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Sensitive method for the determination of baclofen in plasma by means of solid-phase extraction and liquid chromatography–tandem mass spectrometry

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Abstract

A simple and sensitive method for the determination of baclofen in plasma is described. Baclofen and the internal standard, KM 08205, were isolated from plasma by solid-phase extraction using C_{18} material. After separation by reversed-phase liquid chromatography, the analytes were detected with tandem mass spectrometry. The extraction procedure was optimised regarding the solid-phase extraction material, the pH in the conditioning solution and the washing volume. The method was proven to be selective and sensitive with an absolute recovery of about 95%, a relative standard deviation below 5% and a limit of quantification of 10 nmol/l.

Keywords: Pharmaceutical analysis; Baclofen

1. Introduction

Baclofen (4-amino-3-*p*-chlorophenylbutyric acid, Lioresal) (Fig. 1), a chemical analogue of γ-aminobutyric acid (GABA), is used as a muscle relaxant in the treatment of spasticity. In order to carry out pharmacokinetic studies a selective and sensitive analytical method was needed, which allowed for the determination of baclofen in the nanomolar range in plasma samples. Several methods have been described involving techniques such as gas chromatography (GC) after derivatisation [1,2], gas chromatography–mass spectrometry (GC–MS) [3] and high-performance liquid chromatography (HPLC) with ultraviolet [4–6] or fluorescence [7–9] and electrochemical detection [10,11] after derivatisation. Derivatisation with *o*-phtalaldehyde followed by either fluorometric [7] or electrochemical detection [10,11] is based on the reaction of *o*-phtalaldehyde with primary amines in the presence of a thiol. The sensitivity of this method is good. However, endogenous primary amines and amino acids present in the sample matrix may result in interfering peaks, making the chromatograms difficult to interpret. More-

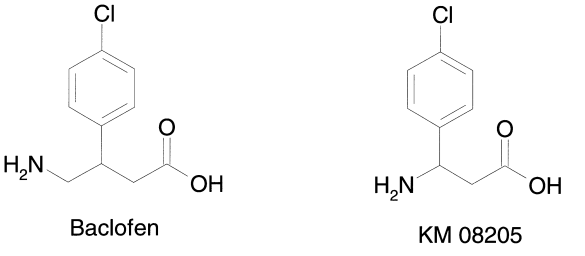

Fig. 1. Structural formulas of baclofen and the internal standard, KM 08205.

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over, with electrochemical detection the reagent itself presents a problem being electroactive.

Liquid chromatography coupled to tandem mass spectrometry (MS–MS) offers a powerful tool in bioanalytical chemistry due to its selectivity. The measurement of a specific fragment from the analyte ion lowers the demand on the chromatographic separation efficiency. However, the existence of co-eluting undetected components present in the sample may cause problems with the mass spectrometric response, due to ion suppression and other effects, leading to less reliable methods [12]. Thus the need for an efficient sample clean-up and chromatographic separation should not be underestimated.

We have developed a simple and sensitive method for the determination of baclofen in plasma, using off-line solid-phase extraction (SPE) followed by LC–MS–MS with atmospheric pressure positive ionization.

2. Experimental

2.1. Reagents and chemicals

Baclofen and the internal standard, KM 08205, (3-amino-3-*p*-chlorophenylpropionic acid) (Fig. 1) were obtained from Sigma (St. Louis, MO, USA) and Maybridge (Cornwall, UK), respectively. Formic acid and ammonium acetate were of analytical grade, Fluka (Buchs, Switzerland). Methanol and acetonitrile were of HPLC grade and obtained from Rathburn (Walkerburn, UK). Water was from an ELGA UHP purification system, ELGA (Wycombe, UK). Bond Elut C_{18} and SCX (strong cation-exchange) SPE columns (100 mg, 1 ml) were from Varian (Harbor City, CA, USA) and Isolute CN and C_2 SPE columns (100 mg, 1 ml) from International Sorbent Technology (Mid Glamorgan, UK).

2.2. Apparatus

The chromatographic system comprised a PE 200 series pump and a PE 200 series autosampler (Perkin-Elmer, Norwalk, CT, USA) with a 200-μl injection loop. The injection volume was 30 μl. The analytical column was a Hypersil BDS C_{18}, 3 μm, (100×4.0 mm I.D.) from Shandon (Astmoor, UK) and the mobile phase consisted of 0.08% (v/v) formic acid and 1.3 mmol/l ammonium acetate in a mixture of acetonitrile and water (4:6), delivered with a flow-rate of 0.75 ml/min, giving a hold-up time (t_0) of ca. 1.0 min and a retention time of approximately 1.5 min for both baclofen and KM 08205. The LC system was coupled to a PE Sciex API 365 quadrupole mass spectrometer with a pneumatically assisted electrospray (turbo ionspray) interface (Sciex, Toronto, Canada). The effluent from the liquid chromatograph was split in a Valco T connection in order to get a liquid flow to the ionspray of about 200 μl/min. The orifice voltage of the mass spectrometer was set at 24 V and the collision energy at 25 V. The turbo temperature was 400°C and the turbo heater gas flow of nitrogen was 7 l/min. Nitrogen was also used as nebulizer, curtain and collision gas. The curtain gas was set at a value of 12 (instrument units) and the collision gas flow setting was 2 (instrument units). Baclofen and the internal standard were monitored at m/z 150.8 (daughter of m/z 214.1) and m/z 140.0 (daughter of m/z 200.1), respectively, with a dwell time of 200 ms. Data were processed using the API 365 standard software Mac Quan from PE Sciex (Foster City, CA, USA).

2.3. Sample preparation procedure

Bond Elut C_{18} SPE columns were conditioned with 1 ml of methanol followed by 1 ml of water and 1 ml of aqueous 0.1% (v/v) formic acid solution. Plasma samples were prepared by mixing 600 μl of the internal standard (8 μmol/l) in 0.1% (v/v) formic acid solution, with 600 μl of centrifuged plasma. After centrifugation for 5 min at 20 000 *g*, 1 ml of the mixture of plasma and internal standard was added to the SPE column. One ml of 0.1% (v/v) formic acid solution was added to the column to wash out plasma residuals before the analytes were eluted with 1 ml of mobile phase directly into the autosampler vial. All liquids were allowed to pass the column by gravity. Plasma reference samples processed in parallel with the authentic samples were prepared in the same way using centrifuged drug-free human plasma and a solution containing both baclofen (2 μmol/l) and the internal standard (8 μmol/l).

3. Results and discussion

3.1. Solid-phase extraction procedure

Four different types of SPE columns were tested, C_{18}, SCX, CN and C_2. For the C_2, CN and the SCX columns, the recoveries of both baclofen and the internal standard were very low, whereas for the C_{18} column the recoveries were about 95% and this material was chosen for further optimisation. The effect of pH in the conditioning solution was investigated. After conditioning with methanol and water, 1 ml of either (1) 0.1% (v/v) formic acid solution, pH 2.7; (2) 75 mmol/l ammonium acetate buffer, pH 3.5; (3) 0.05% formic acid and 50 mmol/l ammonium acetate, pH 5.5 or (4) 100 mmol/l ammonium acetate, pH 6.5 was added to the SPE column. Five hundred μl of reference solution, diluted in buffer 1, 2, 3 or 4, and 500 μl of plasma were added to the column. Plasma residuals were washed out with 1 ml of 0.1% (v/v) formic acid solution. The recoveries were calculated by comparing the peak areas for baclofen and the internal standard, respectively, with those of the same amount injected directly onto the column. The recoveries of baclofen and the internal standard were virtually independent of pH in the range investigated.

An attempt was made to investigate the effect of pH of the washing solution as well. However, using solutions 2, 3 and 4 above as washing solutions resulted in broad peaks for both baclofen and the internal standard. This was probably due to the content of ammonium acetate in the samples, affecting the chromatography or the MS ionization process. The 0.1% (v/v) formic acid solution was chosen as conditioning and washing solution.

The effect of the volume of washing solution on the recovery was evaluated. The volume was varied between 0.5 and 5 ml and as can be seen in Fig. 2, the recovery of the internal standard decreased with a larger washing volume and 1 ml of 0.1% (v/v) formic acid solution was chosen.

No effect on recovery was seen if plasma and internal standard solution were mixed and added to the SPE column, or if either plasma or internal standard was added first to the column. Doubling the plasma and internal standard volumes did not affect the recoveries when the mixing procedure was used. However, when the internal standard was added first the recovery of the internal standard decreased to 60%, while the recovery of baclofen was unaffected. The procedure of mixing plasma and internal standard solution before addition to the column was chosen.

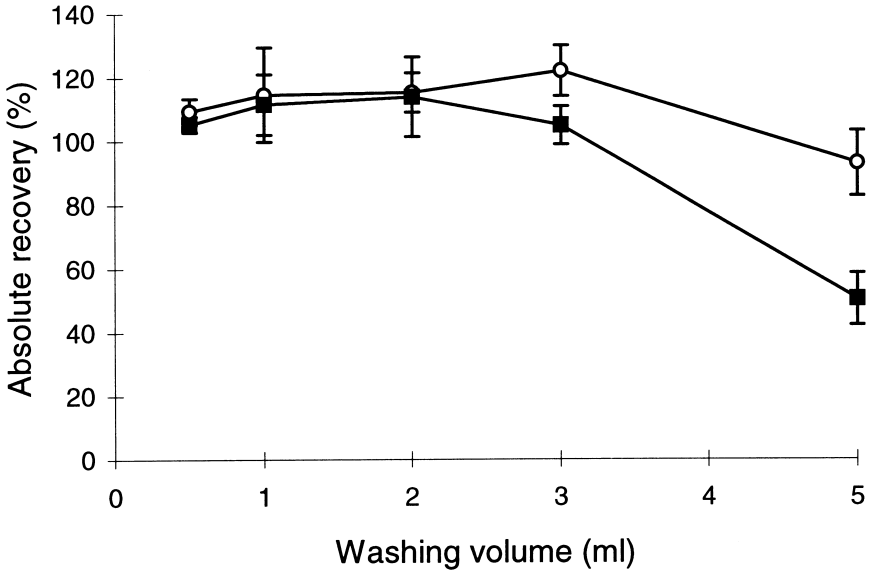

Fig. 2. Effect of washing volume on the recovery of baclofen (○) and the internal standard, KM 08205 (■).

The absolute recoveries at different concentration levels were calculated by comparing peak areas of spiked plasma samples with those obtained for the same amount of baclofen in a solution injected directly onto the column. The recoveries±relative standard deviations (RSDs) of baclofen ($n=8$) at 24, 869 and 2030 nmol/l were 96.1±4.9%, 96.9±4.1% and 93.1±5.7%, respectively. The absolute recovery of the internal standard at 8450 nmol/l was 95.2±3.7% ($n=24$).

The use of an internal standard labelled with a stable isotope would be preferable, as it would show the same behaviour as baclofen both on the SPE column and on the analytical column. However, an isotope-labelled internal standard was not available, why it was crucial to investigate the behaviour of the internal standard relative to baclofen.

3.2. Quantification and accuracy

At least six plasma reference samples were prepared and analysed according to the procedure described above. The ratios of the peak area of baclofen to that of the internal standard in the plasma reference samples were used for calculation of the baclofen concentrations in unknown plasma samples. Intra-day precision was assessed at different concentration levels by analysis of eight replicates. The RSDs were 3.4, 1.7 and 4.5% for samples containing 24, 869 and 2030 nmol/l of baclofen, respectively.

The inter-day variability, determined by performing analyses of plasma samples containing 661 nmol/l of baclofen from a batch of samples stored at −20°C, was 3.2% ($n=10$ days). The intra-day variability of the plasma reference samples used for the daily calibration, containing 1610 or 2030 nmol/l, was 3.8% ($n=6$ samples/day, 10 days). The results were statistically analysed with ANOVA (analysis of variance).

Linearity was studied by preparing at least three replicates of plasma samples at six different concentration levels and processing them according to the analytical procedure. The method was linear up to the tested concentration of 9 μmol/l and the limit of quantification (RSD<20%) was estimated at 10 nmol/l. The regression data for a calibration curve obtained by plotting found concentrations versus nominal concentrations (23–9200 nmol/l) was $y=1.008x+7.200$ ($n=26$); $R^2=0.998$. Full calibration curves were run intermittently during the course of routine analysis to control linearity. Figs. 3 and 4 show chromatograms of an authentic dog plasma sample, containing 25 nmol/l of baclofen and 8230 nmol/l of internal standard, and of a drug-free plasma sample, containing 8230 nmol/l of internal standard, respectively.

3.3. Stability

A stock solution of 80 μmol/l of baclofen prepared in water was stable for at least six months when kept in a refrigerator (+6°C). Plasma samples were stable at −20°C for at least nine months and

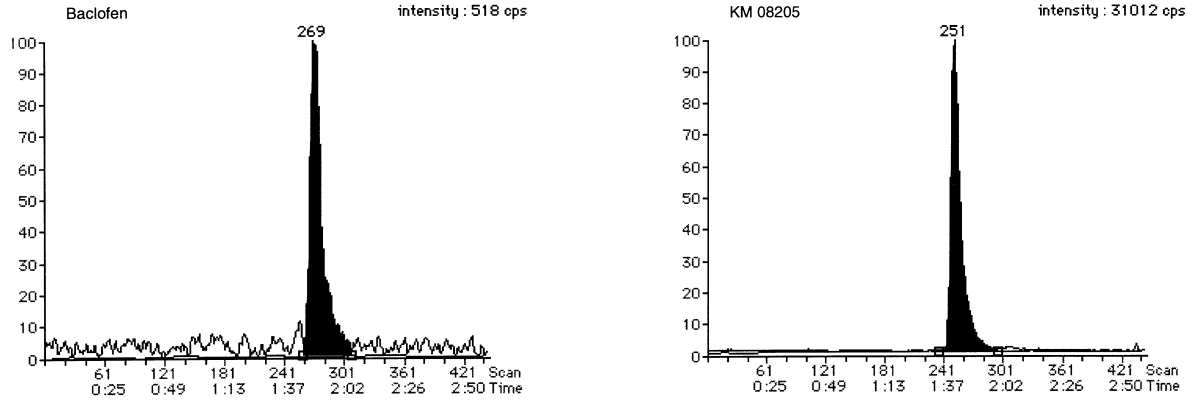

Fig. 3. Chromatogram of an authentic dog plasma sample containing 25 nmol/l of baclofen and 8230 nmol/l of the internal standard, KM 08205.

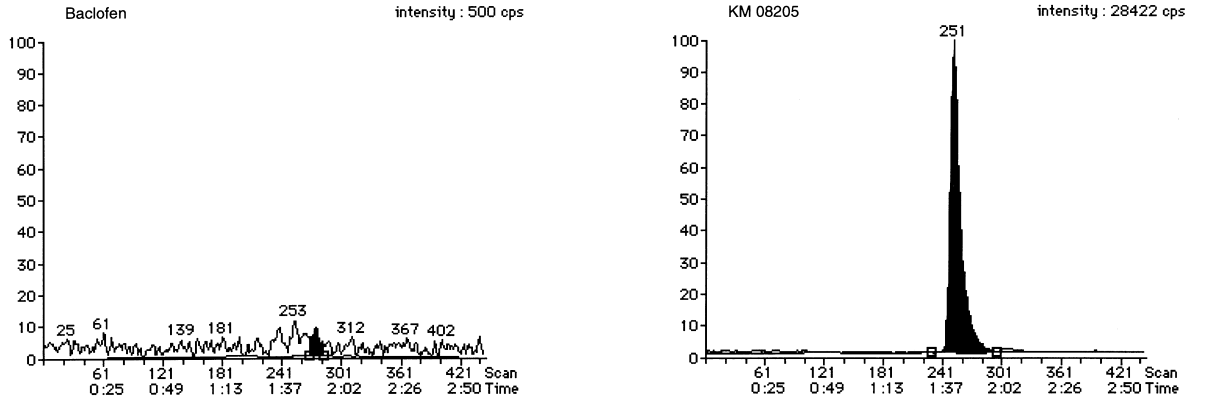

Fig. 4. Chromatogram of a drug-free plasma sample containing 8230 nmol/l of the internal standard, KM 08205.

plasma sample eluates after SPE were stable for at least one week when kept in a refrigerator.

3.4. Matrix effects and ion suppression

The selectivity of the mass spectrometer enables the use of fast chromatography with low separation efficiency. However, co-eluting undetected matrix components may decrease the ion intensity of the analytes and influence the precision and accuracy of the determination. This effect has recently been demonstrated for the PE Sciex turbo ionspray [12]. To investigate the occurrence of matrix effects, small amounts of a concentrated solution of baclofen and the internal standard were added to the eluate after SPE of a blank plasma sample and to the same volume of mobile phase, and the peak areas were compared. The peak areas for baclofen and the internal standard in the plasma eluate compared to those in mobile phase were 99.4% and 96.7%, respectively, showing no matrix effects or ion suppression of the response. The precision (RSD) for baclofen and the internal standard was 1.7% and 2.3% in plasma eluate ($n=6$) and 5.8% and 4.6% in mobile phase ($n=5$), respectively.

4. Conclusions

A simple method, which is both faster and more sensitive than previous methods, has been developed for determination of baclofen in plasma. Utilizing the selectivity and sensitivity of tandem mass spectrometry enabled the use of a simple SPE and a fast liquid chromatographic separation. The method has high recoveries and good precision and has proven to be useful for pharmacokinetic studies.

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