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Determination of propiomazine in rat plasma by direct injection on coupled liquid chromatography columns with electrochemical detection

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Abstract

This method describes the determination of propiomazine by direct injection of rat plasma into a chromatography system based on coupled reversed-phase columns. An extraction column, packed with porous silica particles with covalent-bound α_1-acid glycoprotein (AGP), was used to separate the plasma proteins from the analyte. After isolation the analyte was transferred to the analytical column for separation and detection. Propiomazine was detected by an electrochemical detector and the limit of quantification was 2.0 ng/ml (100 pg injected). The absolute recovery was 80.9±2.4% at 9.0 ng/ml level. The inter-day and intra-day precision was 10.9% (5.6 ng/ml) and 2.8% (9.0 ng/ml), respectively. © 1997 Elsevier Science B.V.

Keywords: Coupled columns; Propiomazine

1. Introduction

Chromatographic analysis of drugs in biological samples requires sample preparation to isolate the drug from the matrix. Usually extraction procedures (solid-phase and liquid–liquid extraction) are used to isolate the drug from the proteins. Protein precipitation with acid, organic solvents or high salt concentration are other methods to separate the protein during preparation. These procedures are often time consuming and tedious. Therefore, a great deal of effort is used to automate these methods.

An optimal way to determine drugs in biological fluids is to inject the fluid without pre-treatment into the liquid chromatography system provided that the chromatographic efficiency and a high recovery of the analyte are maintained. Consequently, the analytes remain unaffected due to the reduction in sample handling steps which leads to an increase in accuracy and precision. During the recent years several packing materials for direct injection of biological samples have become available. Such packing materials are restricted access (RAM), or internal-surface reversed-phase materials (ISRP), and consist of a solid phase with a hydrophilic external surface and a hydrophobic internal phase. The separation principle of such columns is that the pore size of the external surface is small enough to exclude the proteins which are eluted within the internal volume of the column. In contrast, the analyte penetrates through the pores and interacts with the modified silica particles and is retained by hydrophobic interaction. An extraction pre-column based on porous silica particles with covalent bound α_1-acid

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glycoprotein (AGP) on the hydrophilic surface was developed by Hermansson and Grahn [1]. The small size of the column and the AGP tolerance towards high content of organic modifier makes it suitable for chromatographic analysis. In this work the authors demonstrated direct injection of plasma samples on the AGP-column for the analysis of five common drugs. The absolute recovery was in the range of 78–96% and the inter-day precision was below 5%. Recently this group has introduced a larger AGP-column with higher injection capacity of biological samples. Boos et al. [2] introduced a new family of chemical and enzymatic modified packing materials which also are used in pre-columns. On this alkyl diol silica (ADS) the total load of plasma could be over 100 ml. The restricted access materials have been used in different applications. Yu and Westerlund [3] showed large injection volumes during the analysis of 8-methoxypsoralen in plasma, Onnerfjord et al. [4] analyzed triazines in humic-containing water, and Oosterkamp et al. [5] separated free and receptor-bound coumestrol from the oestrogen receptor. The first column with this type of material was introduced by Hagestam and Pinkerton [6]. This column was of analytical size and the protein exclusion and the analyte separation was performed in the same column. The shielded hydrophobic phase (SHP) introduced by Gisch et al. [7] used the same principle. Direct injection of plasma was first described in the late 1970s by Popovitch [8]. Very small volumes, ≤ 3 μl, were injected onto standard analytical columns. The pre-column venting technique based on injection of plasma in an aqueous plug on the pre-column, introduced by Arvidsson et al. [9], stabilized the analytical system.

Propiomazine is a phenothiazine derivate with a sedative effect [10,11]. The substance has a low tendency to cause dependency and tolerance. Furthermore, a low incidence of side effects has been reported [12].

The present work describes the determination of propiomazine by direct injection of rat plasma into a coupled-column reversed-phase liquid chromatography system. The separation of the protein matrix from the analyte was performed using a Biotrap extraction column. The analyte was transferred to a cyano column where further separation and detection took place.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile was purchased from Lab-Scan (Dublin, Ireland), water was purified using the Milli-Q system (Millipore, Bedford, MA, USA), *o*-phosphoric acid cryst., p.a., was purchased from Merck (Darmstadt, Germany), sodium hydroxide, p.a., were obtained from Eka Nobel (Surte, Sweden). Propiomazine-hydrochloride and *N*-demethylated propiomazine-hydrochloride was obtained from Pharmacia & Upjohn (Stockholm, Sweden). Promazine-hydrochloride was purchased from Sigma (St. Louis, MO, USA).

2.2. Instrumentation

The liquid chromatographic equipment used consisted of two pumps: Model 2150 (Pharmacia LKB Biotechnology, Uppsala, Sweden) and a Model Constametric Bio 3000 (Thermo Separation Products, San Jose, CA, USA) equipped with a pulsdampener Model LP-21 (Scientific System Inc., Los Angeles, CA, USA). These two pumps were connected to a degasser unit Model X-Act (Jour Research, Onsala, Sweden). The auto-injector used was a Model CMA/200 (CMA/Microdialysis, Solna, Sweden) equipped with an extra high-pressure electric switching valve (VICI AG Valco, Schenkton, Switzerland) and the detector used was a Model 5100A Coulochem (ESA Inc., Bedford, MA, USA) equipped with an analytical cell Model 5010 and a guard cell Model 5020. The chromatograms were recorded and integrated using a integrator Model CR3-A (Shimadzu, Kyoto, Japan).

The isolation of propiomazine from the proteins was performed on a Biotrap, C_{18}, Amine extraction column (C1), 10×3.0 mm I.D. (Chromtech, Hägersten, Sweden). The extraction buffer used was 0.05 *M* phosphate buffer, pH 3.2, pumped at a flow-rate of 0.6 ml/min. A stainless steel on-line filter with a 10-μm frit (Upchurch Scientific, Oak Harbour, WA, USA) was inserted after the injection valve.

The analytical column (C2) used was a Supelcosil LC-CN, 75×4.6 mm I.D. (Supelco, Bellefonte, PA, USA) packed with 5-μm particles. The mobile phase was 27% acetonitrile in 0.05 *M* phosphate buffer, pH

3.2, pumped at a flow-rate of 1.0 ml/min. Adjustment of the pH to 3.2 was done with 1.0 *M* sodium hydroxide solution. A Brownlee Newguard CN, 15× 3.2 mm I.D. (Applied Biosystem, San Jose, CA, USA) guard column with 7-μm particles was used. The working potentials of the guard cell, pre-oxidation cell and the analytical detection cell to the electrochemical detector were +0.55, +0.30 and +0.55 V, respectively. Propiomazine has a high affinity for PEEK tubing. Therefore, only stainless steel tubing and stainless steel hardware for the columns and the filter were used.

2.3. Sample preparation

Blood samples (300 μl) were taken 0, 2, 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min after nasal and intravenous administration of propiomazine. The samples were drawn into heparinized hematocrit tubes (75 μl), cooled in an ice-bath and centrifuged within 30 min. The plasma fractions were frozen immediately to −70°C and stored until the analysis. The rat plasma samples were thawed and centrifuged prior the injection to remove particular matter. A volume of 50 μl was injected into the analytical system.

2.4. Calibration and quantification

Propiomazine-HCl was dissolved in 0.05 *M* phosphoric acid, pH 3.5, to make a stock solution. Aliquots of this solution were diluted with rat plasma to give a calibration graph with five concentration levels in the range of 2.3–70.0 ng/ml. Calibration was performed every day together with quality control plasma samples (stored at −20°C). Linear least-squares regression analysis was used to fit a line between the standard points with peak height versus concentration added, and unknown samples were quantified from the intercept and slope of the fitted line.

2.5. Absolute recovery

For the determination of the absolute recovery, pooled rat plasma samples were spiked with a known amount of propiomazine to give samples at two concentration levels, 9 and 114 ng/ml, respectively. These samples were injected into the analytical system and compared with propiomazine samples diluted in 0.05 *M* phosphoric acid, pH 3.2, which were injected directly on the analytical column. The peak area of propiomazine was recorded. The absolute recovery was determined as the ratio between the rat plasma samples and the samples injected in the buffer solution.

2.6. Determination of the capacity factor of propiomazine on the extraction column

Propiomazine was diluted in 0.05 *M* phosphoric acid, pH 3.2. The samples were injected and chromatographed exclusively on the extraction column with mobile phases containing acetonitrile in different concentrations. Log k' was plotted as a function of percent acetonitrile in the mobile phase.

3. Results and discussion

Analysis of propiomazine in plasma and urine has been reported earlier and involved GC [11], LC–MS [13] and GC–MS [14]. Liquid–liquid extraction was used as sample preparation to isolate propiomazine from the matrix in these methods. However to develop an analytical procedure with high sample through-put alternative methods were investigated. Coupled-column chromatography is a powerful technique which has been used for many years in liquid chromatography [15]. This approach is especially useful in bioanalytical work where the analyte needs to be separated from complex biological fluids [16–18]. The use of a restricted-access column in a coupled-column system gives an analytical method where the sample handling has been minimized. Therefore, the analyte remains unaffected due to the reduction in sample handling steps, which leads to a method with good accuracy and precision [1,19].

The described analytical system (Fig. 1) is based on a pre-column packed with a biocompatible packing material. The plasma samples were injected onto the pre-column which was conditioned with phosphate buffer. The analyte penetrates the small pores in the packing material and interacts with the C_{18} chains and is retained. The plasma proteins are excluded from the packing material and eluted within

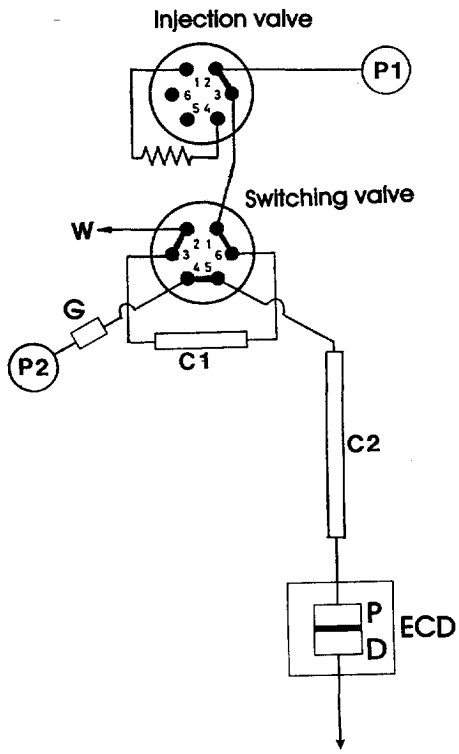

Fig. 1. Scheme of the switching configuration. P1 and P2 represent the LC pumps. The extraction column and the analytical column are labelled C1 and C2, respectively, and the electrochemical detector is labelled ECD, including guard cell (G), pre-oxidation cell (P) and detection cell (D).

the internal volume of the column. When all plasma proteins are washed out from the column the valve is switched. The mobile phase from the analytical column transfers (backflush mode) propiomazine from C1 to C2 where the separation and detection take place. The next sample could be injected after 14 min. The column switching time-table is presented in Table 1.

3.1. Stability of propiomazine

Propiomazine was determined in the quality control plasma samples which were stored at −20°C for 2 months. The concentration of propiomazine in the plasma samples did not change during that period. Furthermore, no degradation of propiomazine could be observed during the storage in the auto-injector at ambient temperature for 4 h.

3.2. Stability of the extraction column

The lifetime of the extraction column was dependent on the sample matrix, the injected volume, the composition of the mobile phases, and the manner in which the extraction column was rinsed by the extraction mobile phase and by the analytical mobile phase. The size of the extraction columns was 10× 3.0 mm I.D. Nevertheless, large plasma volumes could be injected before the column had to be replaced. The total maximum plasma volume that could be injected was 6.5 ml. Usually, the column could receive 4–5 ml (80–100 injections) of plasma before exchange was necessary due to high back pressure and reduced chromatographic efficiency. Initially, during the method development a 3-min extraction time was used. As a result, the extraction column had to be changed more frequently, every 30–40 injections, due to deterioration of the column which leads to high back pressure. An increase in the extraction time to 5 min increased the plasma capacity of the extraction column by more than two-fold. The absolute recovery at the 9 ng/ml level was decreased from 100.1% (R.S.D. 4.0%) to 80.6%

Table 1
Column switching time-table

Time (min)	Valve operated	Event
0	Injector	The sample is injected on C1. Propiomazine is isolated from the proteins.
5	Valve	Propiomazine is transferred from C1 to C2
8.3	Valve	The elution from C1 is completed and the separation continues on C2. The extraction column is conditioned with buffer
14	Injector	Next sample is injected

(R.S.D. 3.0%) when the large extraction volume was used.

3.3. Selectivity

Electrochemical detection gave the selectivity and the sensitivity needed for determination of propiomazine in rat plasma. The guard cell of the electrochemical detector was placed before the switching valve to produce an electrochemically 'clean' analytical mobile phase, which increased the sensitivity five times. The analytical cell consisted of two electrodes. The first was set to +0.3 V to pre-oxidize plasma components and the second was used for detection. A chromatogram obtained after injection of a blank plasma sample is shown in Fig. 2A. A representative chromatogram where the concentration of propiomazine was determined to be 65 ng/ml is presented in Fig. 2B. The plasma sample was taken 10 min after an i.v. administration of propiomazine [20]. The selectivity of the method was verified by injection of the main metabolite *N*-demethylated propiomazine [11,14], promazine and the barbiturate (thiobutabarbital) used to anaesthetize the rats. Separation between propiomazine and these compounds was obtained (data not shown).

3.4. Absolute recovery

The pH of the mobile phase on the analytical column was chosen to be 3.2 in order to get good chromatography without any tailing. Consequently, the same pH was chosen for the extraction mobile phase to avoid system peaks in the chromatogram due to valve switching. To obtain maximum retention on the pre-column, the pH of the extraction buffer should exceed the pK_a value of the analyte. The pH was increased temporarily to physiological pH during the injection of the plasma samples. This means that the extraction was maximized when the protein plug was eluting through the column. The injection volume was approximately equal to the internal volume of the extraction column. The k' of propiomazine in the extraction buffer was extrapolated to 730 (Fig. 3). This k' value was used to calculate the k' needed to retain propiomazine in the presence of proteins according to [1]. The phenothiazine derivate has an high protein binding value in plasma, values between 90–95% have been reported [21]. If the assumed protein binding level is 95%, the calculated k' value for propiomazine in the protein plug was 38. Using the k' and the plate number

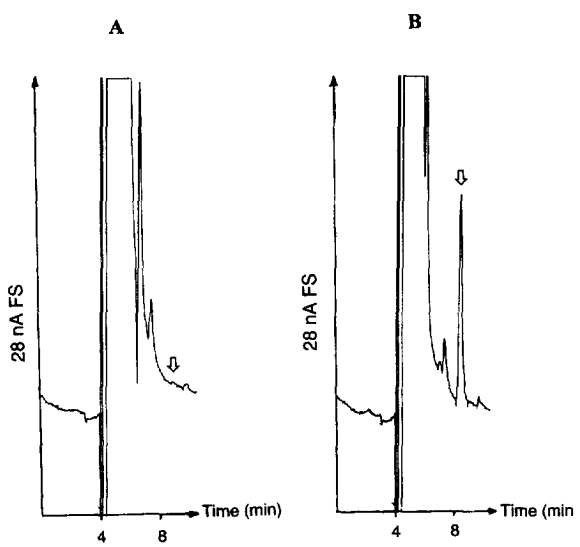

Fig. 2. Chromatograms obtained after a 50-μl direct injection of rat plasma. (A) Blank plasma. (B) Plasma sample containing 65 ng/ml of propiomazine collected 10 min after i.v. administration.

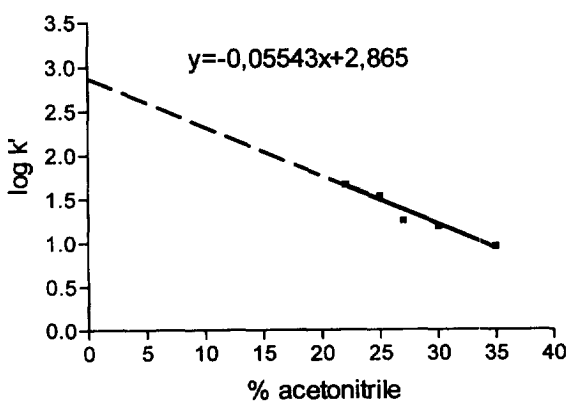

Fig. 3. The graph shows the capacity factors of propiomazine chromatographed in 0.05 *M* phosphate buffer with various percentages of acetonitrile on the extraction column (C1). The k' value in pure buffer without organic modifier was extrapolated to be 730.

Table 2
Inter-day and intra-day precision

Inter-day precision			Intra-day precision		
Conc. (ng/ml)	R.S.D. (%)	n	Conc. (ng/ml)	R.S.D. (%)	n
5.6	10.9	10	9.0	2.8	10
63.2	4.5	11	114.0	0.7	10

(n=29) gives a break-through volume of 1.45 ml. In other words, 1.45 ml plasma can be injected onto the extraction column before propiomazine breaks through at pH 3.2. The break-through volume for propiomazine in buffer, pH 3.2, was 26 ml. According to these calculations propiomazine could be recovered in quantitative amounts from the extraction column. Nevertheless, the absolute recovery was decreased with increased rinsing volume on the extraction column. Plasma contains a broad range of endogenous compounds such as proteins, peptides, lipids and other organic compounds in high concentrations [22] which will compete with propiomazine for adsorption on the internal reversed-phase surface. As a result, the absolute recovery will be decreased with increased extraction time. This effect could probably be minimized if a more effective ion-pairing reagent (phosphate ions have a low tendency to form ion-pairs), such as trifluoro acetic acid (TFA), was present in the extraction mobile phase. The analytical mobile phase containing 27% acetonitrile did not have the capacity to completely desorb all the plasma components from the extraction column between each injection. A washing step with a stronger eluent like propanol may increase the lifetime of the columns, but this was not investigated since 130 injections was considered to be an acceptable number for analysis.

3.5. Precision and linearity

The intra-day and inter-day precisions were determined to evaluate the reliability of the analytical system. The data are listed in Table 2. The calibration graphs showed a linear relationship. The mean value of the correlation coefficients was 0.9985 (n=10).

4. Conclusion

A reproducible, sensitive and fast method for determination of propiomazine in rat plasma has been developed. By using a Biotrap extraction column, the plasma samples could be injected directly into the chromatographic system, after centrifugation. This approach with an minimum sample pretreatment gave a method with good accuracy and precision. A total volume of 6.5 ml (130 injections) could be injected onto the extraction column before replacement was required. Electrochemical detection gave a limit of quantification of 2 ng/ml (100 pg injected).

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