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Determination and quantification of clonidine in human blood serum

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Abstract

Clonidine ((2-[2,6-dichlorophenyl]amino)-2-imidazoline) preferentially stimulates central α_2-adrenoceptors, which leads to inhibition of sympathetic tone, resulting in a lowering of arterial pressure and of heart rate. Additionally, many other desirable and undesirable effects are described, including analgesia, sedation and withdrawal reactions, which consist of a sudden rise in arterial pressure, nervousness, agitation and increased heart rate.

The present study has the goal to develop a simple and effective method for the analysis of trace amounts of clonidine in human blood serum. Special emphasis is necessary to make application of electron impact ionization and separation of the analyte fragments in a quadruple mass analyzer suitable. The procedure comprises solid phase extraction followed by formation of the pentafluorobenzyl derivative. Further purification is achieved by phase transfer extraction into an acidic aqueous solution succeeded by re-extraction into dichloromethane. After solvent exchange, an aliquot is injected into the gas chromatograph equipped with a DB5 MS capillary column and a mass spectrometric detector. Chromatograms are recorded in single ion monitoring mode. Quantification is accomplished by internal standardization with moxonidine [4-chloro-5-(2-imidazolin-2-yl-amino)-6-methoxy-2-methylpyridine].

Keywords: Clonidine; Moxonidine; Pentafluorobenzylbromide; Gas chromatopgraphy; Derivatization

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1. Introduction

Clonidine ((2-[2,6-dichlorophenyl]amino)-2-imidazoline) is a potent drug, which is used since more than three decades in the treatment of arterial hypertension [1]. The interaction of clonidine with a group of central α-adrenergetic receptors is supposed to be the reason for lowering blood pressure [2]. The active agent is administered orally in tablets or in infusions as its hydrochloride, which is traded under the brand name "Catapresan", in a dose of 50 to 200 μg/day. Due to side effects, which are attributed to the oral uptake, transdermal therapeutic systems were developed [3]. Clonidine was also used for therapy of glaucoma, soothing of withdrawal syndromes of morphine addicts and as a prophylaxis against migraine. Within the last 10 years, clonidine moved again into the interest of medical research, due to the discovery of its strong analgesic property [4]. Meanwhile, it is investigated towards its analgesic, respectively anxiolytic, effects in premedication of various surgeries [5,6]. A promising application is the local administration at the place of operation. The advantage of this form of medication is that the cardiovascular system is a priori not strained by the active agent. Therefore, undesirable side effects should be reduced. Presently, the analgesic effect of Catapresan at intra-articular dosage after athroscopic knee operations is investigated. The transition rate of clonidine of the knee joint into blood is supposed to be very low. Even at oral or intravenous administration of the mentioned dose, analyte concentration is in the range of nanogram to picogram per millilitre plasma [7]. For that reason, sensitive methodologies are required for the determination of the drug in human body fluids.

A variety of methods has been reported including radio immunoassay (RIA) [8], high performance liquid chromatography [9] and gas chromatography [7,11–14]. The deficiency of the liquid chromatography/UV-detection procedures is the lack of sensitivity. Limits of detection were in the microgram per millilitre range [10]. Gas chromatography showed to be more effective, but sample preparation is more laborious. Generally, separation of the analyte from the complex matrix and enrichment is inevitable. This is preferably done by solid phase extraction on octadecyl-cartridges [11]. Solvent extraction of alkaline plasma samples is described too [12]. In order to enhance gas chromatographic properties of the target compound, the secondary amino groups of the imidazoline function are derivatized to tertiary amines or amides [7,13]. Further purification is necessary to remove excess reagent. Concentrated samples are injected into the gas chromatograph either by on-column or splitless injection. Analyte detection is accomplished mainly by mass spectrometry. The most dominant ionization method for this analysis purpose is negative chemical ionization. Recording the chromatograms in selected ion monitoring mode gives high selectivity and sensitivity [11]. Electron capture detection was also used for the measurement of perfluoro-derivatives of clonidine [14]. Quantification was generally accomplished by the method of internal standardization. Deuterated clonidine-D_4 molecules as well as other imidazolines with similar structure were used as internal standards.

The task of the present work was to develop a method for the measurement of low levels of clonidine in human blood serum, to control the concentration of clonidine in serum after a single intravenous dosage, which was done at half of the test persons, respectively to elucidate the transference rate from intra-articular administered drug into

the cardiovascular system of the other part of patients. Special regard was set to widespread applicability, which implied analysis with standard instrumentation. The developed method is critically discussed and compared with previously published sample treatments. Limits of detection and quantification are evaluated by means of statistical methods.

2. Materials and methods

2.1. Chemicals and reagents

Clonidine was purchased as its hydrochloride from Fluka (Buchs, Switzerland), the quality was better than 98%, while moxonidine [4-chloro-5-(2-imidazolin-2-yl-amino)-6-methoxy-2-methylpyrimidine], which was used as internal standard, was thankfully supplied by Eli Lilly Company (Indianapolis, IN, USA). Water was prepared with a Barnstead Nanopure Ultrapure Water System from International PBI (Milan, Italy) and IKA distillation unit (Janke&Kunkel, IKA Labortechnik, Staufen, Germany). Methanol picograde, acetone picograde and dichloromethane (for HPLC) were obtained from Promochem (Wesel, Germany). Potassium dihydrogenphospate (pro analysis) and potassium hydroxide (pro analysis) as well as absolute ethanol (pro analysis) were delivered from Merck (Darmstadt, Germany). Dimethyldichlorosilane with purity better than 98% and di-sodiumhydrogenphosphate (free of water) were purchased from Fluka. The derivatization reagent pentafluorobenzylbromide was obtained from Sigma-Aldrich (Vienna, Austria). Toluene was ordered in the quality Ultra-Resi-analyzed at Baker (Phillipsburg, NJ, USA).

2.2. Preparation of standards and reagents

For calibration purposes, the method of internal standardization has been applied. Standards were prepared by appropriate dilution of concentrated stock solutions of clonidine hydrochloride (523 μg/ml) and moxonidine (506 μg/ml). The active agent was dissolved in 10 ml of a mixture of acetone and 5% (v/v) triethylamine, while pure acetone was used for dissolution of moxonidine. Standards were prepared in distilled water as well as in pooled blank serum. Analyte concentrations between 44.9 and 371 pg clonidine and 5.06 ng moxonidine/ml were used for calibration purposes. The pH-value of the standard solutions was adjusted to about 12 by addition of potassium hydroxide solution.

Standard volumes up to 100 μl were handled by means of calibrated capillaries (Brand, Wertheim/Main, Germany), while the derivatization reagent and organic solvents were transferred with adjustable Transferpettors (Brand). Aqueous sample volumes were pipetted with fixed volume Transferpettes (Brand).

Dimethyldichlorosilane was used in a concentration of 5% (v/v) in toluene for deactivation of glassware.

The buffer solution (pH = 7) was prepared by weighing 1.76 g potassium dihydrogensulfate and 3.63 g of disodium hydrogenphosphate into a 100-ml volumetric flask, which was filled up to the ring mark with distilled water after dissolution of the salts.

2.3. Solid phase extraction

OASIS™ HLB extraction cartridges with an adsorbent quantity of 60 mg were purchased from Waters (Vienna, Austria) and were preconditioned prior to sample adsorption with 2 ml of methanol and 2 ml of alkaline, aqueous solution (pH = 12). One millilitre of a standard solution was pipetted onto the extraction cartridge, followed by rinsing of the cartridge with 2 ml of alkaline water. For the removal of excess water, the cartridges were centrifuged (Z510, Hermle, Germany) at 90 × *g* for 3 min and purged with dry air through the adsorbent bed for a period of 20 min. For this purpose, as well as for the loading of the cartridges, a Baker SPE 12G solid phase extraction unit was applied. Analyte elution was accomplished with 1.5 ml of methanol. The eluate was collected in a deactivated 4 ml glass vial, which was tightly sealed with a PTFE-butylrubber septum in a screw cap.

2.4. Derivatization

The imidazoline function needs to be derivatized in order to enhance volatility and chromatographic properties. This was achieved by the reaction of the target compounds with pentafluorobenzylbromide (Fig. 1). The reaction solution was prepared by blowing first the methanolic sample extracts to dryness in a gentle stream of nitrogen and reconstitution of the residue in 1 ml of toluene. Fifty microlitres of triethylamine were added to guarantee alkaline conditions. The derivatization reaction was initiated by addition of 200 μl of the pentafluorobenzylbromide solution (5% (v/v) in toluene) and kept at 65 °C overnight.

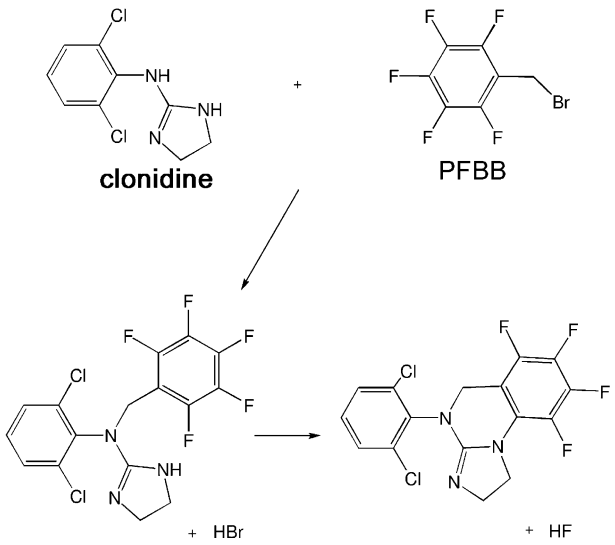

Fig. 1. Reaction of clonidine with pentafluorobenzylbromide.

2.5. Clean up

The derivatives were isolated by phase transfer extraction into 2 ml sulphuric acid (0.3 M). The organic phase was withdrawn and the aqueous solution was extracted again with a 1-ml aliquot of toluene. After removal of the toluene, the pH-value of the aqueous solution was adjusted by the addition of 300 μl of a 5-M potassium hydroxide solution to about 12. Additionally, 10 μl of *n*-butanol was added to prevent adsorption of the derivatives on the surface of the vial. The analytes were extracted as their free bases into 1 ml of dichloromethane and the aqueous phase was discarded. Excess KOH was removed from the dichloromethane phase by first extracting it with 2 ml of the pH 7 buffer solution, followed by extraction with 1 ml of distilled water. Finally, 30 μl of toluene were added and the volume of sample was reduced to about 100 μl and transferred into deactivated 100 μl micro inserts for 2 ml auto injector vials. Further reduction of the solvent volume (to about 30 μl) was achieved by a gentle stream of inert gas.

All extractions were performed for a period of 3 min by the use of a Heidolph Vortex vibratory mixer (Heidolph, Gobheim, Germany) and phase separations were accomplished by centrifugation of the samples for 2 min at $90 \times g$. Aliquots of 2 μl were injected for gas chromatographic analysis.

2.6. Instrumentation and conditions of analysis

A Hewlett Packard 6890 gas chromatograph was equipped with a HP 7683 automatic injector, a split/splitless injection port and connected to an Hewlett Packard 5973A mass selective detector (Hewlett Packard, Wilmington, DE, USA). The derivatives were separated on a DB 5 capillary column (J&W Scientific, Folson, CA, USA), 30-m length, 0.25 mm internal diameter and 0.25 μm film thickness. Target analytes were ionized by electron impact ionization at 70 eV and detected in single ion-monitoring mode. Together with the most characteristic fragment ions, one qualifier fragment was recorded for each compound simultaneously to check for potential interferences. This could be accomplished automatically during data analysis by calculation of the ratios of the abundance of the target ion signals to the qualifier ion signals. Target ions had a mass to charge ratio (m/z) of 354 for clonidine and 366 for moxonidine, while $m/z = 356$ in the case of clonidine, respectively $m/z = 401$ for moxonidine were recorded as qualifier ions. Data acquisition was carried out with a G1701BA Hewlett Packard MS ChemStation, version B.00.00. The injection port of the gas chromatograph was operated at 270 °C. A pulsed splitless injection with a pulse pressure of 100 kPa for 1.0 min was selected. The oven temperature program was chosen as follows: 70 °C for 1.0 min, 25 °C/min to 200 °C for 0 min, and 10 °C/min to 300 °C for 1 min.

3. Results and discussion

3.1. Reduction of adsorption phenomena

During the course of sample preparation, special care has to be taken to avoid adsorption of the analytes on raw glass surfaces. Therefore, all glassware was deactivated

by silylation with dichlorodimethylsilane as has been described elsewhere [15]. Nevertheless, some possibility of adsorption due to residual hydroxyl groups on the inner surface of the vials may remain. For the analysis of amines, further reduction of adsorption phenomena was attained by the addition of small amounts of an alcohol to the samples [15]. In the present study, *n*-butanol and triethylamine were added to the sample to displace the derivatives from the adsorption positions.

3.2. Differences compared to already published methods

The application of OASIS™ HLB-cartridges, which contain a special copolymer as adsorbent, allows to increase the pH-value above the pK_a value of clonidine, in order to shift equilibrium towards the free basis. Thus, the adverse effect of partial hydrolysis of the adsorbent, which could happen with the application of silica base C18-material, can be prevented.

In previously published methods, clonidine was analyzed as its di-trifluoromethylbenzoyl- or pentafluorobenzoyl-derivative [7,11,13]. In this work, pentafluorobenzylbromide (PFBB) was chosen as a reagent offering the advantage of a lower molecular weight of the resulting amines compared to those of the diamides, which are formed in reactions with the specified acyl bromides. The better volatility of the derivatives allows an analysis under more moderate conditions.

Instead of acetone, which has been proposed by Yamahata et al. [11], toluene was chosen as solvent for the derivatization step. The higher boiling point of toluene diminishes the risk of solvent losses during the reaction period. Potassium carbonate, which had been used to catch the hydrogen bromide and hydrogen fluoride [14], has the disadvantage of insolubility in the reaction mixture. Since the solution formed two phases and was not further agitated, improper removal of the reaction by-products could occur. For this reason, the inorganic salt was replaced by addition of the strong organic base triethylamine, which provides for a proper reaction environment due to a pK_a value of about two units higher than that of clonidine.

A further modification concerns the solvent used for re-extraction of the sample components for clean up purposes. In contrast to all of the previously published methods, which proposed solvents with a density lower than water, dichloromethane was used for this purpose in this work. This solvent permits for an ease of phase separation and the glass tubes need not be changed for discarding the aqueous layers. As a result, improved precision can be observed.

3.3. Statistical evaluation of the method

Aqueous standards were applied for method development. The established procedure was evaluated by measurement of spiked serum samples. Recovery from the serum samples was between 70% and 80%, which indicates strong matrix effects. Therefore, internal standardization is absolutely necessary. Blank values of the pooled serum, which was used for sample preparation, were found to be below the limit of detection. At least four replicate samples were measured for calibration purposes at nine concentration levels between 45.1 and 375.5 pg/ml serum. Due to the laborious sample preparation, calibration

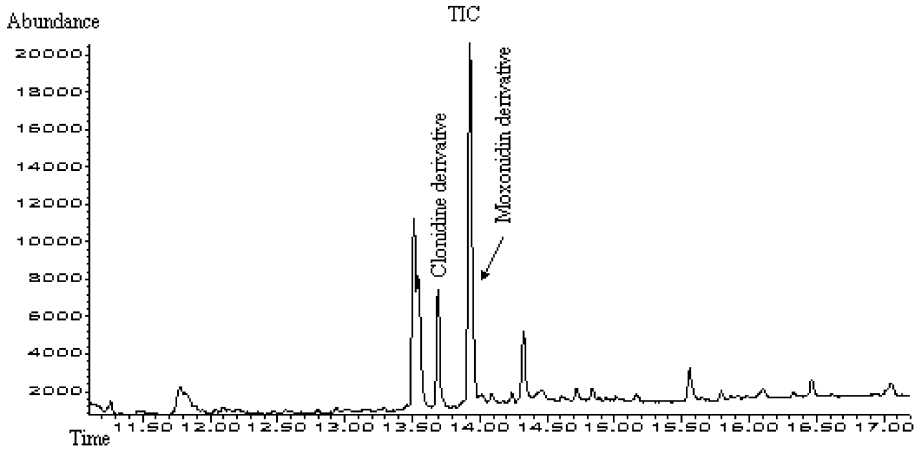

Fig. 2. Representative total ion chromatogram (TIC) of a spiked serum sample containing 228 pg/ml clonidine and 5.06 ng/ml moxonidine.

standards were prepared at three consecutive days. A representative total ion chromatogram of a sample containing 228 pg/ml of clonidine is depicted in Fig. 2. Integration was accomplished by an automatic routine. Additionally, all chromatograms were checked for proper integration settings.

As can bee seen easily, the applied clean up and measurement procedure has proved to be suitable for selective isolation of the derivatives. Analyte peaks are clearly separated from potential interference. For identification of the peaks eluting with retention times close to the analytes, measurements were performed by operating the instrument in full scan mode. The peak eluting in front of clonidine was identified as cholesterol. The peak, which is eluting immediately after clonidine, could not be reliably identified, due to its low intensity. Raw data of the spiked serum samples were used for the determination of the first order calibration graph and the limit of detection of the method. Calculation was performed according to Eurachem guide with the software "ValiData Excel-Makro zur

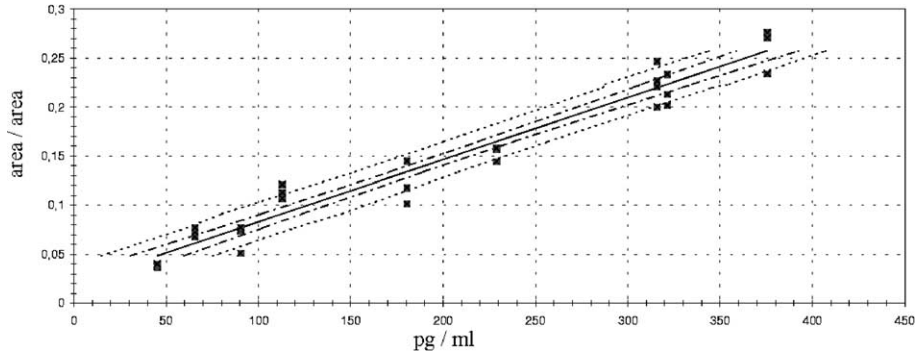

Fig. 3. First order calibration graph of the measurements of clonidine in human blood serum.

Methodenvalidierung, Wegscheider, Rohrer and Neuböck, Version 3.02.54 ger'' at the 95% confidence interval [16]. Fig. 3 shows the resulting calibration graph, including the limits of the 99% respectively 95% confidence region. The limit of detection for clonidine was determined with 26.3 pg/ml and limit of quantification with 87.5 pg/ml serum sample. The coefficient of variation of the method was calculated with all calibration data and found to be 13.8%. Thus, the method is suitable to detect even the low amounts of clonidine, which are expected in human blood serum after a single intravenous, respectively, intra-articular administration of 150 μg of the drug.

4. Concluding remarks

The goal of the present study was the development of a method for the determination of clonidine in human blood serum by application of electron impact ionization and separation of the analyte fragments in a quadruple mass analyzer. The procedure consists of a solid phase extraction on a polymeric adsorbent followed by formation of the pentafluorobenzyl derivatives, which are purified by phase transfer extraction. After solvent exchange, the toluene solution is injected into the gas chromatograph equipped with unipolar capillary column. Statistical evaluation of the applied sample preparation and analysis procedure has shown that the described method is suitable for this analytical task. Hence, this method will be applied for the evaluation of a clinical study, which concerns the pharmacokinetic properties of intra-articular administered clonidine.

Acknowledgements

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