow titration [24], FIA based on magnesium ion-selective electrode [25] and a multi-component flow injection based analysis with diode array detection [26]. Nevertheless, these methods have not been applied on determination of magnesium in pharmaceuticals.

2.2.2. Determination of Potassium

Czech officinal analytical method is atomic emission spectrometry [3].

Several procedures were published for the determination of potassium in pharmaceuticals. These are FIA using a potentiometric electrode based on ionophore nonactin occluded in EVA membrane [27] and capillary electrophoresis with conductivity detection [28].

Other methods analysing potassium in non-pharmaceutical matrices are atomic absorption spectrometry [29], liquid chromatography [30] and potentiometry [31].

2.2.3. *Determination of Ascorbate*

Czech pharmacopoeia analytical method for determination of ascorbic acid is direct iodometric titration in acetic conditions with the addition of starch solution [3].

A great variety of articles were published describing determination of ascorbate in pharmaceutical formulations. Ascorbate was analysed by liquid chromatography with electrochemical detection [32]; by flow injection analysis with spectrophotometrical detection [33, 34, 35]; by FIA with fluorometric detection [36]; by FIA with amperometrical detection [37]; by potentiometrical determination using a copper based mercury film electrodes [38]; by iodometric potentiometric titration [39]; by automatic potentiometric flow titration [40]; by spectrophotometric determination [41]; by spectrophotometric determination using copper(II)-neocuproine reagent [42]; by spectrophotometric determination using extraction with potassium iodate [43]; by solid-phase UV spectrophotometry [44]; by near infrared reflectance spectroscopy [45] and by capillary zone electrophoresis [46].

Other methods were published, determining ascorbic acid in non-pharmaceutical matrices. These include voltametry [47], fluorometry [48] and atomic absorption spectrometry [49]. These methods have not been applied on analysis of ascorbic acid in pharmaceuticals.

HPLC is mostly coupled with UV, fluorescence or electrochemical detection. However, the coupling of HPLC with ELS detector adds a new option to the determination of ascorbate.

2.2.4. *Determination of Aspartate*

Czech officinal method for determination of aspartate is alcalimetric titration with sodium hydroxide [3].

2.3. Evaporative Light Scattering Detection

2.3.1. *Introduction*

About twenty years have been past since the introduction of the first commercially available Evaporative Light Scattering Detector (ELSD) in early 1980s (Mass Detector,

Applied Chromatography System Limited, Macclesfield, Cheshire, UK), and nowadays ELSDs have moved into the mainstream of HPLC detection techniques. The inherent advantage of ELSD to detect any analyte, regardless of the optical (ie UV absorptivity), electrochemical or other analyte properties, is the main reason for ELSD expanded applicability. ELSD is considered to be a quasi-universal rather than a fully universal detector, since analytes with higher volatility than the mobile phase can not be detected. It is mainly considered to be an LC detector, however, it also appears compatibility with countercurrent (CCC) and supercritical fluid chromatography (SFC) [4].

Beyond the common usefulness, which any universal detector appear [eg refractive index detector (RID) and mass spectrometers (MS)], the increasing interest for ELSD is additionally attributed to some special characteristics: a) compatibility with gradient elution and insensibility to temperature variation (unlike RID), b) much better detectability than RID for most molecular classes, similar to conventional LC detectors (regular detection limit is in the nanogram range, depending on analyte volatility and relative molecular mass) and c) low cost and easy operation (unlike mass spectrometers). However, it should be clarified that until now, ELSD is mainly considered to be a good alternative or supplemental detector rather than a substitute for the conventional HPLC detectors (UV/Vis, fluorimeters e.t.c.), while it lacks the huge identification potential of the wide range of LC-MS techniques [4].

Operation principle of ELSD mainly consists of three successive processes, depicted in 0 nebulization of the chromatographic effluent, evaporation of the mobile phase and detection of the non-volatile residual particles, by means of the measurement of the scattered light [4].

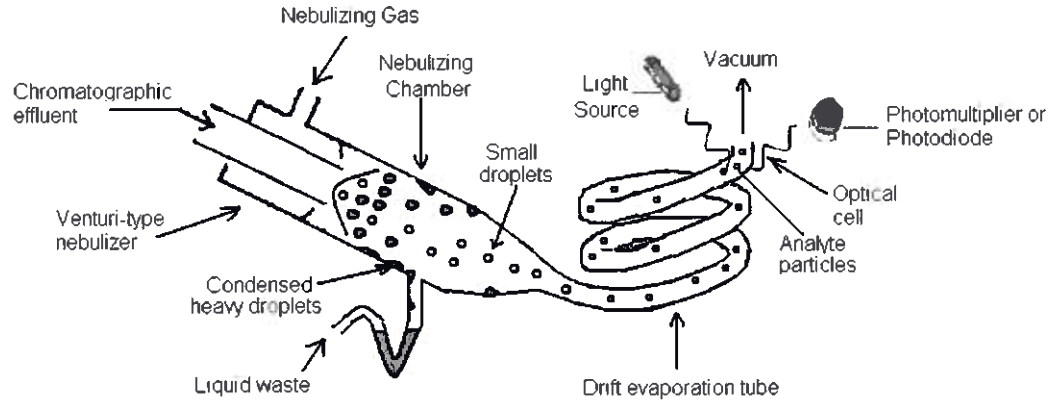

Fig. 2.1: Depiction of the main steps of the ELSD operation. Design of ELSD type B.

2.3.2. ***Nebulization***

In the first step of ELSD detection mechanism, the effluent from a chromatographic column enters a Venturi-type nebulizer, where it is transformed into an aerosol. These nebulizers create a high flow of carrier gas (air or inert gas, such as nitrogen, carbon dioxide, argon or helium) over the liquid surface producing a high amount of droplets with remarkably uniform size [4].

Distribution and mean values of droplets diameter are considered to be very critical parameters, which strongly influence the analytical characteristics (ie detectability, sensitivity and repeatability) of the ELSD methods. The formation of uniform, reproducible and stable aerosols depends on the relation of the nozzle diameter and the flow rates of mobile phase and nebulizing gas. For constant diameter of the nozzle, stable aerosols are formed only for a limited range of flow rates, and further, the flow rate of the nebulizer gas must be adjusted in relation to the flow rate of the mobile phase. For a mobile phase flow rate of 1 ml min^{-1}, the usual consumption of the nebulizing gas must be in the range of 2-5 l min^{-1} [4].

Furthermore, it has been indicated that mean diameter of aerosol droplets strongly influences ELSD response and in fact an increase of the mean diameter of aerosol droplets results in ELSD response enhancement [4].

If the gas flow rate is too low, mobile phase would not be completely nebulized and/or it would not be completely vaporized, which would result in an excessive noise or baseline with spiked sharp peaks [4].

ELSDs are classified in two types according to their structure after the nebulization unit. In ELSD of type A (non aerosol splitting), the entire aerosol immediately enters the heated evaporation tube (drift tube), where the evaporation process begins. In ELSD of type B (aerosol splitting, Fig. 2.1), the aerosol, before the evaporation step, enters a glass chamber or a focusing cone (nebulization chamber), in which the droplets of high size are condensed on the walls of the chamber and diverted to waste. The proportion of the wasted aerosol depends on mobile phase volatility and varies from >90% (aqueous mps) to <10% (highly volatile organic solvents). Each type appears its own benefits, while the appropriate choice depends on the nature of the analyte and the composition of the mobile phase. ELSD of type B requires lower evaporation temperature than type A and thus it is more sensitive for volatile, semi-volatile or thermo-sensitive analytes. On the other hand, for non-volatile analytes, ELSD of type A appears to be more sensitive, since the entire quantity of analyte reaches the optical cell. Considering the composition and flow rate of the mobile phase, ELSD of type A is incompatible with gradient elution and requires low flow rates and highly volatile mps (non-aqueous or low water portion), while ELSD of type B appears wider compatibility [4].

2.3.3. Evaporation of Mobile Phase

In this stage, the size of the aerosol droplets is reduced, due to the evaporation of the mobile phase, which is performed in a heated drift tube. Ideally, the purpose of this stage is to completely vaporise the mobile phase, without any analyte loss (due to evaporation or thermal decomposition). The completeness of the mobile phase evaporation and the extent of loss of analyte is mainly determined by the evaporation temperature, which should be selected in accordance to the mobile phase and analyte volatility, to the mobile phase flow rate and to the ELSD type (A or B). Inappropriate selection of the evaporation temperature results, in case of low temperature, in an excessive noise or baseline with spiked sharp peaks, or in case of high temperature, in reduced sensitivity. Apart from the analyte loss, high evaporation temperature causes rigorous solvent evaporation, which destroys uniformity of particle size, and favours the formation of liquid rather than solid particles. Both effects

result in decrease of ELSD sensitivity. The evaporation temperature is usually set between 30 and 100 °C. Decrease of the required evaporation temperature can be obtained with nebulizing gas of high thermal conductivity (helium was found to require at least 30 °C lower evaporation temperature than carbon dioxide), which in cases of volatile and thermosensitive compounds results in enhancement of detector sensitivity. On the other hand, for stable and non volatile compounds, the ELSD response factor has been found to be independent of the nature of the nebulizing gas [4].

2.3.4. Light Scattering

The aerosol, after the evaporation process, ideally composed by solid particles of analyte, enters the optical cell and passes through a light beam. The scattered light is measured by a photomultiplier or a photo diode, providing the output signal [4].

Light scattering processes are classified in two types: elastic scattering, in which the scattered radiation is of the same frequency as the incident radiation, and inelastic scattering, in which the scattered radiation is of a different frequency. In ELSDs, inelastic scattering is considered to be negligible and it is not further examined. Elastic scattering is classified in three types: Rayleigh, Debye and Mie. Refraction-reflection mechanism, which has its origin in the induced secondary emission of particles in the path of the incident beam, has also been reported as a potential mechanism of scattering in the ELSD optical cell [4].

Since scattering and not absorbing phenomenon is intended to occur when the light interacts with the analyte particles, a tungsten filament or halogen lamp that produces a continuous spectrum of wavelengths, rather than a monochromatic laser-emitting diode, is favored as a light source. In some instruments a secondary gas, independent of the nebulizing gas, is used to concentrate the particles in the center of the detection cell and to prevent the deposition on the cell inner surfaces [4].

The power of scattered light is controlled by the particle diameter, the light wavelength and the angle of scattered light. It has been observed that the ELSDs sensitivity is higher, but the dynamic range narrower, for low detection angle, with wide angular acceptance and the use of vertically polarized or unpolarized light [4].

2.3.5. *Limitations*

Apart from the fact that ELSDs appear nearly no selectivity, an inherent characteristic of most 'universal' detectors, some additional requirements may limit their applicability. The main difficulty for the development of LC-ELSD analytical methods is the restriction on the mobile phase volatility. Non-volatile modifiers, ion-pairing reagents, acids, bases and buffers cannot be used with ELSD. Therefore, a very useful part of the mobile phase chemistry is not compatible with ELSD, making quite difficult to convert an LC-UV method to an LC-ELSD method or to achieve efficient chromatographic separations for some type of analytes. Some acceptable volatile reagents are trifluoroacetic, heptafluorobutyric, nonafluoropentanoic, acetic and formic acid and their ammonium salts in low concentrations (<0.1 M) [4].

ELSD is a destructive detector, therefore it must be last in line if it is used in series with other detectors. In cases that it is used in line with another destructive detector (eg MS), a line splitter should be added and the flow rate of the nebulizating gas should be accordingly adjusted [4].

Generally, it appears relatively low detectability, inadequate for the direct analysis of compounds (eg impurities, residues) at ng ml^{-1} concentration level (quantitation limit is usually above 0.1 μg ml^{-1}). In these cases, preconcentration steps should be developed, in order to enrich the under analysis samples. However, development of a preconcentration procedure is quite difficult, mainly for two reasons: firstly, non-volatile reagents can not be applied (eg precipitation reagents and buffers) and secondly, preconcentration procedure may simultaneously enrich some of the matrix components resulting in excessive interferences due to the low ELSD selectivity [4].

2.3.6. *Applications*

ELSD has been effectively used for the determination of a wide variety of compounds in various synthetic or natural matrices. The main application areas of ELSD concern pharmaceuticals, foods and beverages, natural products and biological samples and polymers. A wide range of column types and mobile phase polarity have been utilized and various procedures for sample preparation have been developed depending on the analyte

nature and the sample matrix. Beyond the differences of analyte and matrix nature, a common characteristic of all ELSD methods is the conformity with the following rule: "non volatile analytes are determined utilizing a volatile mobile phase" [4].

3. EXPERIMENTAL PART

3.1. Chemicals and Reagents

All chemicals were of analytical reagent grade.

3.1.1. Water

HPLC grade water was prepared in three steps: a) deionization, b) distillation and c) purification of HPLC grade. Tap water passed deionizating column Zalion 2.004 purchased from IONEL A.E.B.E. company (N. Irakleio Attikis, Greece), then it was distilled in "Mega-Pure automatic" distilling instrument manufactured by CORNING (New York, USA). Purification of HPLC grade was performed by Milli-Q R6 system made by MILLIPORE Corporation (Billerica, MA, USA). HPLC water was prepared within 15 days before use.

3.1.2. Mobile Phase

Trifluoracetic acid (TFA) in purity of >98% (Sigma-Aldrich, St. Louis, USA), pentafluoropropionic acid (PFPA) in purity of >97%, nonafluoropentanoic acid (NFPA) in purity of >97% (both from Aldrich, Steinheim, Germany), acetic acid in purity of >99.5%, oxalic acid dihydrate in purity of >99.5% and acetonitrile of HPLC grade (all three from Merck, Darmstadt, Germany) were used for preparation of aqueous mobile phase.

The examined mobile phases were prepared by appropriate dilutions of the following storage solutions: TFA 4.0 ml l^{-1}; PFPA 0.5 ml l^{-1}; NFPA 8 ml l^{-1}; Acetic acid 10 ml l^{-1}; Oxalic acid 22 μg ml^{-1}.

For the reason that TFA, PFPA and NFPA are highly volatile substances, their storage solutions were prepared by adding appropriate volumes of acid into the volumetric flask (250 ml) almost full of already degassed HPLC grade water and filling with the same diluent to the punch mark. All storage solutions were kept in refrigerator in airtight bottles.

3.1.3. ***Standards***

Standard solutions were prepared from the following substances: Magnesium acetate tetrahydrate in purity of >99% (Hopkin&Williams LTD, Chadwell Health, Essex, England); Pottasium bromide p.a. in purity of >99% (The British Drug Houses LTD, Poole, England); L-Ascorbic acid in purity of >99%; DL-Aspartic acid in purity of >99%; (both from Sigma-Aldrich, Steinheim, Germany).

Magnesium

100.02 μg ml^{-1} standard stock solution of magnesium was prepared from 0.0893 g of magnesium acetate tetrahydrate diluted by the mobile phase in 100 ml volumetric flask. From the stock solution of magnesium, volumes 0.6 ml, 1.2 ml, 1.6 ml, 2.0 ml and 2.4 ml were taken by automatic pipette to 10 ml volumetric flasks and diluted by the mobile phase, in order to gain solutions of 6.01 μg ml^{-1}, 12.02 μg ml^{-1}, 16.03 μg ml^{-1}, 20.04 μg ml^{-1} and 24.05 μg ml^{-1}. From these five dilutions a calibration curve was constructed.

Potassium

99.37 μg ml^{-1} standard solution of potassium was prepared from 0.0306 g of potassium bromide diluted by mobile phase in 100 ml volumetric flask. From the stock solution of potassium 2.0 ml, 4.0 ml, 6.0 ml and 8.0 ml were taken by automatic pipette to 10 ml volumetric flasks and diluted by the mobile phase, in order to gain solutions of 19.87 μg ml^{-1}, 39.75 μg ml^{-1}, 59.62 μg ml^{-1} and 79.50 μg ml^{-1}. From these four dilutions a calibration curve was constructed.

Aspartate

493.02 μg ml^{-1} standard solution of aspartate was prepared from 0.0498 g of DL-Aspartic acid diluted by mobile phase in 100 ml volumetric flask. From the stock solution of aspartic acid 0.4 ml, 0.8 ml, 1.2 ml, 1.6 ml and 1.9 ml were taken by automatic pipette to 10 ml volumetric flasks and diluted by the mobile phase, in order to gain solutions of 19.72 μg ml^{-1}, 39.44 μg ml^{-1}, 59.16 μg ml^{-1}, 78.88 μg ml^{-1} and 93.67 μg ml^{-1}. From these five dilutions a calibration curve was constructed.

Ascorbate

508.86 μg ml^{-1} standard solution of ascorbate was prepared from 0.0514 g of L-ascorbic acid and 0.4500 g of sodium bisulphite for stabilising the solution, diluted by mobile phase in 100 ml volumetric flask. From the stock solution of ascorbic acid 1.0 ml, 1.4 ml, 1.8 ml, 2.2 ml and 2.6 ml were taken by automatic pipette to 10 ml volumetric flasks and diluted by the mobile phase, in order to gain solutions of 50.89 μg ml^{-1}, 71.24 μg ml^{-1}, 91.59 μg ml^{-1}, 111.95 μg ml^{-1} and 132.30 μg ml^{-1}. From these five dilutions a calibration curve was constructed.

3.1.4. ***Other Chemicals***

Calcium hydroxide in purity of >96%; Aluminium nitrate nonahydrate in purity of >98.5% (both from Merck, Darmstadt, Germany); Calcium lactate (Mallinckrodt Chemical Works); Zinc acetate dihydrate in purity of >99.5% (Pancreac Quimica SA); Citric acid in purity of >99.5% (Sigma-Aldrich, Steinheim, Germany); Calcium chloride + aq. (The British Drug Houses LTD, Poole, England).

For stabilising solutions of ascorbic acid were used Sodium meta-bisulphite heptahydrate and Phosphorous acid in purity of >30% (Merck, Darmstadt, Germany).

3.2. Instrumentation

3.2.1. ***HPLC System***

Modular Shimadzu HPLC system (Tokyo, Japan) consisting of a LC-10AD VP pump; a FCV-10AD VP flow-channel selection valve and a RHEODYNE 7725i by Perkin Elmer (Wellesley, MA, USA) manual injector with a built-in position sensing switch, which provided the chromatograph start signal. The complete filling method with 20 μl loop was used. Syringe containing excess of sample was required to completely flush mobile phase from the loop and the volume of the loop was injected.

3.2.2. ***Chromatographic Column***

The chromatographic analytical column used was the endcapped C_{18} Waters Spherisorb 5 μm ODS2 (4.6 × 250 mm), (Milford, MA, USA). Its filling was based on silica

particles of spherical shape; pore size 80 Å; surface area 220 $m^2 g^{-1}$; pore volume 0.50 cc g^{-1}; percentage of carbon load 11.5%.

3.2.3. *ELS Detector*

The evaporative light scattering detector (ELSD) was the Sedex 75 (Sedere, Alfortville Cedex, France). The nebulizing gas was nitrogen. Appropriate pressure drop was applied at the end of the flow line in order to ensure the complete removal of the gas wastes.

3.2.4. *Softwares*

The data from HPLC system were compiled by Class VP Chromatography Data System, version 4.3, Shimadzu (Germany).

Statistics were performed by StatMost Analysis and Graphics, version 2.50 (DataMost Corporation, Dataxiom Software Inc., LA, USA).

3.3. Procedures

3.3.1. *General Procedures*

Analytical column, which was utilized, is not compatible with pH below 1.5. Since the applied mobile phases contained a strong acid (TFA), pH was measured before usage and analytical column was very carefully washed with acetonitrile at the end of each working day and stored in the same solvent. Before measurements, flow path was rinsed with mobile phase for about 15 min, until baseline noise became negligible (less than 5 mV at detector gain 11).

3.3.2. *Optimum Mobile Phase and Chromatographic Parameters*

The optimum composition of mobile phase was aqueous solution of 0.2 ml l^{-1} TFA for the analysis of Cardilan® and Magnesii lactici 0.5 tbl.®. For the analysis of Magnesium 250 mg® gradient elution was used: from 0 min to 2.5 min aqueous NFPA 0.5 ml l^{-1}; from 2.5 min to 3.5 min linear gradient to aqueous TFA 1.0 ml l^{-1} up to 11.5 min. Flow of mobile phase during the analyses was 1.0 ml min^{-1}, corresponding to a back pressure of 1490 psi.

Sensitivity of the detector was 11. Higher sensitivity would cause a higher response of the detector but also a bigger noise. Detector temperature used for the analysis was 60 °C for Cardilan® and 70 °C for other formulations. High temperature was used for the reason that mobile phases contain a high portion of water which is not as volatile as organic solvents. The temperature was increased in order to minimalise the noise. Pressure of the nebulizing gas was 3.5 bar.

There is a big noise in chromatograms where aspartic acid was measured. That was possibly caused by some impurities in the standard substance. For this reason, it was necessary to clean the detector often by increased flow of acetonitrile 2 ml min^{-1} at the temperature of 100 °C.

Due to lamp aging, it was necessary to switch on the detector at least one and half hour before measurements in order to stabilise the light intensity. When measuring sooner, there was a higher response of the detector which caused bigger areas of peaks and significant inaccuracy of results.

3.3.3. Validation Procedures

The LC-ELSD analytical method was validated in terms of resolution and symmetry of chromatographic peaks, precision (repeatability and reproducibility), concentration range, correlation coefficient of calibration curve, detectability and accuracy.

Since ELSD is not a linear detector, double logarithmic relations were established and correlation coefficients were determined for magnesium, potassium, aspartate and ascorbate by triplicate measurements of the corresponding chromatographic peaks of five standard solutions.

Repeatability of the method was evaluated by replicate measurements of standard solutions (n=3) and repeated analyses of pharmaceutical formulations (3 independent sub-samples × 3 measurements).

Reproducibility of the method was evaluated by the estimation of %RSD of the peak area of standard solutions and the slope of calibration curves obtained at three different days within a week, with 3 replicates per day.

Detection limit was determined as the analyte concentration for which the area of the chromatographic peak was equal to 3.3 times the standard deviation of the most dilute standard and was practically equal to the concentration having S/N ratio equal to 3.3. **Quantification limit** was determined as the analyte concentration for which the area of the chromatographic peak was equal to 10 times the standard deviation of the most dilute standard and was practically equal to the concentration having S/N ratio equal to 10.

Accuracy was evaluated by recovery experiments. Formulation samples were fortified by standard solutions at three concentration levels, with three samples being prepared at each level and measured in triplicate. Mean recovery and range of recovery values were calculated. Accuracy was also evaluated by comparison of the results for magnesium, potassium, aspartate and ascorbate, analyzed by the Pharmacopoeia and the proposed method.

3.4. Analyses of Formulations

Three formulations were analysed. All of them are registered in the Czech Republic.

3.4.1. ***Cardilan®***

effective sustances:	Potassium hydrogenaspartate hemihydrate 175 mg (i.e. 37.97 mg K^+, 0.97mmol K^+)	in 1 tablet
	Magnesium hydrogenaspartate hemihydrate 175 mg (i.e. 11.8 mg Mg^{2+} , 0.49 mmol Mg^{2+})	in 1 tablet
excipients:	Silica oxid coloidial Silica oxid methylated Povidon Sodium carboxymethylstarch Talc Magnesium stearate Natrium benzoate	
manufacturer:	Zentiva	
dosage form:	tablets	

10 tablets were weighted and the weight of an average tablet was calculated, which is 0.6568 g. The tablets were powdered in order to gain a fine powder. 0.8271 g of the powder was dissolved in mobile phase in 100 ml volumetric flask. The suspension was

sonicated in ultrasonic bath for 15 min in order to discorporate the aggregates. The volumetric flask was filled with mobile phase up to the punch mark. The suspension was centrifuged for 15 min in order to remove the excipients. For analysis of magnesium and potassium, 0.5 ml, 1.0 ml and 1.5 ml of supernatant solution was taken by micro-pipette. The taken volumes were diluted by mobile phase in 10 ml volumetric flasks in order to gain solutions corresponding to 7.43 μg ml^{-1}, 14.86 μg ml^{-1} and 22.29 μg ml^{-1} of magnesium and 23.91 μg ml^{-1}, 47.82 μg ml^{-1} and 71.73 μg ml^{-1} of potassium. These two ions can be determined in one injection, for aspartate it was necessary to make separate dilutions. From the same supernatant solution 0.15 ml, 0.25 ml and 0.35 ml were taken in the 10 ml volumetric flasks and diluted by mobile phase. For every day, a new two-point calibration curve was constructed.

3.4.2. ***Magnesii Lactici 0.5 tbl.*** ®

effective substance:	Magnesium lactate dihydrate 0.500 g (i.e. 51 mg Mg^{2+}) in 1 tablet
excipients:	Lactose monohydrate
	Potato starch
	Gelatina
	Talc
	Magnesium stearate
manufacturer:	Medicamenta
dosage form:	tablets

10 tablets were weighted and the weight of an average tablet was calculated, which is 0.7838 g. The tablets were powdered in order to gain a fine powder. 0.3994 g of the powder was dissolved in mobile phase in 100 ml volumetric flask. The suspension was sonicated in ultrasonic bath for 10 min in order to discorporate the aggregates. The volumetric flask was filled with mobile phase up to the punch mark. The suspension was centrifuged for 15 min in order to remove the excipients. The supernatant solution was filtered through a membrane filter (Millipore, 0.45 μm) in order to remove the fine non-centrifuged excipients. For analysis of magnesium, 0.4 ml, 0.6 ml and 0.8 ml of the filtrate were taken by micro-pipette. The taken volumes were diluted by mobile phase in 10 ml volumetric flasks in order to gain solutions corresponding to 10.40 μg ml^{-1}, 15.59 μg ml^{-1} and 20.79 μg ml^{-1} of magnesium. For every day, a new two-point calibration curve was constructed.

3.4.3. *Magnesium 250 mg®*

effective substances:	Magnesium oxide 420 mg (i.e. 250 mg Mg^{2+}) in 1 tablet
	Ascorbic acid 150 mg in 1 tablet
excipients:	Sodium saccharine
	Strawberry flawour
	Azorubine 85
	Corn starch
	Saccharose
	Sodium hydrogencarbonate
	Citric acid anhydride
	Macrogol
manufacturer:	Pharmavit
dosage form:	effervescent tablets

10 tablets were weighted and the weight of an average tablet was calculated, which is 3.4652 g. The tablets were powdered in order to gain a fine powder. 0.5051 g of the powder was dissolved in mobile phase in 100 ml volumetric flask. The suspension was sonicated in ultrasonic bath for 5 min in order to discorporate the aggregates. The volumetric flask was filled with mobile phase up to the punch mark. The suspension was filtered through a membrane filter (Millipore, 0.45 μm) in order to remove the excipients. 0.30 ml, 0.45 ml and 0.60 ml of the filtrate were taken by micro-pipette. The taken volumes were diluted by mobile phase in 10 ml volumetric flasks in order to gain solutions corresponding to 10.93 μg ml^{-1}, 16.40 μg ml^{-1} and 21.86 μg ml^{-1} of magnesium. During the analysis of magnesium, retention times fluctuate within the range of about 0.8 min. This problem was caused by damaged pump of the HPLC instrument. For other measurements, the pump was repaired. This fluctuation doesn't have a significant influence on the results.

Using this mobile phase, it was not possible to determine ascorbat because its peak was not separated into baseline. Therefore another method was developed.

To 0.5005 g of formulation sample 0.2002 g of sodium bisulphite was added. The sample was consequently adjusted by usual way. From the filtrate 3.0 ml and 4.0 ml were taken to 10 ml volumetric flasks by micropipette. These volumes were diluted by the mobile phase in order to gain dilutions corresponding to 65.00 μg ml^{-1} and 86.66 μg ml^{-1} of ascorbic acid. For every day, a new two-point calibration curve was constructed.

4. RESULTS AND DISCUSSION

4.1. Method Optimisation

4.1.1. Separation of Magnesium, Potassium and Aspartate

For finding optimal composition of mobile phase, aqueous acetic acid was tried first. In concentrations from 1.25 up to 10% (v/v), the retention time was close to void time (1.7 min) (Table 4.1). For 20% solution, retention time of magnesium was slightly increased (3.2 min), probably due to increase of the lipophilicity of [Mg-(acetic)$_n$] complexes as a result of a higher grade of complexation.

Since it was not possible to further increase the concentration of acetic acid (because of analytical column sensitivity to low pH), mobile phases of aqueous TFA was tried. In comparison to [metal-(acetic)n] complexes, [metal-(TFA)n] complexes are of higher molecular weight (and so of lipophilicity), therefore lower concentration of TFA is sufficient to gain longer retention time (Table 4.1). Opposite to acetic acid, decrease of TFA concentration resulted in increase of retention times of metals. By decreasing the concentration of TFA, retention time was longer which is caused by decreasing acidity of the mobile phase. This behaviour can be attributed to the diprotonation of the free silanolic groups of stationary phase, resulting in enhancement of cation-exchange interactions with positively charged metal cations. Optimal concentration found was 0.02% (v/v) (Table 4.1). A typical chromatogram is depicted on Fig. 4.1.

Table 4.1: Effect of mobile phase composition on retention time of magnesium, potassium and aspartate.

Mobile Phase Composition	Retention time, min			Resolution	
	Magnesium	Potassium	Aspartate	Aspartate-Potassium	Magnesium-Potassium
Acetic acid, % v/v					
1.25	1.6				
2.5	1.6				
5.0	1.6				
10.0	1.7				
20.0	3.2				
TFA, % v/v					
0.10	3.0				
0.05	3.5				
0.02	5.2	4.1	2.9	3.2	3.4
0.01	11.2				

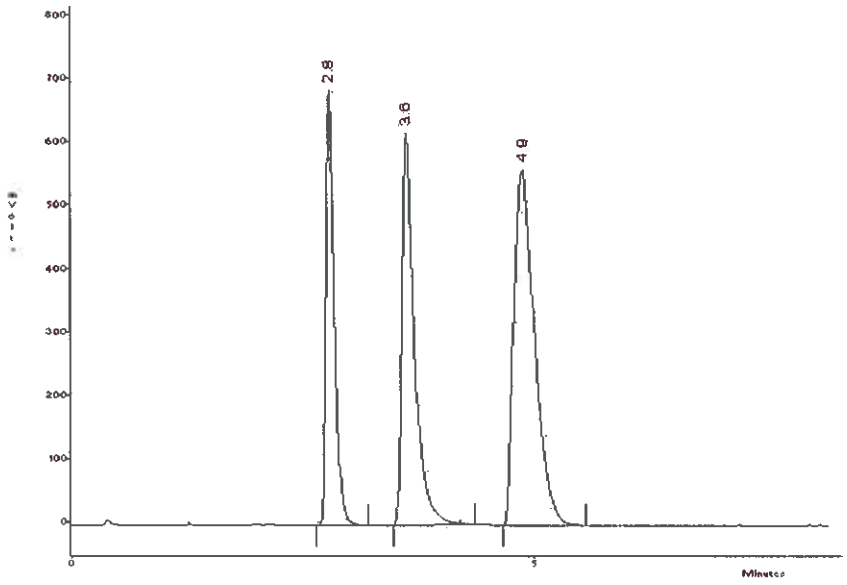

Fig. 4.1: Typical chromatogram of aspartate (Rt=2.8 min), potassium (Rt=3.6 min) and magnesium (Rt=4.9 min), analytical column Waters ODS-2, mobile phase aqueous TFA 0.02% (v/v).

4.1.2. Separation of Magnesium, Calcium, Zinc, Aluminium, Ascorbate, Aspartate and Citrate

By using mobile phase of aqueous 0.02% TFA differentiated retention time and good 'baseline to baseline' separation were obtained for organic anions, but not for inorganic cations (Table 4.2, 4.3). Furthermore, separation of cations was tried to be obtained by aqueous mobile phases of PFPA, NFPA and oxalic acid, due to formation of more lipophilic or more stable metal-complexes. Acetonitrile was also examined in order to improve the peak symmetry. Complete separation of all metal cations was not finally achieved. When using low concentrations of acids, peaks were splitted and tailed. By increasing acetonitrile portion or the concentration of the acid, retention times of magnesium and calcium were almost similar. For analysis of formulations containing both of these cations, a different column type cation-exchange has to be used.

Table 4.2: Retention time and resolution of magnesium, calcium, zinc, aluminium, ascorbate, aspartate and citrate using mobile phase aqueous TFA 0.02% v/v.

	Aspartate	Ascorbate	Zinc	Magnesium	Calcium	Aluminium	Citrate
Retention time, min	2.9	4.2	4.8	5.1	5.1	5.9	6.3
Resolution	4.1	0.8	0.4	0.2	0.6	0.2	
Asymmetry	1.6	1.3	1.6	1.7	1.5	6.3	1.2

Table 4.3: Effect of mobile phase composition on retention time of magnesium, potassium and aspartate.

Mobile phase	Retention time, min		Resolution	Asymmetry	
	Magnesium	Calcium		Magnesium	Calcium
PFPA 0.02 %	8.7	9.0	0.3	1.7	1.4
PFPA 0.03%	6.5	6.4	0.1	1.4	1.5
NFPA 0.03 %	9.6	10.1	-	2.4	-
NFPA 0.036% Acetonitrile 50%	7.2	7.5	0.3	1.4	1.3
NFPA 0.12% acetonitrile 30%	5.2	5.2	0	1.7	1.6
NFPA 0.28% Acetonitrile 30%	4.6	4.7	0.2	1.9	1.7
NFPA 0.2% Acetonitrile 30%	4.9	4.9	0	1.5	1.7
NFPA 0.34% Acetonitrile 30%	4.7	4.7	0	1.7	1.9
NFPA 0.03% TFA 0.005% Acetonitrile 50%	6.2	6.2	0	1.2	1.3
NFPA 0.04% TFA 0.005% Acetonitrile 50%	5.2	5.3	0.2	1.2	1.2
NFPA 0.05% TFA 0.005% Acetonitrile 45%	4.5	4.6	0.4	1.4	1.5
TFA 0.024% Oxalic acid 4.4 $\mu g\ ml^{-1}$ Acetonitrile 20%	4.8	4.9	0.1	1.4	1.4

4.1.3. ***Separation of Sodium, Ascorbate, Magnesium and Citrate***

By using aqueous mobile phase containing 0.02% TFA, it was not possible to achieve adequate separation among ascorbate, sodium and magnesium (Table 4.4, 4.5). Decrease of mobile phase TFA concentration resulted in a prolonged retention time of magnesium (not eluted until 20 min), while increase of mobile phase TFA concentration resulted in reduced retention of sodium and magnesium and lower resolution. A mobile

phase of low TFA concentration containing a portion of polar organic solvent (acetonitrile) was also found not to be efficient for the adequate separation of ascorbate.

Mobile phase of NFPA 0.5 ml l^{-1} resulted in good separation of ascorbate from sodium; magnesium was not eluted from the column (due to higher molecular weight and so lipophilicity of NFPA). Neither increasing the portion of NFPA nor using a mixture of TFA and NFPA as a mobile phase was successful for eluting magnesium from the column in a reasonable time.

Efficient separation of the analytes was finally achieved with gradient elution. Mobile phase of NFPA was appropriate for the separation of sodium from ascorbate, but retention time of magnesium was prolonged and so a change of mobile phase composition to a high concentration of TFA was required. The gradient time programme was: from 0 min to 2.5 min aqueous NFPA 0.5 ml l^{-1}; from 2.5 min to 3.5 min linear gradient to aqueous TFA 1.0 ml l^{-1} up to 11.5 min. A typical chromatogram is depicted on Fig. 4.2.

Table 4.4: Effect of mobile phase composition on retention time of sodium, ascorbate, magnesium and citrate.

Mobile phase	Retention time, min			
	Magnesium	Sodium	Ascorbate	Citrate
TFA 0.02%	4.9	3.7	4.1	5.9
TFA 0.015%	-	4.9	4.0	5.2
TFA 0.025%	4.5	3.4	-	6.4
TFA 0.015% Acetonitrile 10%	7.0	4.1	-	3.0
TFA 0.02% Acetonitrile 10%	5.5	3.6	3.3	3.1
NFPA 0.05%	-	7.0	4.0	5.9
NFPA 0.05% TFA 0.01%	-	7.1	4.0	5.9
NFPA 0.005% TFA 0.02%	8.7	4.5	-	6.0
NFPA 0.05% TFA 0.015%	12.0	7.0	4.0	5.8

Table 4.5: Effect of mobile phase composition on resolution and asymmetry of sodium, ascorbate, magnesium and citrate.

Mobile phase	Resolution			Asymmetry			
	Na/Asc	Asc/Mg	Mg/Cit	Mg	Na	Asc	Cit
TFA 0.02%	-	-	-	-	0.81	-	-
TFA 0.01%	-	-	-	-	-	1.6	-
TFA 0.04%	-	-	1.9	0.9	0.9	-	0.9
	Asc/Na	Na/Cit	Cit/Mg				
NFPA 0.005% TFA 0.02%	-	1.8	-	-	0.9	-	0.9
	Asc/Cit	Cit/Na	Na/Mg				
TFA 0.015% Acetonitrile 10%	-	2.1	2.6	2.4	0.8	-	0.8
TFA 0.02% Acetonitrile 10%	-	1.2	2.3	1.2	0.9	-	1.2
NFPA 0.05%	4.2	1.9	-	-	1.3	1.5	1.0
NFPA 0.05% TFA 0.015%	4.1	2.1	-	-	1.3	1.5	1.2
NFPA 0.05% TFA 0.01%	4.2	2.1	-	-	1.3	1.5	1.0

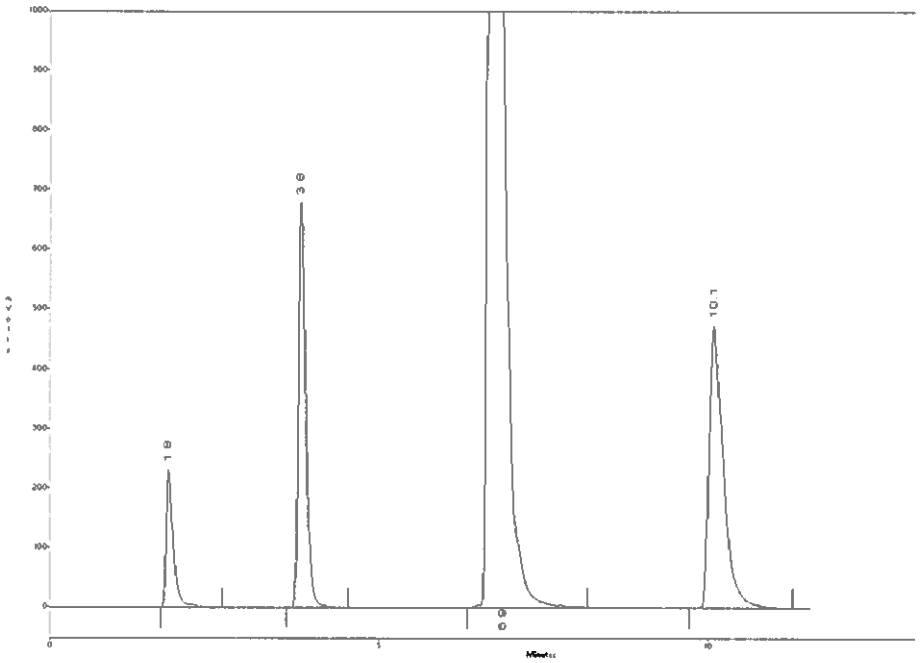

Fig. 4.2: Typical chromatogram of ascorbate (Rt=3.9 min), citrate (Rt=6.9 min) and magnesium (Rt=10.1 min), analytical column Waters ODS-2, gradient elution: from 0 min to 2.5 min aqueous NFPA 0.5 ml l-1; from 2.5 min to 3.5 min linear gradient to aqueous TFA 1.0 ml l-1 up to 11.5 min.

4.1.4. ***Stabilisation of Ascorbic Acid***

Ascorbic acid is very instable in solution. It's easily oxidised to its lacton-form. This lacton is more hydrophilic, is less retented in the C_{18} column and so it has a shorter retention time. Ascorbic acid is more stable in acidic environment. Therefore, it's better to dissolve the samples in mobile phase than in water. Furthermore, it's less stable in presence of Mg^{2+} which could maybe work as catalyser. For this reason it's better to prepare separate standard solutions of magnesium and ascorbate and mix them just before injecting.

For stabilisation of ascorbate solution, samples containing sodium bisulphite and phosphorous acid were examined. Sodium bisulphite proved to be a more effective antioxidant. It was used in an excess of five times over ascorbate, for which stabilisation of magnesium-ascorbate mixed solutions (i.e. Magnesium 250 mg®) was confirmed for one hour.

4.2. Validation

4.2.1. Resolution and Asymmetry

Resolution (R) of two neighboring peaks was studied on standard solutions, according to the equation 4.1:

$$R = 2(t_{RB} - t_{R\,A}) / (w_A + w_B) \qquad \textbf{eq. 4.1}$$

where tr_A and tr_B are the retention time of the peaks and w_A and w_B the corresponding peak width at 10% of the peak height.

Asymmetry factor (A_s) determined according to the equation 4.2 in 0.1 high (h) of the peak:

$$A_s = \frac{a}{b} \qquad \textbf{eq. 4.2}$$

where a is the front part and b is the tail path of the peak width in 0.1 high, splitted by the middle line of the peak.

Results are presented in Table 4.6. Adequate resolution was achieved for all analytes.

Table 4.6: Resolution and asymmetry of peaks of magnesium, potassium, aspartate and ascorbate.

	Ascorbate	Magnesium	Magnesium	Potassium	Aspartate
Mobile phase	*	*	**	**	**
Concentration (μg ml^{-1})	92	16	18	60	62
Rt (min)	3.85	10.13	4.78	3.62	2.82
Width at 10%	0.23	0.43	0.39	0.30	0.20
Resolution		19.0		3.4	3.2
Assymetry	1.3	1.7	1.7	1.9	1.6

* aqueous solution of 0.2 ml l^{-1} TFA

** gradient elution was used: from 0 min to 2.5 min aqueous NFPA 0.5 ml l^{-1}; from 2.5 min to 3.5 min linear gradient to aqueous TFA 1 ml l^{-1}

4.2.2. Linearity and Range

Very good correlation was achieved with the well-established exponential relationship between peak area (A) and analyte mass (m) (eq. 4.3). The linearity of the method was calculated by using the linear least squares regression technique of the logarithm of peak area (A) versus the logarithm of analyte concentration (c) (eq. 4.4).

$$A = a \times m^b \qquad \textbf{eq. 4.3}$$

$$\log A = b \log c + \log a \qquad \textbf{eq. 4.4}$$

where a and b are coefficients depending on the ELSD instrumentation and on nebulization and evaporation processes (flow rates of the nebulization gas and mobile phase, composition of the mobile phase, evaporation temperature, e.t.c.)

Standard solutions were measured in triplicate. Regression data are presented in Table 4.7. The calibration curve of aspartate is depicted on Fig. 4.2.

Table 4.7: Regression data of magnesium, potassium, aspartate and ascorbate.

	Concentration range, μg ml^{-1}	Intercept (±SD)	Slope (±SD)	Correlation coefficient
Magnesium	6 - 24	5.353 (±0.020)	1.255 (±0.017)	**0.9997**
Potassium	20-80	2.898 (±0.116)	2.112 (±0.070)	**0.998**
Aspartate	20-94	3.478 (±0.010)	1.642 (±0.005)	**0.99998**
Ascorbate	50-132	3.438 (±0.159)	1.636 (±0.081)	**0.996**

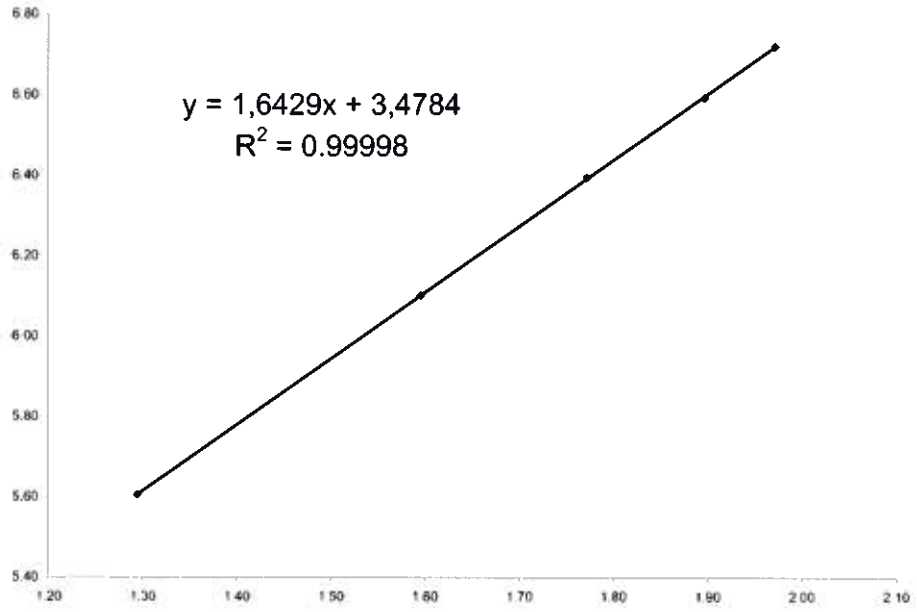

Fig. 4.2: Calibration curve of aspartate, analytical column Waters ODS-2, mobile phase aqueous TFA 0.02% (v/v).

4.2.3. ***Precision***

Repeatability of the method was evaluated by replicate measurements of standard solutions, n=5-6 (Table 4.8). Reproducibility of the method was evaluated by the estimation of %RSD of the slope of calibration curves obtained at three different days within a week, with 3 replicates per day (Table 4.9).

Table 4.8: Precision of HPLC-ELSD determination of magnesium, potassium, aspartate and ascorbate.

	Concentration level (μg ml^{-1})	RSD, %	n
Magnesium	6.01	4.28	5
Potassium	19.87	8.36	6
Aspartate	19.72	5.94	6
Ascorbate	50.89	5.38	6

Table 4.9: %RSD of slope of HPLC-ELSD calibration curves of magnesium, potassium, aspartate and ascorbate.

	Day 1	Day 2	Day 3	R.S.D., %
Magnesium	1.2554	1.3130	1.3602	4.01
Potassium	2.1120	2.0708	2.1487	1.85
Aspartate	1.6429	1.8146	1.7900	5.31
Ascorbate	1.6369	1.6717	1.6217	1.56

4.2.4. Detectability

Detection and quantification limits are presented in Table 4.10.

Table 4.10: Detectability of HPLC-ELSD determination of magnesium, potassium, aspartate and ascorbate.

	LOD (μg ml^{-1})	LOQ (μg ml^{-1})
Magnesium	1.26	3.04
Potassium	10.66	18.03
Aspartate	9.05	17.51
Ascorbate	17.10	33.66

4.3. Results of Analyses

4.3.1. Cardilan®

The formulation was analysed according to the new isocratic HPLC-ELSD method. The results obtained from the assay of magnesium and potassium revealed conformance to the common European Pharmacopoeia for content within the range of 95.0 – 105.0 % ($r > 0.95$) of the labeled content. Individual values are presented in Table 4.11. Content of aspartate is not issued on the label, however, according to the HPLC-ELSD method it was determined to be 213.52 mg in 1 tablet. A typical chromatogram of the analysis is depicted on Fig. 4.3.

Table 4.11: Comparison of the HPLC-ELSD results of the assay of Cardilan® tablets to the label content.

Analyte (formulation content)	Run Concentration ($\mu g\ ml^{-1}$)	Tablet Content mg	%Percentage of label concentration	n
Magnesium	7.43	12.32	104.37	2
(11.8 mg per	14.86	12.10	102.55	3
tablet)	22.29	12.22	103.58	3
Potassium	23.91	41.20	108.51	2
(37.97 mg per t	47.82	38.92	102.51	3
tablet)	71.73	39.03	102.79	3

The accuracy of the new HPLC-ELSD method was evaluated by recovery experiments. Samples were fortified by adding known amounts of standards of magnesium, potassium and aspartate. For each component, five spiked samples were prepared. The good accuracy of the proposed method was confirmed since the individual recovery values are within the range of 95.0 – 105.0 % ($r > 0.95$) (except of two measurements) (Table 4.12).

Table 4.12: Spiked samples of Cardilan®.

	Formulation Concentration $\mu g\ ml^{-1}$	Spiked Concentration $\mu g\ ml^{-1}$	Recovery (%)	Mean Recovery (%)	n
Magnesium	7.5	3.5	106.51	101.91	2
	7.5	7.5	104.01		3
	7.5	12.5	101.31		3
	11.5	3.5	96.50		3
	16.5	3.5	101.24		3
Potassium	25	10	108.26	105.19	2
	25	25	103.32		2
	25	40	104.22		3
	40	10	103.85		2
	55	10	106.30		2
Aspartate	40	12	97.88	98.90	3
	40	25	95.67		3
	40	40	96.28		2
	53	12	99.75		3
	68	12	104.95		3

Further study of the matrix effect on the determination was carried out by dilution experiments (determination of magnesium, potassium or aspartate content using a varying dilution factor D (Vinitial/Vfinal) at three different levels). The correlation curve of the concentration found (in the diluted solution) versus D was linear ($r > 0.95$) with a slope equal to the content of the formulation and a statistically (proven by t-test) zero intercept (except of aspartate). Similarly, the correlation curve of formulation content found versus D was very linear with statistically (proven by t-test) zero slopes. These results confirmed the absence of any constant or proportional determinate error due to matrix (excipients) effect (Tables 4.13, 4.14, 4.15; Fig. 4.4, 4.5).

Table 4.13: 1/dilution, run concentration and concentration of formulation of magnesium, potassium and aspartate in Cardilan®.

	1/dilution	Run concentration ($\mu g\ ml^{-1}$)	Concentration of formulation ($\mu g\ ml^{-1}$)
Magnesium	0.050	7.750	0.01874
	0.100	15.24	0.01843
	0.149	23.09	0.01870
Potassium	0.050	25.94	0.06273
	0.100	49.02	0.05927
	0.015	73.73	0.05973
Aspartate	0.015	41.49	0.33458
	0.025	66.82	0.32315
	0.035	92.53	0.31996

Table 4.14: Dependence of run concentration on dilution factor of Cardilan®.

	Parameter a	**Standard deviation a**	**t_a***	**Parameter b**	**Standard deviation b**
Magnesium	4.18	4.02	**1.040**	154.93	3.00
Potassium	1.46	1.32	**1.101**	482.65	12.32
Aspartate	3.11	0.37	**8.259**	2555.17	14.30

* For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95%.

Table 4.15: Dependence of concentration of formulation on dilution factor of Cardilan®.

	Parameter a	**Standard deviation a**	**Parameter b**	**Standard deviation b**	**t_b***
Magnesium	0.01	0.0004	0.0004	0.0034	**0.117**
Potassium	0.06	0.0024	0.0304	0.0227	**1.339**
Aspartate	0.34	0.0062	0.7321	0.2373	**3.085**

* For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95%.

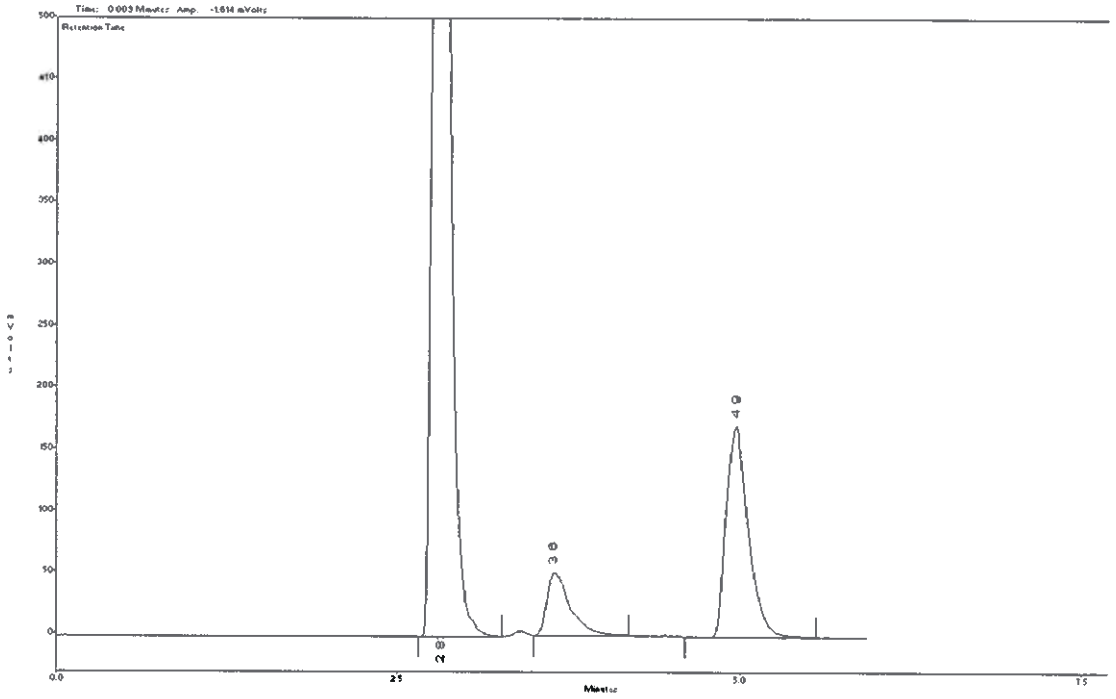

Fig. 4.3: Chromatogram of analysis of aspartate (Rt=2.8 min), potassium (Rt=3.6 min) and magnesium (Rt=4.9 min) in Cardilan®, analytical column Waters ODS-2, mobile phase aqueous TFA 0.02% (v/v).

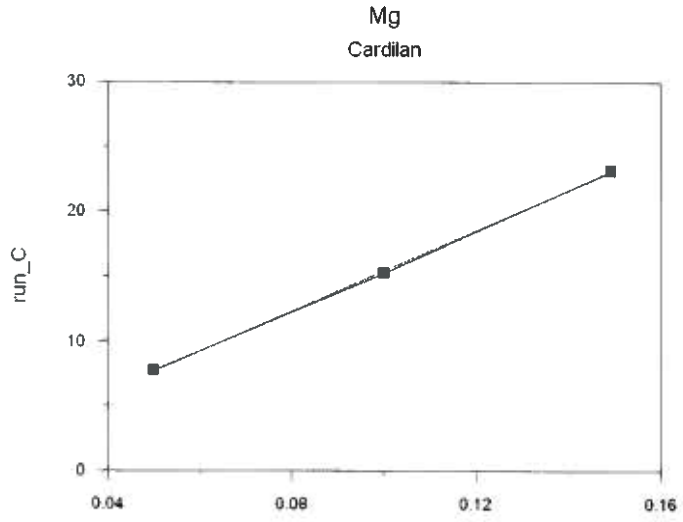

Fig. 4.4: Dependence of run concentration on dilution factor, magnesium in Cardilan®, separation conditions see Fig. 4.3.

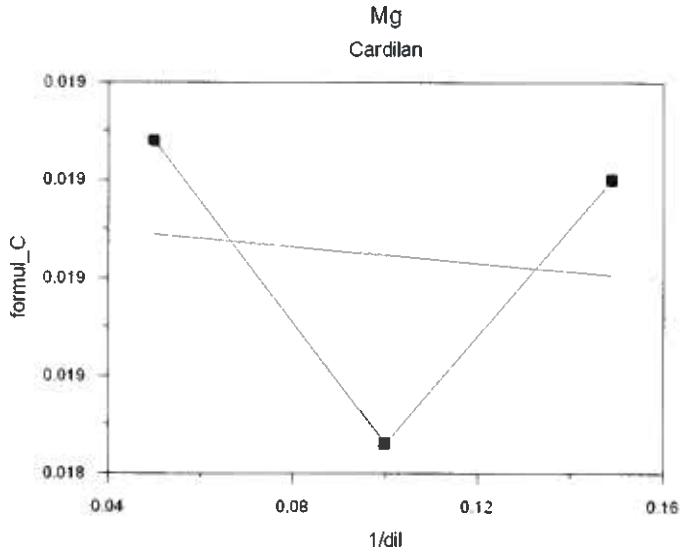

Fig. 4.5: Dependence of concentration of formulation on dilution factor, magnesium in Cardilan®, separation conditions see Fig. 4.3.

4.3.2. ***Magnesii Lactici 0.5 tbl.*** ®

The formulation was analysed according to the new HPLC-ELSD method. The results obtained from the assay of magnesium revealed conformance to the common European Pharmacopoeia for content within the range of 95.0 – 105.0 % ($r > 0.95$) of the labeled content. Individual values are presented in Table 4.16. There is no peak of lactate appearing at the chromatograms. Lactate anions are therefore supposed to volatilize at the temperature of 70 °C and cannot be analyzed by ELS detector. A typical chromatogram of the analysis is depicted on Fig. 4.6.

Table 4.16: Comparison of the HPLC-ELSD results of the assay of Magnesii lactici 0.5 tbl® tablets to the label content.

Analyte (formulation content)	Run Concentration ($\mu g\ ml^{-1}$)	Tablet Content mg	%Percentage of label concentration	n
Magnesium	10.40	50.64	**99.28**	3
(51 mg per	15.59	49.02	**96.11**	3
tablet)	20.79	49.02	**96.11**	3

The accuracy of the new HPLC-ELSD method was evaluated by recovery experiments. Samples were fortified by adding known amounts of standard of magnesium. Five spiked samples were prepared. The good accuracy of the proposed method was confirmed since the individual recovery values are within the range of 95.0 – 105.0 % ($r > 0.95$) (except of one value) (Table 4.17).

Table 4.17: Spiked samples of Magnesii lactici 0.5 tbl®.

	Formulation Concentration μg ml^{-1}	Spiked Concentration μg ml^{-1}	Recovery (%)	Mean Recovery (%)	N
Magnesium	5.2	4.0	**99.87**	**98.35**	3
	5.2	12.0	**98.35**		3
	5.2	16.0	**100.12**		3
	13.0	4.0	**93.65**		3
	18.2	4.0	**99.78**		3

Further study of the matrix effect on the determination was carried out by dilution experiments (determination of magnesium content using a varying dilution factor D (Vinitial/Vfinal) at three different levels). The correlation curve of the concentration found (in the diluted solution) versus D was linear ($r > 0.95$) with a slope equal to the content of the formulation and a statistically (proven by t-test) zero intercept. Similarly, the correlation curve of formulation content found versus D was very linear with statistically (proven by t-test) zero slopes. These results confirmed the absence of any constant or proportional determinate error due to matrix (excipients) effect (Tables 4.18, 4.19, 4.20; Fig. 4.7, 4.8).

Table 4.18: 1/dilution, run concentration and concentration of formulation of magnesium in Magnesii lactici 0.5 tbl.®.

	1/dilution	Run concentration (μg ml^{-1})	Concentration of formulation (μg ml^{-1})
Magnesium	0.04	10.32	64603.64
	0.06	14.99	62538.76
	0.08	19.98	62539.65

Table 4.19: Dependence of run conconcentration on dilution factor of Magnesii lactici 0.5 tbl. ®.

	Parameter a	Standard deviation a	t_a^*	Parameter b	Standard deviation b
Magnesium	0.60	0.28	**2.112**	241.50	4.61

* For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95% .

Table 4.20: Dependence of concentration of formulation on dilution factor of Magnesii lactici 0.5 tbl. ®.

	Parameter a	Standard deviation a	Parameter b	Standard deviation b	t_b^*
Magnesium	66323.33	1854.08	51599.75	29816.82	**1.730**

*For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95% .

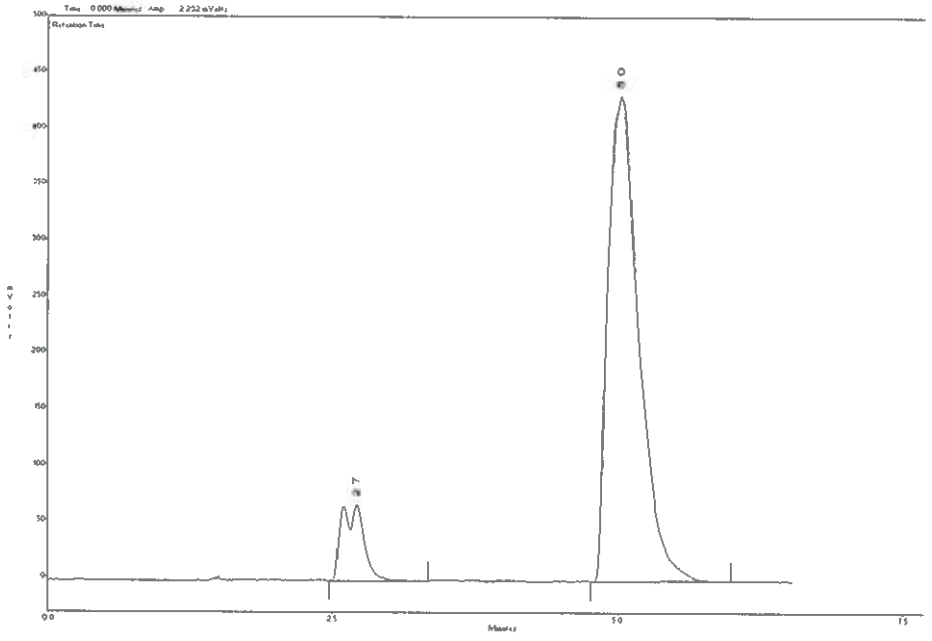

Fig. 4.6: Chromatogram of analysis of magnesium (Rt=5.0 min) in Magnesii lactici 0.5 tbl. ®, analytical column Waters ODS-2, mobile phase aqueous TFA 0.02% (v/v).

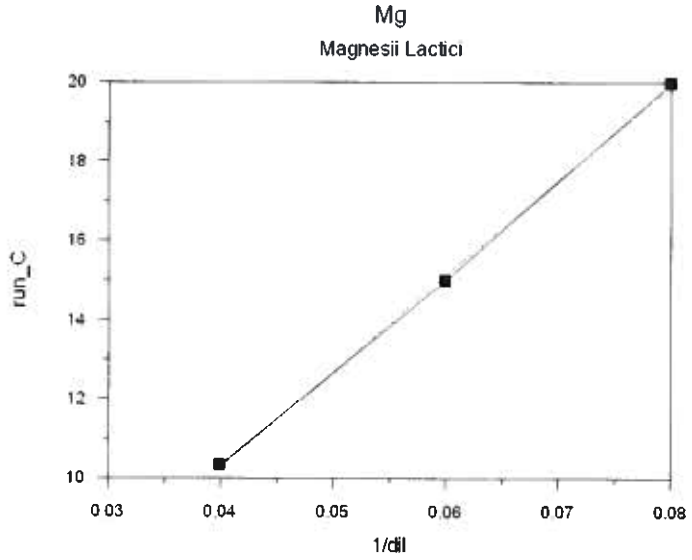

Fig. 4.7: Dependence of run conconcentration on dilution factor, magnesium in Magnesii lactici 0.5 tbl. ®, separation conditions see Fig. 4.6.

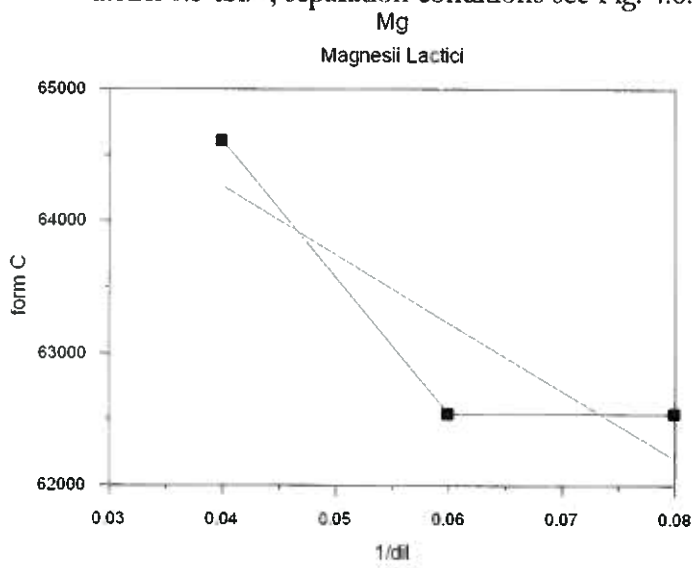

Fig. 4.8: Dependence of concentration of formulation on dilution factor, magnesium in Magnesii lactici 0.5 tbl. ®, separation conditions see Fig. 4.6.

4.3.3. ***Magnesium 250 mg®***

Magnesium in the formulation was analysed according to the new HPLC-ELSD method. Ascorbate in the formulation was stabilised by natrium bisulphite and analysed according to the new HPLC-ELSD method using gradient elution. The results obtained from the assay of magnesium and ascorbate revealed conformance to the common European Pharmacopoeia for content within the range of 95.0 – 105.0 % of the labeled content (r >

0.95). Individual values are presented in Tables 4.21. Typical chromatograms of the analyses are depicted on Fig. 4.9, 4.10.

Table 4.21: Comparison of the HPLC-ELSD results of the assay of Magnesium 250 mg® tablets to the label content.

Analyte (formulation content)	Run Concentration ($\mu g\ ml^{-1}$)	Tablet Content (mg)	%Percentage of label concentration	n
Magnesium (250 mg per tablet)	10.93	251.05	**104.42**	3
	16.40	254.45	**101.78**	3
	21.86	253.98	**101.59**	3
Ascorbic acid (150 mg per tablet)	65.00	149.96	**100.32**	4
	86.66	146.00	**97.37**	2

The accuracy of the new HPLC-ELSD methods was evaluated by recovery experiments. Samples were fortified by adding known amounts of standards of magnesium and ascorbic acid. Five spiked samples for magnesium and two spiked samples for ascorbate were prepared. The good accuracy of the proposed method was confirmed since the individual recovery values are within the range of 95.0 – 105.0 % ($r > 0.95$) (Table 4.22).

Table 4.22: Spiked samples of Magnesium 250 mg®.

	Formulation Concentration $\mu g\ ml^{-1}$	Spiked Concentration $\mu g\ ml^{-1}$	Recovery (%)	Mean Recovery (%)	n
Magnesium	7.2	4.0	**96.85**	**101.55**	3
	7.2	7.0	**100.18**		3
	7.2	10.0	**102.97**		3
	12.6	4.0	**103.15**		3
	18.0	4.0	**104.58**		3
Ascorbic acid	21.7	50.7	**100.61**	**99.78**	2
	21.7	101.4	**98.94**		2

Further study of the matrix effect on the determination was carried out by dilution experiments (determination of magnesium or ascorbate content using a varying dilution factor D (Vinitial/Vfinal) at three different levels). The correlation curve of the concentration found (in the diluted solution) versus D was linear ($r > 0.95$) with a slope equal to the

content of the formulation and a statistically (proven by t-test) zero intercept. Similarly, the correlation curve of formulation content found versus D was very linear with statistically (proven by t-test) zero slopes. These results confirmed the absence of any constant or proportional determinate error due to matrix (excipients) effect (Tables 4.23, 4.24, 4.25; Fig. 4.11, 4.12).

Table 4.23: 1/dilution, run concentration and concentration of formulation of magnesium and ascorbate in Magnesium 250 mg®.

	1/dilution	Run concentration ($\mu g\ ml^{-1}$)	Concentration of formulation ($\mu g\ ml^{-1}$)
Magnesium	0.030	11.42	75337.08
	0.045	16.69	73427.01
	0.060	22.21	73292.47
Ascorbate	0.30	65.20	43425.21
	0.40	84.39	42150.84

Table 4.24: Dependence of run concentration on dilution factor of Magnesium 250 mg®.

	Parameter a	**Standard deviation a**	**t_a**	**Parameter b**	**Standard deviation b**
Magnesium	0.58	0.22	**2.622***	359.66	4.81
Ascorbate	18.01	16.70***	**1.078****	256.00	-

*For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95%.
** For a 2-point curve, the limit of t-test value is 12.706 with the confidence 95%.
***Standard deviation of the peak area of the most diluted sample was used instead of standard deviation of the intercept.

Table 4.25: Dependence of concentration of formulation on dilution factor of Magnesium 250 mg®.

	Parameter a	**Standard deviation a**	**Parameter b**	**Standard deviation b**	**t_b**
Magnesium	77085.76	1593.58	68153.66	34170.09	**1.994***
	C_1	**SD_1**	**C_2**	**SD_2**	
Ascorbate	149.96	15.22	146.00	5.33	**0.347****

*For a 3-point curve, the limit of t-test value is 4.303 with the confidence 95%.
**For a 2-point curve, the limit of t-test value is 12.706 with the confidence 95%; t-test was calculated according to the equation $t = (C_1 - C_2)\sqrt{2} / \left(\sqrt{SD_1^2 + SD_2^2}\right)$ where C1 and C2 are the mean formulation contents of the two different dilution levels, SD are the corresponding standard deviation of each content.

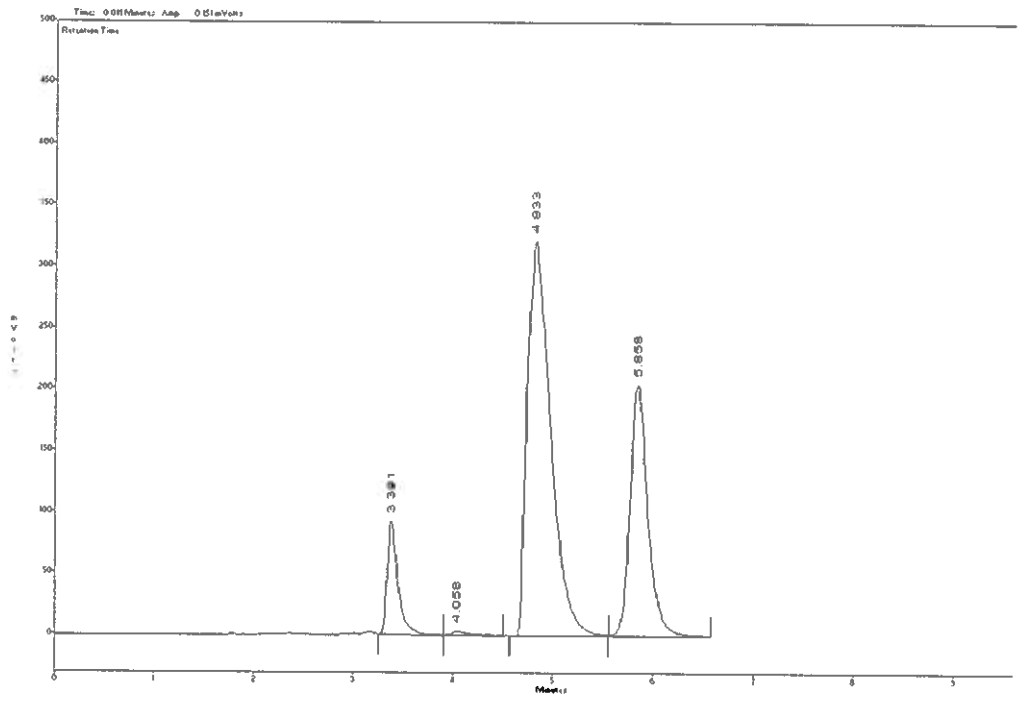

Fig. 4.9: Chromatogram of analysis of magnesium (Rt=4.9 min) in Magnesium 250 mg®, analytical column Waters ODS-2, mobile phase aqueous TFA 0.02% (v/v).

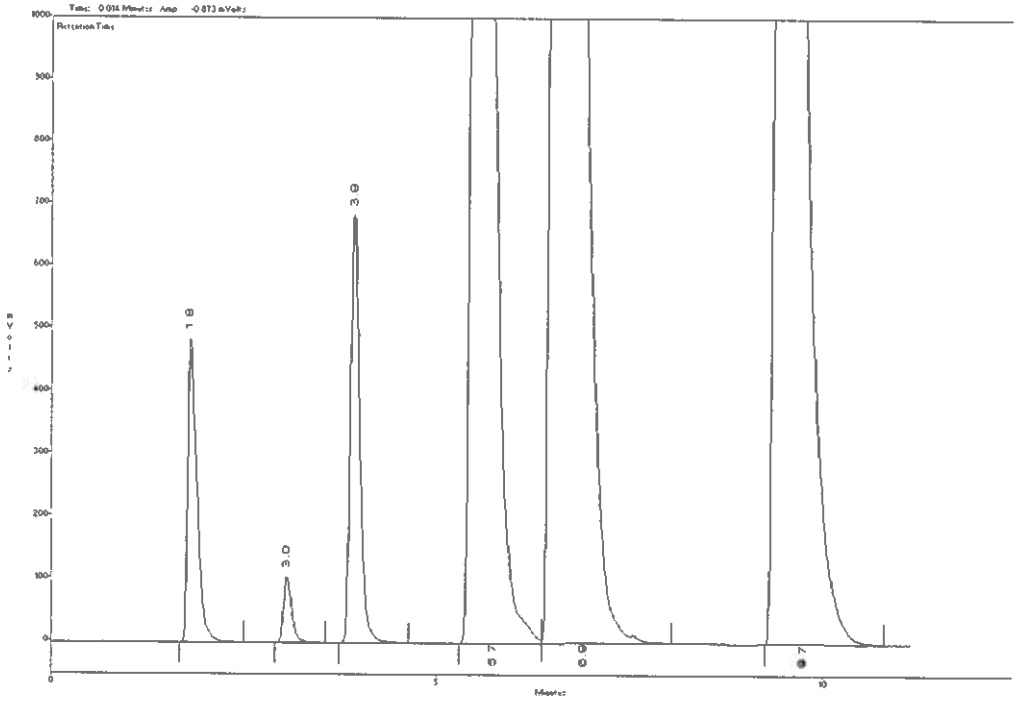

Fig. 4.10: Chromatogram of analysis of ascorbic acid (Rt=3.9 min) in Magnesium 250 mg®, analytical column Waters ODS-2, gradient elution: from 0 min to 2.5 min aqueous

NFPA 0.5 ml l^{-1}; from 2.5 min to 3.5 min linear gradient to aqueous TFA 1.0 ml l^{-1} up to 11.5 min.

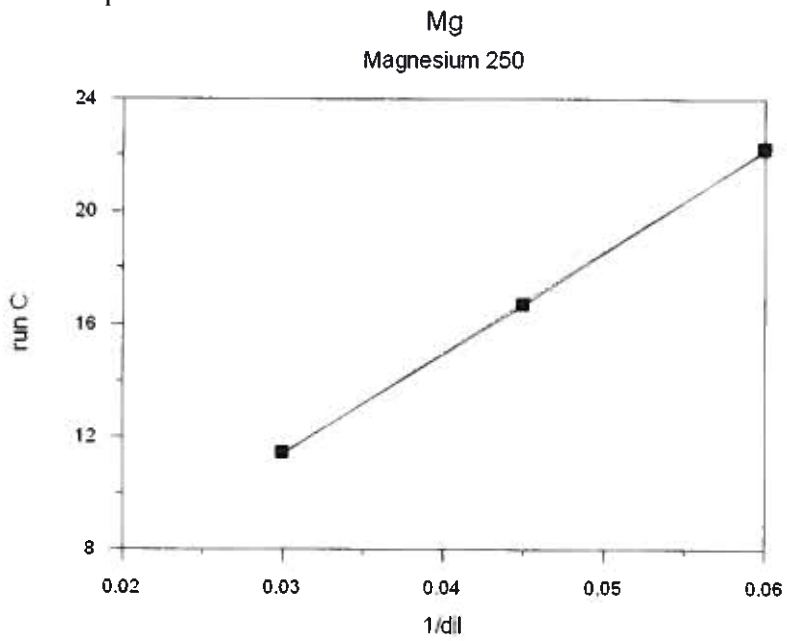

Fig. 4.11: Dependence of run concentration on dilution factor, magnesium in Magnesium 250 mg®, separation conditions see Fig. 4.9.

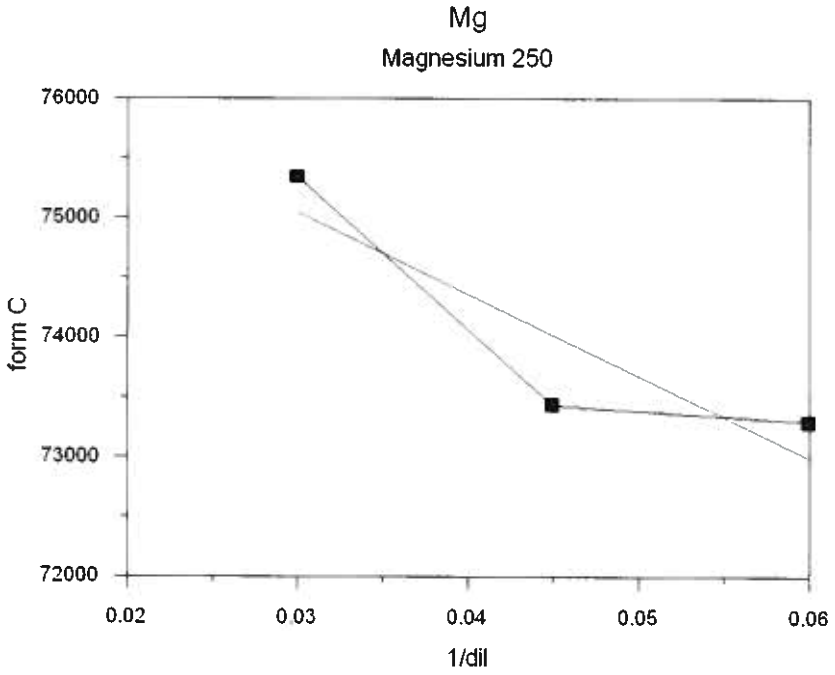

Fig. 4.12: Dependence of concentration of formulation on dilution factor, magnesium in Magnesium 250 mg®, separation conditions see Fig. 4.9.

5. CONCLUSIONS

- Metal cations in form of organic salts are commonly contained in pharmaceutical formulations. According to Pharmacopoeia [3], two or more individual methods are required for determination of these two components in one formulation. No method simultaneously determining both metal cation and organic anion has been published. Reversed phase HPLC-ELSD isocratic and linear gradient methods developed and validated in this paper show, that simultaneous determination of magnesium, potassium and aspartate (isocratic method) or magnesium and ascorbate (linear gradient) can be reliably achieved.

- The proposed HPLC-ELSD methods are able to determine several analytes in one step, simply, rapidly and for low cost. The pre-treatment of the samples is very easy, requiring no derivatization of analyte as it is common for spectrometric methods. Direct determination saves the time and lowers the expenses for analyses.

- Validation data of the HPLC-ELSD methods are fully acceptable for analysis of active substance in pharmaceutical formulations. Validation data include: resolution of two neighboring peaks (Table 4.6); asymmetry of peaks (Table 4.6); linearity and range of five-point calibration curves (Table 4.7); precision - repeatability (Table 4.8) and reproducibility (Table 4.9); detection and quantification limits (Table 4.10).

- Validated methods were applied on three pharmaceutical formulations. Magnesium, potassium and aspartate in Cardilan®, magnesium in Magnesii lactici 0.5 tbl.® and magnesium in Magnesium 250 mg® were determined by isocratic method. Ascorbate in Magnesium 250 mg® was determined by linear gradient method. For ascorbate, two dilutions of the sample were measured; for all the other analytes, three dilutions were measured. The results obtained from the assays revealed conformance to the European Pharmacopoeia for content within the range of 95.0 – 105.0 % of the labeled content ($r > 0.95$) (Tables 4.11, 4.16, 4.21).

- The accuracy of the new HPLC-ELSD method was evaluated by recovery experiments. Samples were fortified by adding known amounts of standards. For ascorbate, two spiked samples were prepared; for all of the other components, five spiked samples were prepared. The good accuracy of the proposed methods was confirmed since most of the individual recovery values were within the range of 95.0 – 105.0 % ($r > 0.95$) (Tables 4.12, 4.17, 4.22).

- A study of the matrix effect on the determination was carried out by dilution experiments at three different levels (two for ascorbate). The correlation curve of the concentration found (in the diluted solution) versus dilution factor (D) was linear ($r > 0.95$) with a slope equal to the content of the formulation and a statistically (proven by t-test) zero intercept (Tables 4.14, 4.19, 4.24). Similarly, the correlation curve of formulation content found versus D was very linear with statistically (proven by t-test) zero slopes (Tables 4.15, 4.20, 4.25). These results confirmed the absence of any constant or proportional determinate error due to matrix (excipients) effect.

- The main advantage of the proposed HPLC-ELSD methods is the rapidness, easy pretreatment of the samples, good precision and accuracy and low costs of the instrumentation.

6. SOUHRN

Hořčík a draslík hrají esenciální roli v existenci živých organismů. Hořčík, čtvrtý nejčastější minerál v lidském těle, se účastní kolem 300 základních enzymatických reakcí, podílí se výrazně na transkripci DNA a na proteosyntéze, je důležitý pro energetický metabolismus [8]. Draslík je v lidském těle hlavním intracelulárním kationtem. Má zásadní význam pro elektrické děje na buněčných membránách, zejména nervů a svalů včetně srdce [9, 10]. Rovnováha těchto minerálů v těle je důležitou podmínkou potřebnou k udržení zdraví. Jejich nedostatek nebo naopak nadbytek v organismu je spojen s řadou vážných syndromů a onemocnění. Hořčík a draslík jsou využívány ve formě různých solí, oxidů, hydroxidů nebo komplexů při výrobě mnoha léčivých přípravků nebo potravinových doplňků. Často se tyto kovy vyskytují ve formě solí s organickými anionty, např. jako acetát, aspartát, citrát, glukonát, pidolát, salicylát, stearát, etc. [1, 3].

Askorbová kyselina je ve vodě rozpustný vitamin, který je důležitý pro správnou funkci a stavbu pojivové tkáně a cévní stěny, činnost enzymů a metabolismus některých látek (např. cholesterolu). Podílí se na redukci železa při jeho resorpci. Uvádí se také jeho podíl na zvýšení celkové odolnosti organismu a antioxidační působení [14]. Kyselina askorbová je součástí mnoha léčiv a potravinových doplňků, buď samotná nebo se také často vyskytuje v kombinaci s některými minerály jako je např. hořčík, železo, selen nebo zinek. Asparagová kyselina je neesenciální aminokyselina, účastnící se např. metabolismu močoviny. Její sůl se nazývá aspartát, podílí se rovněž na nervovém přenosu [15].

Lékopis [3] používá ke stanovení hořčíku chelatometrickou titraci edetanem disodným v alkalickém pufru; další popsané metody jsou plamenová atomová absorpční spektrometrie [7], sekvenční injekční analýza [17] a v současné době multikomutativní průtokový systém pro multielementární analýzu [29]. Oficinální metoda pro stanovení draslíku je atomová emisní spektrometrie [3]; mezi další metody patří FIA s potenciometrickou detekcí [27] a kapilární elektroforéza s konduktometrickou detekcí [4]. Askorbovou kyselinu lékopis stanovuje přímou iodometrickou titrací v prostředí kyseliny

sírové a s přídavkem roztoku škrobu [3]; další publikované metody jsou kapalinová chromatografie s elektrochemickou detekcí [32]; průtoková injekční analýza se spektrofotometrickou [33, 34, 35], fluorimetrickou [36] a amperometrickou detekcí [37]; potenciometrické stanovení [38]; iodometrická potenciometrická titrace [39]; spektrofotometrické stanovení [41, 42, 43] nebo kapilární zónová elektroforéza [46]. Oficinální metoda pro stanovení asparagové kyseliny je alkalimetrická titrace hydroxidem sodným [3].

Kovové kationty se často vyskytují ve léčivých přípravcích společně s organickými anionty. Přesto nebyla publikovaná žádná metoda stanovující obě komponenty současně. Každý z iontů by bylo potřeba kvantifikovat odděleně. Cílem této práce bylo vyvinout a validovat metodu stanovující v jednom nástřiku anorganický kation i organický anion a tuto metodu aplikovat na léčivé přípravky.

Metoda je založena na spojení vysokoúčinné kapalinové chromatografie s ELSD (Evaporative Light Scattering Detection). ELS detektor je polouniverzální detektor, který v současné době nachází stále širší uplatnění, a to především u analytů, které nemají ve své molekule chromoforové skupiny a nemohou být tudíž bez derivatizace detekovány spektrofotometricky. ELSD je převážně považován za detektor kapalinové chromatografie, nicméně lze ho s úspěchem použít i při protiproudové (CCC) a superkritické fluidní chromatografii (SFC). Oproti ostatním univerzálním detektorům, jako je např. refraktometrický (RID) nebo hmotnostní (MS), ELSD vykazuje určité přednosti: a) kompatibilita s gradientovou eluci (na rozdíl od RID); b) podstatně lepší citlivost ve srovnání s RID (běžný limit detekce se pohybuje v nanogramových množstvích, v závislosti na těkavosti a molekulové hmotnosti); c) nízké náklady a snadná obsluha (na rozdíl od hmotnostního spektrometru). Nicméně je třeba uvést také určité nevýhody ELS detektoru. Tou hlavní je požadavek na těkavost mobilní fáze. Nesmí být použity netěkavé reagenty, pufry ani jiné složky mobilní fáze. Výběr vhodných kyselin a bazí se tím značně omezuje; mezi často používané patří kyselina octová, mravenčí, trifluoroctová, pentafluoropropionová a heptafluoromáselná a jejich amonné soli v nízkých koncentracích (<0.1M). ELSD je destruktivní detektor, proto musí být poslední v řadě, pokud je použit v sérii s jinými detektory. Vykazuje také nedostatečnou citlivost pro analýzu např. nečistot a reziduí, které se vyskytují v množstvích ng ml^{-1} (LOQ je obvykle vyšší než 0,1 μg ml^{-1}). Tyto vzorky je

většinou třeba upravit prekoncentrací, což bývá obtížné, neboť není možné použít netěkavé reagencie [4].

Základní princip detektoru sestává ze tří následných kroků: a) nebulizace chomatografického eluentu; b) vypaření mobilní fáze a c) detekce netěkavých částic měřením rozptylu světla (Fig.2.1). V prvním kroku eluent z kolony vstupuje do nebulizéru Venturiho typu, kde proudem nosného plynu vzniká velké množství kapiček téměř stejné velikosti. Tento aerosol je v druhém kroku unášen do vyhřívané trubice, kde dojde k vypaření těkavé mobilní fáze a zůstanou oddělené částice netěkavého analytu. V následující fázi tyto částice vstupují do optické cely a prochází světelným paprskem. Rozptyl světla je měřen fotonásobičem nebo fotodiodou [4].

Pro separaci byla použita analytická reverzní kolona C_{18} Waters ODS-2.

Pro separaci hořčíku, draslíku a aspartátu v přípravku Cardilan® se nejlépe osvědčila izokratická eluce s vodnou mobilní fází obsahující 0.02% trifluoroctové kyseliny (v/v) (Table 4.1, Fig 4.1). Tato mobilní fáze byla též použita pro analýzu přípravku Magnesii lactici 0.5 tbl. ®. Laktátový aniont zde nebylo možné stanovit, neboť při použité vysoké teplotě v detektoru vytěkal. Pro separaci askorbátu a hořčíku v přípravku Magnesium 250 mg® bylo třeba použít gradientovou eluci. Nejvhodnější složení mobilní fáze bylo zjištěno od 0 min do 2.5 min vodná nonafluoropentanová kyselina 0.5 ml l^{-1}; od 2.5 min do 3.5 min lineární změna gradientu na vodnou TFA 1.0 ml l^{-1} až do 11.5 min (Tables 4.4, 4.5, Fig 4.2). Snaha o separaci hořečnatých kationtů od vápenatých, zinečnatých a hlinitých skončila neúspěšně (Tables 4.2, 4.3). Pro tuto separaci by bylo třeba použít jiný typ kolony, například iontovýměnnou.

Bylo třeba stabilizovat askorbovou kyselinu v přípravku Magnesium 250 mg®. Nejlepších výsledků bylo dosaženo s hydrogensiřičitanem sodným přidaným v pětinásobném nadbytku. Askorbová kyselina zůstala po této úpravě stabilní v roztoku po dobu jedné hodiny.

Pro obě metody byly určeny validační parametry: rozlišení dvou sousedících píků (Table 4.6); asymetrie píků (Table 4.6); linearita a rozsah pětibodových kalibračních křivek (Table 4.7); přesnost z hlediska opakovatelnosti (Table 4.8) a reprodukovatelnosti (Table 4.9); limity detekce a kvantifikace (Table 4.10).

Značnou výhodou HPLC-ELSD metod je velice snadná úprava vzorku. Na rozdíl od spektrálních metod není zde potřeba provádět derivatizaci ani jiné často složité a zdlouhavé postupy. Přímé stanovení šetří čas a snižuje náklady na analýzu.

Validované metody byly použity k analýze tří léčivých přípravků registrovaných v České republice. Jednalo se o: Cardilan® (účinná látka hydrogenaspartát hořečnatý hemihydrát 175 mg v 1 tabletě, hydrogenaspartát draselný hemihydrát 175 mg v 1 tabletě); Magnesii lactici 0.5 tbl.® (účinná látka laktát hořečnatý dihydrát 500 mg v 1 tabletě); Magnesium 250 mg® (účinná látka oxid hořečnatý 420 mg v 1 tabletě, kyselina askorbová 150 mg v 1 tabletě). Hořčík, draslík a aspartát v přípravku Cardilan®, hořčík v Magnesii lactici 0.5 tbl.® a hořčík v Magnesium 250 mg® byly stanoveny izokratickou metodou. Askorbát v přípravku Magnesium 250 mg® byl stanoven metodou lineárního gradientu. Askorbát byl měřen ve dvou zředěních po dvou replikacích, všechny ostatní analyty ve třech zředěních po třech replikacích. Zjištěný obsah se pohyboval v rozmezí 95.0 – 105.0 % obsahu udávaného výrobcem ($r > 0.95$) (Tables 4.11, 4.16, 4.21). Dalším předmětem analýzy byla recovery - měření pěti vzorků s přídavkem známého množství standardu příslušného kationtu. Stanovení proběhlo většinou ve třech replikacích a opět vyhovovala většina vzorků (Tables 4.12, 4.17, 4.22). Z naměřených údajů byla pomocí t-testu hodnocena významnost odchylky u parametru *a* od nulové hodnoty pro závislost koncentrace vzorku na obrácené hodnotě jeho zředění (Tables 4.14, 4.19, 4.24) a významnost odchylky u parametru *b* od nulové hodnoty pro závislost koncentrace formulace na obrácené hodnotě jeho zředění (Tables 4.15, 4.20, 4.25). Všechny hodnoty kromě jedné byly menší než 4,303; což je maximální hodnota předepsaná pro tři zředění zajišťující konfidenční interval 95%. Pro askorbát měřený pouze ve dvou zředěních byly hodnoty t-testu nižší než limitní hodnota 12.706.

Hlavní výhodou uvedených HPLC-ELSD metod je rychlost, snadná úprava vzorků, značná přesnost, dostatečná citlivost a nízké náklady na přístrojové vybavení.

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ERRATA

Str. 17 kap. 2.3.1 …depicted in Fig. 2.1.
Str. 24 kap. 3.1.3 …dissolved by mobile phase
Str. 60 citace [22] Int. J. M. Spectrom.