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Electrochemical characterization of repaglinide and its determination in human plasma using liquid chromatography with dual-channel coulometric detection

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ABSTRACT

A simple, fast and sensitive HPLC method employing dual-channel coulometric detection for the determination of repaglinide in human plasma is presented. The assay involved extraction of repaglinide by ethyl acetate and isocratic reversed-phase liquid chromatography with dual-channel coulometric detection. The mobile phase composition was 50 mM disodium hydrogen phosphate/acetonitrile (60:40, v/v), pH of the mobile phase 7.5 set up with phosphoric acid. For all analyses, the first cell working potential was +380 mV, the second was +750 mV (*vs.* Pd/H_2). Calibration curve was linear over the concentration range of 5–500 nmol L^{-1}. Rosiglitazone was used as an internal standard. The limit of detection (LOD) was established at 2.8 nmol L^{-1}, and the lower limit of quantification (LLOQ) at 8.5 nmol L^{-1}. The developed method was applied to human plasma samples spiked with repaglinide at therapeutical concentrations. It was confirmed that the method is suitable for pharmacokinetic studies or therapeutic monitoring.

1. Introduction

Diabetes mellitus type 2 (T2DM), or non-insulin-dependent diabetes mellitus (NIDDM), is one of the most common metabolic disorders affecting humans. The disorder is characterized by depressed insulin secretion associated with increased resistance to the action of insulin, a hormone that regulates glucose absorption.

Peroral antidiabetics (PADs) are an integral part of NIDDM complex treatment. The occurrence of this metabolic disorder has been growing explosively over the last years in the population with still younger age groups being affected [1,2]. Recently, PADs have become, as a consequence of increasing amount of information about this metabolic disorder, subject of an intense development.

Today, much effort is devoted to the research and development of new, more effective drugs. At the same time – with the tendency to decrease therapeutic dosages – the demands on sufficiently selective and sensitive analytical methods are constantly increasing.

The determination of drugs in plasma at low concentration levels is a very challenging analytical task and only highly sensitive instrumental techniques can meet such requirements.

Repaglinide is a synthetic peroral antidiabetic drug of the meglitinide class, used in the therapy of T2DM [3–5].

Repaglinide (RPG), chemically *S*-(+)-2-ethoxy-4-[2[[3-methyl-1-[2-(1-piperidinyl) phenyl] butyl] amino)-2-oxoethyl] benzoic acid (for structure see Fig. 1), can be characterized as a weak diacid. Various pK_a values (pK_{a1} 3.96–4.19; pK_{a2} 5.78–6.20) are reported [6,7]. RPG is produced as a single enantiomer. Thus the (*R*)-enantiomer, exhibiting only weak hypoglycemic activity, should be considered as a chiral impurity [8].

RPG acts as an effective blood glucose-lowering drug. Its mechanism of action is partly similar to that of the sulphonylureas: the release of insulin from the pancreatic beta cells is stimulated by closure of ATP-dependent potassium channels, but, unlike other PADs, not in the absence of glucose [9]. Therapeutic doses are lower compared to those of other PADs, RPG has a rapid onset of dose dependent blood glucose lowering effect, making it suitable for co-administration with food. The drug is rapidly eliminated from the blood stream with a half-life of approximately 1 h [10,11]. After oral administration, RPG is fast and completely absorbed from the gastrointestinal tract [12]. The reported mean absolute bioavailability is about 60% [13]. RPG is metabolized by oxidative biotransformation involving the hepatic CYP3A4 enzyme. Only 8% of an administered dose is excreted in the urine [11].

As the protein binding and binding to human serum albumin is reported to be >98% [11,14] free-drug plasma levels are typically very low and beyond quantification limits of most analytical methods. Therefore, for its reliable analytical determination, preliminary procedures eliminating the protein binding are essential.

Currently, only few analytical methods for the determination of RPG in biological fluids are reported. Liquid chromatography–tandem mass spectrometry (LC–MS–MS) was used for the determination of 10 PADs in equine plasma and urine.

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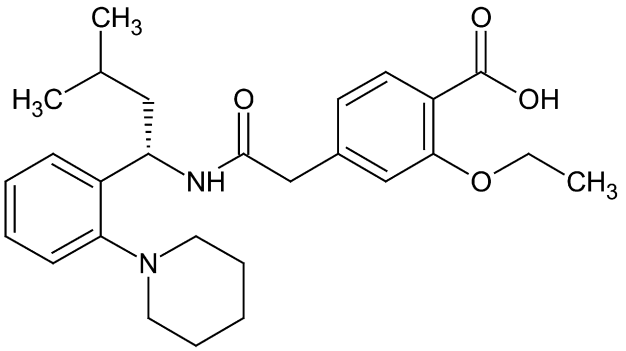

Fig. 1. Structure of repaglinide.

The published method can be used to detect the abuse of anti-diabetic drugs in racehorses [15].

An HPLC method for the separation and simultaneous determination of six anti-diabetic drugs (glibenclamide, gliclazide, glipizide, pioglitazone, repaglinide and rosiglitazone in pharmaceutical formulations employing UV detection at 260 nm was published [16] Celecoxib was used as an internal standard. The assay has been applied to support quantification of those six anti-diabetic drugs in human plasma.

A simultaneous determination of six selected antidiabetic drugs (tolbutamide, gliclazide, glimepiride, glibenclamide, repaglinide, and glipizide) by micellar electrokinetic chromatography employing non-ionic surfactant was published [17]. An on-line pre-concentration method based on reversed electrode polarity switching was employed for the determination of these antihyperglycemic drugs in blood serum after acetonitrile protein precipitation. The reached limits of detection ranged from 20.8 nmol L^{-1} to 6.5 nmol L^{-1}.

High-performance liquid chromatography coupled with electrochemical detection (HPLC-ED) is a powerful analytical technique, well known for its very high sensitivity and selectivity, and widely used in pharmaceutical analysis [18]. In this respect, especially multi-electrode coulometric systems with electrochemical conversion degrees up to 100% have significant advantages over conventional amperometry-operated detectors [19,20]. Thanks to these inherent unique properties, some preliminary purification processes connected with a sample manipulation and thus with the risk of loss or decomposition of the analyte, can often be avoided or at least simplified. Coulometric cells are also less susceptible to electrode passivation and provide much more stable and reproducible response.

In spite of the fact that RPG is electroactive, and its electrochemical behavior at carbon paste electrode was described and direct voltammetric method for its analysis in plasma was reported [7] electrochemical detection is not widely used for its determination.

A sensitive, but complicated and complex HPLC-ED method for the RPG determination in human plasma, employing column-switching system was published by Greischel et al. [21]. Plasma samples were acidified by hydrochloric acid and injected into the multi-column system. After on-line C2 pre-column purification, an isocratic analysis using the main analytical C18 column was performed by column switch. A splitter was used in front of the detector to switch the effluent into the amperometric cell only for the time interval necessary to detect the eluting RPG peak. Quantification based on an external standard calibration curve was carried out and LOD of 5 ng mL^{-1} in plasma was calculated.

The aim of this work was to develop a fast, simple and sensitive method for determination of RPG in plasma using HPLC with coulometric detection. In context with this goal, an optimization of separation conditions and electrochemical detection was performed and the influence of several experimental parameters on both chromatographic and electrochemical process was studied. The developed method was applied on human plasma samples.

2. Materials and methods

2.1. Reagents

The standard of RPG was purchased from Sigma (Sigma, St. Louis, MO, USA) and rosiglitazone (RSG) was obtained from Alexis Biochemicals (Alexis Corp., Lausen, Switzerland). Stock solutions (0.1 mg mL^{-1}) were prepared by dissolution of standards in methanol and stored at −20 °C. Work standard solutions were prepared from the stock solutions by diluting in the mobile phase. For the mobile phase preparation, disodium hydrogen phosphate and phosphoric acid purchased from Fluka (Fluka AG, Buchs, Switzerland) of trace select purity, and gradient grade acetonitrile, methanol and ethyl acetate (Merck, Darmstadt, Germany), were used.

2.2. Voltammetric experiments

Standards of RPG and RSG were dissolved in methanol at a final concentration of 1 mmol L^{-1}. Supporting electrolytes were prepared using sodium dihydrogen phosphate and disodium hydrogen phosphate aqueous solutions (50 mmol L^{-1}) mixed with acetonitrile (for HPLC, gradient-grade, Sigma–Aldrich) in volume ratio of 60/40. The pH value of the mixture was adjusted by concentrated phosphoric acid. All chemicals for supporting electrolyte preparation were of analytical-reagent grade (Fluka AG, Buchs, Switzerland). Ultrapure water from an ELGA system (average specific resistance 18.2 MΩ cm^{-1}, i.e. conductivity 0.055 μS cm^{-1}) was used.

A computer controlled Eco-Tribo Polarograph (Polaro-Sensors, Prague, Czech Republic), equipped with Polar.Pro v. 4 software was used for all voltammetric measurements. A three-electrode system consisted of a working glassy carbon electrode (disk diameter 3.0 mm, Bioanalytical Systems, USA), a platinum auxiliary and a saturated calomel reference electrode (SCE). The working electrode was polished using 0.05 μm alumina slurry on wet micro cloth (Buehler, USA) and sonicated in distilled water for 10 s prior to each measurement. Differential pulse voltammograms were recorded using a scan range of 20 mV s^{-1} with a pulse amplitude of 50 mV and a pulse width of 80 ms. Measurements by cyclic voltammetry were performed at a scan rate of 100 mV s^{-1}. All experiments were performed at 22 °C.

2.3. Chromatographic system

The HPLC system consisted of an ESA isocratic pump (Model 582), (ESA Inc., Chelmsford, MA, USA) with a pulse damper, manual injector (Rheodyne, Cotati, CA, USA) equipped with a 10 μL loop and an ESA coulometric detector Coulochem III with a dual-electrode standard analytical cell (Model 5010A) combined with a guard cell (Model 5020) prior to the injector, (all ESA Inc., Chelmsford, MA, USA).

The chromatographic station Clarity (DataApex, Prague, Czech Republic) was used for simultaneous dual-channel chromatogram recording.

The samples were introduced into the system by a glass 25 μL syringe (Hamilton, Reno, NV, USA). All fittings, ferules and tubings were of PEEK™. Polaris 3 μm C18-A 150 × 3.2 mm I.D., (Varian, Inc., Palo Alto, CA, USA) HPLC column was used.

Mobile phase composition: 50 mM disodium hydrogen phosphate/acetonitrile (60/40, v/v), final pH 7.5 set up with concentrated phosphoric acid. The mobile phase was vacuum-filtered

through a 0.2 μm porous filter (Supelco, Bellefonte, PA, USA) and degassed by helium sparkling prior to use. The flow rate was 0.4 mL min^{-1}.

For all analyses, the first cell working potential was +380 mV, the second was +750 mV (*vs.* Pd/H_2). The set-up gain ranged from 100 nA V^{-1} to 1 μA V^{-1}, according to particular sample type. The guard cell potential was set to +780 mV (*vs.* Pd/H_2) in order to electrochemically remove eventual impurities from the mobile phase prior detection.

2.4. Extraction procedure for plasma samples

Three different deproteination approaches were tested in order to determine the most effective method for the plasma sample preparation:

Direct deproteination using plasma centrifugation through the Microcon YM-10 centrifugal filter devices (Millipore, Billerica, MA, USA) was carried out as follows: 100 μL of a human plasma spiked with RPG and internal standard were transferred into the Microcon filter vial insert and centrifuged at 7200 *g* for 1.5 h. The obtained de-proteinated filtrate was directly injected into the HPLC system.

Protein precipitation by acetonitrile was performed by mixing 100 μL plasma spiked by RPG and RSG (internal standard) with 100 μL acetonitrile. The mixture was thoroughly vortexed, sonicated and centrifuged at 7200 *g* for 5 min. The resulting supernatant was separated from the protein precipitate by transferring into a new vial and directly analyzed.

Liquid–liquid extraction by ethyl acetate procedure: ethyl acetate (550 μL) was added to 250 μL of spiked plasma and properly shaken, sonicated and finally centrifuged. The upper organic layer (500 μL) was transferred into a new vial and evaporated to dryness under a nitrogen stream at room temperature. The dried sample was re-constituted with 50 μL mobile phase and consequently subjected to HPLC analysis as described above.

2.5. Calibration curve, LOD, LLOQ, precision and accuracy

The method was calibrated using internal standardization by adding the internal standard (RSG) of concentration 500 nmol L^{-1} to the re-constituted plasma sample containing calibrant (RPG) of desired concentration. The calibration graph was plotted as the peak area ratio of RPG/RSG *vs.* RPG concentrations. The calibration was measured for the concentration range expected in plasma (5–500 nmol L^{-1}). The calibration curve was constructed using linear regression analysis of the calibration points (five points total; number of replicated measurements for each point: $n = 5$) and slope, intercept and correlation coefficient were calculated. The lower limit of quantification (LLOQ) and the limit of detection (LOD) were estimated using equation LLOQ = $10\sigma/S'$ or LOD = $3.3\sigma/S'$ [22] respectively, where σ is the standard deviation of the intercept and S' is the slope of a calibration regression straight line. Estimation of LLOQ was verified by replicated ($n = 5$) injections of the corresponding concentration giving an accuracy between 80% and 120% and a coefficient of variation (CV) < 20%. The estimated value is in good agreement with the control statistical method.

The precision of the assay was determined from the plasma sample spiked with standards of RPG and RSG (500 nmol L^{-1}), respectively. Run-to-run precisions were determined as relative standard deviations (RSD) of 12 replicates of a single homogenous sample within the same day, and between-day precision was calculated from five different analyses for five consecutive days.

The drug recovery from plasma was determined by comparing the peak areas obtained with standards from spiked plasma (10, 100 and 1000 nmol L^{-1}) to the peak areas obtained with the corresponding unextracted standards prepared in mobile phase at the same concentrations.

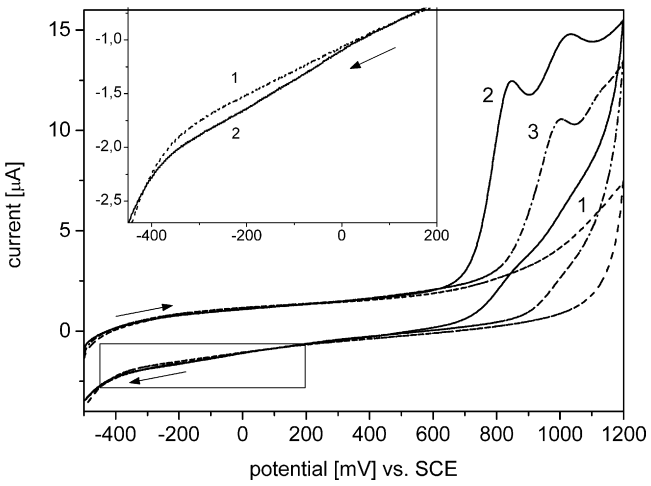

Fig. 2. Cyclic voltammograms of repaglinide (2) and rosiglitazone (3) in concentration of 0.2 mmol L^{-1}. Supporting electrolyte (1) – 50 mM phosphate buffer/acetonitrile (60/40, v/v) at pH 7.5, scan rate 100 mV s^{-1}.

3. Results and discussion

3.1. Optimization of the electrochemical detection

Oxidation of RPG was studied using two voltammetric methods – cyclic voltammetry and differential pulse voltammetry at a glassy carbon working electrode. Figs. 2 and 3 show cyclic and differential pulse voltammograms of RPG (0.2 mmol L^{-1}) recorded in a mixed medium of aqueous phosphate buffer and acetonitrile 60/40 (v/v) at pH 7.5. The voltammograms exhibit two well defined anodic peaks corresponding to the oxidation of RPG. The reverse scan of cyclic voltammogram shows a slight current wave (see the inset of Fig. 2) in consequence of reduction of some repaglinide oxidation product. The height of the cathodic wave is 1.5% compared to the first anodic RPG peak. Oxidation of RPG occurred similarly in the entire tested pH range 2–9 giving two anodic current responses. RSG has similar electrochemical characteristics, however, its anodic waves are lower and rather shifted towards the more positive potentials (Figs. 2 and 3, curve 3).

An electrochemical characterization of RPG under hydrodynamic conditions was also carried out. As can be seen from

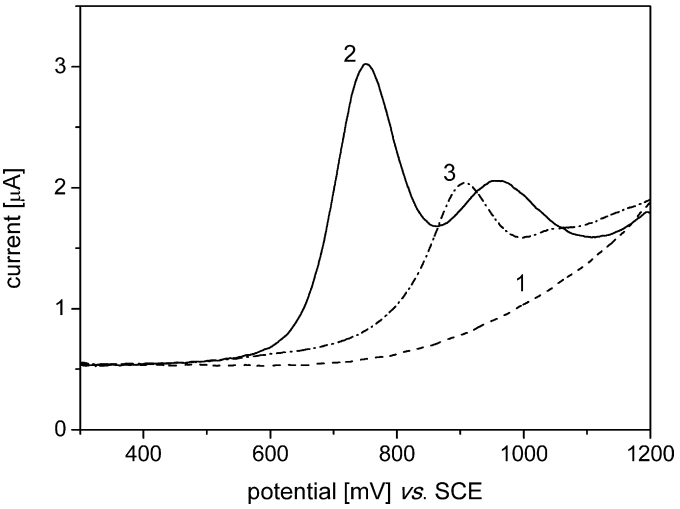

Fig. 3. Differential pulse voltammograms of repaglinide (2) and rosiglitazone (3) in concentration of 0.2 mmol L^{-1}. Supporting electrolyte (1) – 50 mM phosphate buffer/acetonitrile (60/40, v/v) at pH 7.5, scan rate 20 mV s^{-1}, modulation amplitude 50 mV, pulse width 80 ms.

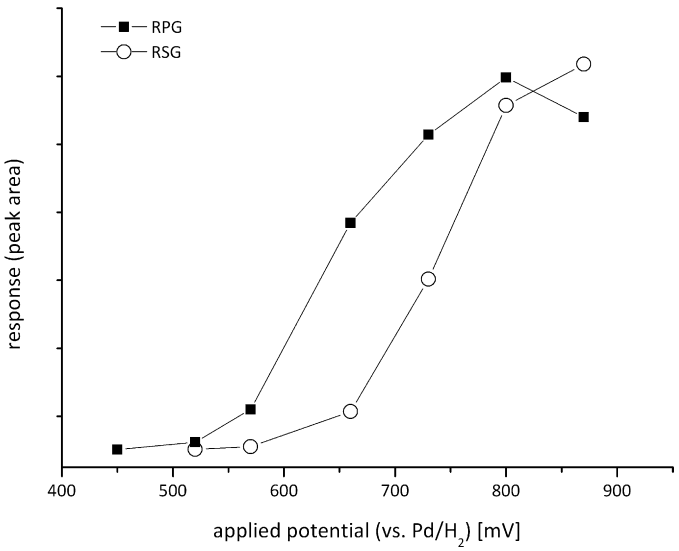

Fig. 4. Hydrodynamic voltammograms of repaglinide and rosiglitazone. Other conditions see Section 2.

hydrodynamic voltammograms (Fig. 4), RPG can be easily oxidized in the flow-measurement system at porous-carbon electrodes enabling its direct electrochemical detection: above the electrode potential of about +500 mV (*vs.* Pd/H_2) the oxidation of RPG occurs whereas maximum anodic currents (and thus maximum electrochemical conversion efficiency) at working potential of about +800 mV (*vs.* Pd/H_2) are achieved. The internal standard (RSG) exhibits a very similar voltammetric behaviour with current response growing from +600 up to +900 mV (*vs.* Pd/H_2).

We used the dual-channel coulometric detection in a *screen mode* in this study. In this arrangement, the first series coulometric sensor acts as a screening electrode with the working potential slightly below the effective value necessary for the conversion of the analyte. The eluting interferents with lower oxidation potential than the compound of interest are electrochemically "removed" by their transformation into electrochemically inactive products, not affecting the response at the second (analytical) sensor. Thus the plasma sample is partially pre-purified before the analytes enter the cell.

A dual-channel mode using subsequent reversed reduction of the oxidized RPG was also tested. In this so-called *redox mode*, a molecule of the analyte is oxidized at the first sensor and the resulting product is directly reduced at following serial electrode. In this case, oxidized RPG provided a cathodic peak at the second channel at −350 mV (*vs.* Pd/H_2), similarly as observed with cyclic voltammetry. The reverse redox reaction can be used to confirm identity of the target analyte. The relative area of the reduction peak was 2.8%.

3.2. Plasma preparation

To evaluate drug recovery from the human plasma, three different deproteination methods were compared (see Section 2.4) – ultra-centrifugation through the Microcon centrifugal filter unit (1) and two common precipitation/extraction procedures – direct precipitation using acetonitrile (2) and liquid–liquid extraction into ethyl acetate (3). The found recoveries of RPG from human plasma for the each method were as follows: not detected (1), 62 ± 3% (2) and 104 ± 4% (3).

Extraction by ethyl acetate yielded high recovery. This corresponds well with similar findings of other authors [23]. On the contrary, ultra-filtration through membrane filters provided recoveries below detection limits of the method (for the RPG concentrations tested). Hence this approach proved to be useless for our purposes. The obtained results can be explained by the known strong protein binding of RPG: in untreated plasma, the analyte exists predominantly in a bound form whereas the addition of the less polar organic solvent breaks the associates, resulting in higher extraction recoveries [11].

Precipitation of plasma with acetonitrile is one of the most common and frequently used methods of protein removal. The procedure is also known to be sufficiently effective, particularly if higher ratios acetonitrile/plasma are used [24]. As a rule, acetonitrile well matches typical mobile phases used in HPLC. This approach is, however, inevitably connected with some additional sample dilution which can be undesirable if trace levels are to be determined. Moreover, dilution of the sample by buffered mobile phase is advisable for the robust electrochemical response.

Liquid–liquid extraction by ethyl acetate followed by reconstitution by mobile; phase is a rather more complex procedure; on the other hand, it provides simultaneous protein removal and purification/pre-concentration resulting in cleaner chromatogram backgrounds as compared to direct protein precipitation. For larger plasma samples than those used in this study (250 μL) further analyte enrichment can be achieved.

3.3. Chromatographic method

To obtain sufficient resolution as well as reasonable retention times, the mobile phase composition was optimized by testing the influence of pH, type of the organic modifier (methanol, acetonitrile) and its content. The type of organic solvent in the mobile phase affects the separation selectivity by specific interactions as well as peak width and asymmetry. As expected, the use of acetonitrile resulted in much narrower and symmetrical peaks. Moreover, the electrochemical response in acetonitrile environment was significantly higher compared with that in methanol. Acetonitrile is a stronger eluent than methanol. Thus its percentage content in the mobile phase is generally lower to keep the analyte retention constant.

As supposed, due to the weak acidic character of RPG [6,7], the pH of the mobile phase has a significant influence on both the chromatographic and electrochemical process. The mobile phase pH range examined was within pH 5–8 in 0.5 steps. Although acidic conditions (pH 2.5–5.0) were also preliminarily tested, the observed electrochemical response was diminished and retention characteristic of RPG and RSG was dissimilar in this pH area (in respect of isocratic elution limitations). It is also beneficial if the pH of the mobile phase is as close as possible to that of plasma.

Three different HPLC columns were tested for the separation of RPG: Agilent Zorbax Eclipse XDB-C8 4.6 × 150 mm (5 μm), Phenomenex Gemini C18 3.0 × 150 mm (5 μm) and Polaris C18-A 3.2 × 150 mm (3 μm). The latter is a HPLC column which is specifically designed for the highly-aqueous mobile phases typically used in connection with electrochemical detection. Its narrower inner diameter (3.2 mm) and smaller sorbent particles (3 μm) contributes to reduce the mobile phase consumption while maintaining satisfactory chromatographic efficiency. As Polaris column provided the best peak symmetry and chromatographic performance, it was chosen for the experiment. Final HPLC conditions were as follows: column – Polaris C18-A 3.2 × 150 mm (3 μm), mobile phase composition – 50 mM disodium hydrogen phosphate/acetonitrile (60/40, v/v), final pH 7.5 set up with phosphoric acid; flow rate 0.4 mL min^{-1}. A typical chromatogram of RPG using the best conditions is shown in Fig. 5.

Table 1
Results of the partial validation of the method.

	Within-day[a]		Inter-day[b]		LOD (nmol L^{-1})	LLOQ (nmol L^{-1})	Slope	Intercept	r^2
	Area RSD (%)	t_R RSD (%)	Area RSD (%)	t_R RSD (%)					
RPG	2.0	1.5	2.5	2.1	2.9	8.5	2.7498	−0.0658	0.9997
RSG	4.1	0.9	4.7	1.1					

[a] $n = 12$, $c = 500$ nmol L^{-1}.
[b] $n = 5$, $c = 500$ nmol L^{-1}.

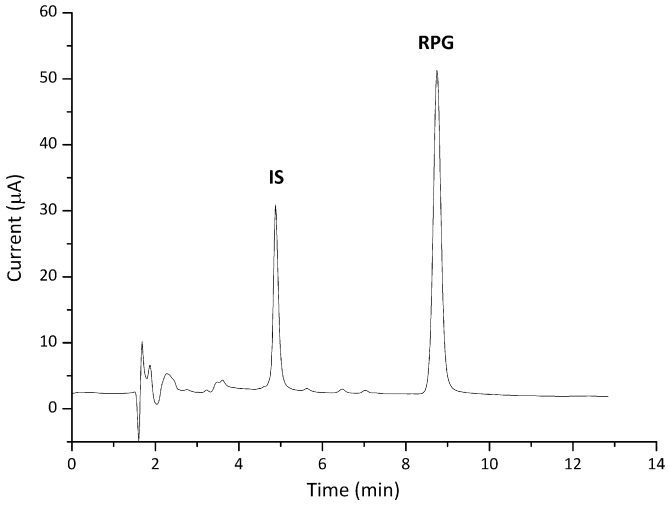

Fig. 5. A chromatogram showing separation of RPG and internal standard (RSG). The chromatographic conditions used were as follows: Stationary phase – a Varian Polaris™ AQ 3 μm 150 × 3.2 mm I.D. column, mobile phase – 50 mM sodium phosphate dibasic/acetonitrile (55/45, v/v), adjusted with phosphoric acid, final pH 7.5. Flow rate 0.40 mL min^{-1}, manual injection, sample size 10 μL (0.02 mg mL^{-1} RPG +0.02 mg mL^{-1} IS, 1:1). Detection – coulometric, applied voltage +750 mV (*vs.* Pd/H^2) (first channel).

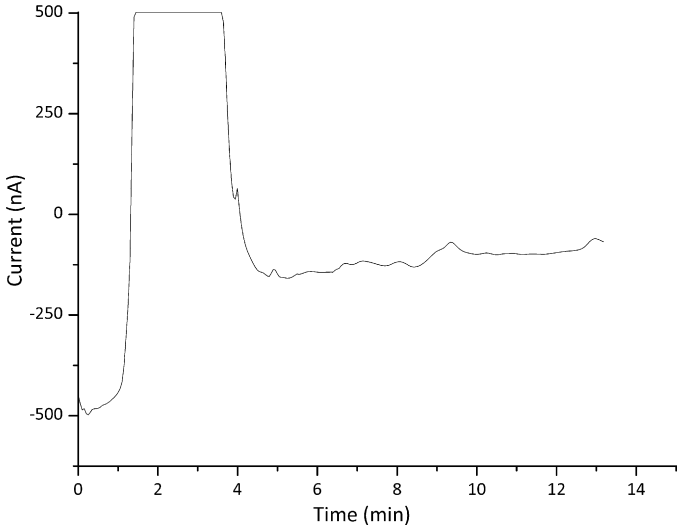

Fig. 6. A typical chromatogram of blank plasma. For conditions see Section 2.

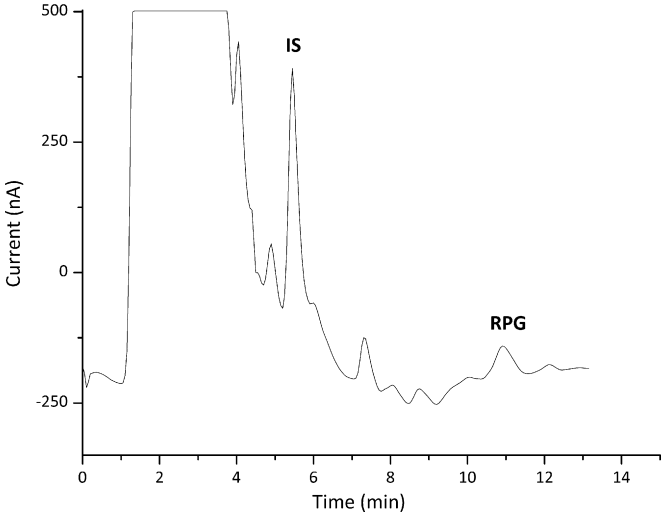

Fig. 7. A chromatogram of ethyl acetate extract of human plasma; RSG used as an internal standard (5×10^{-7} mol L^{-1}). Plasma spiked with RPG (3×10^{-8} mol L^{-1}). For other conditions see Section 2.

3.4. Determination of RPG in plasma

We used RSG as an internal standard. RSG, a synthetic thiazolidinedione derivative (glitazone) is another PAD used for NIDDM treatment [25]. It proved to be a good candidate as it is naturally electroactive, exhibits similar plasma recovery and does not interfere with other peaks eluting from plasma. The calibration using internal standardization method provided significantly better linearity and correlation compared with direct calibration of analyte spiked plasma without internal standard. The calibration curve of RPG in human plasma (measured as peak area ratios RPG/IS) was found to be linear over the concentration range measured. The calibration parameters, LOD, LLOQ, intra-day and inter-day precisions for retention times as well as peak areas are summarized in Table 1.

Typical chromatograms of blank plasma and a plasma sample spiked with RPG and internal standard, processed by liquid–liquid extraction, are shown in Figs. 6 and 7.

As the reported therapeutic average plasma concentration level achieved 2 h after administration is about 11 nmol L^{-1} (5 μg L^{-1}), proportionally growing after multiple doses [13] the described method meets the limits required for the determination of RPG in plasma. For the higher concentration levels than normally occurring in plasma, RPG can also be detected in the redox mode as discussed in Section 3.1. Such arrangement offers enhanced detection selectivity, and the analyte peak identity in a sample can be additionally confirmed by the presence of the corresponding reduction peak. It should be mentioned that the presented method can be also used vice versa for the analysis of RSG in plasma with RPG as an internal standard.

4. Conclusion

In the present study, a simple and sensitive HPLC method based on dual-channel coulometric detection for the determination of the peroral antiabetic drug RPG was developed.

RPG exhibits electrochemical activity – two well defined anodic waves can be observed using cyclic and differential pulse voltammetry at the glassy-carbon electrode. This property is advantageous for the sensitive direct determination of RPG using a HPLC-ED method.

We demonstrated that HPLC with coulometric detection is a very useful method for the determination of RPG in human plasma at therapeutic concentration levels. It offers high sensitivity in virtue of high conversion degrees inherent for coulometric principles, and lacks typical electrode response instability, common for the amperometric sensors. The developed HPLC-ED method requires 250 μL of plasma, employs only facile and fast deproteination based on liquid–liquid extraction into ethyl acetate, and with the reached LLOQ (8.5 nmol L^{-1}) it is suitable for either pharmacokinetic studies or therapeutic monitoring of the drug in targeted treatment of patients, suffering from type 2 diabetes mellitus.

Acknowledgments

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