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HPLC study on Fenton-reaction initiated oxidation of salicylic acid. Biological relevance of the reaction in intestinal biotransformation of salicylic acid.

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Running title: In vitro - in vivo hydroxilation of salicylic acid.

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Keywords

Fenton reaction
Reactive oxygen species (ROS)
Salicylic acid
Non-steroidal anti-inflammatory agents (NSAIDs)

Diabetes

Abbreviations:
HPLC: high performance liquid chromatography
UV-Vis: ultraviolet-visible
MS: mass spectrometry
µl: microliter
mM: millimol/liter
µM: micromol/liter
ROS: reactive oxygen species
CAT: catechol
SA: salicylic acid
2,3-DHBA: 2,3 dihydroxybenzoic acid
2,4-DHBA: 2,4-dihydroxybenzoic acid

2,5-DHBA: 2,5-dihydroxybenzoic acids
2,6-DHBA: 2,6-dihydroxybenzoic acid
4-NP: 4-nitrophenol
STZ: streptozotocin
NADPH: reduced nicotinic acid adenine dinucleotide phosphate

Abstract:

Fenton-reaction initiated *in vitro* oxidation and *in vivo* oxidative biotransformation of salicylic acid was investigated by HPLC-UV-Vis method. By means of the developed HPLC method salicylic acid, catechol and all the possible monohydroxylated derivatives of salicylic acid can be separated. Fenton oxidations were performed in acidic medium (pH 3.0) with two reagent molar ratios: (1) salicylic acid: iron: hydrogen peroxide 1:3:1 and (2) 1:0.3:1. The incubation samples were analyzed at different time points of the reactions. The biological effect of elevated reactive oxygen species concentration on the intestinal metabolism of salicylic acid was investigated by an experimental diabetic rat model. HPLC-MS analysis of the *in vitro* samples revealed presence of 2,3- and 2,5-dihydroxybenzoic acids. The results give evidence of non-enzyme catalyzed intestinal hydroxylation of xenobiotics.

1. Introduction

There is an increasing number of lines of epidemiological evidence that reactive oxygen (ROS) and nitrogen (RNS) species are implicated in the pathogenesis of several chronic diseases, such as cancer, cardiovascular and neurodegenerative diseases [1-3]. These reactive species can react with sensitive endogenous and exogenous molecules to form characteristic products. It is well documented in the literature that several acute and chronic diseases are accompanied by elevated level of reactive oxygen species [4]. Among others, diabetes and acut or chronic inflammations are those ones that can cause increased non-enzymatic oxidation of both endogenous compounds and xenobiotics [5].

A large number of drug molecules containing aromatic rings are administered in the above disorders in which involvement of oxidative damage has been suggested. Anti-inflammatory drugs have been reported to scavenge hydroxyl radicals generated in solution at almost diffusion-controlled rates (rate constants about 10^{10} M^{-1} s^{-1}) [6]. Based on the latter reactivity, aromatic hydroxylation can be used for measuring *in vitro* hydroxyl radical production [7].

Acetylsalicylic acid (aspirin) is a widely used medication to treat pain, fever, and inflammation. Salicylic acid (SA) is the main primary metabolite of acetylsalicylic acid (ASA) [8]. The hydrolytic transformation is rather fast; the halflife of ASA is about 20 minutes [9, 10]. Accordingly, pharmacological effects of aspirin substantially involve those of salicylic acid [8]. The major route of metabolism of ASA and SA in humans is conjugation with glycin and glucoronic acid [10, 11]. Two oxidative metabolites of salicylic acid are 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) [10, 12, 13]. These products can be attributed to oxidation by cytochrome P450 enzymes as well as Fenton-type non-enzymatic hydroxylation. Attack by hydroxyl radicals has been reported to form the 2,3-DHBA and is speculated to form some of the 2,5-DHBA product as well [5, 7, 12-15].

In our earlier study, *in vivo* intestinal absorption and oxidative metabolism of salicylic acid (SA) was investigated in rats. In the intestinal perfusate low levels of 2,3-DHBA and 2,5-DHBA were detected and identified by HPLC-MS [16]. As a continuation of our earlier study here we report on the similar investigations performed with hyperglycemic rats. Hyperglycemia is one of the leading factor of diabetic complications. It can result oxidative stress and accompanied by increased formation of reactive oxygen species (ROS) [17].

Fenton reaction is a tool for modeling oxidative stress by forming hydroxyl radicals in a reaction of hydrogen peroxide and some transitional metals. The key features of the reaction are believed to be reaction conditions, such as reagent concentrations, pH and temperature [18]. The exact nature of the oxidizing intermediates involved in Fenton reaction is still to be identified, but there are several views about the mechanism and the nature of intermediate products which

may have effect on the outcome of the reaction [19]. One of the important processes during the reaction is the iron(II)/iron(III) redox cycling because the iron(II) regeneration is the rate limiting step of the Fenton system [20]. The optimal pH for this reaction and for the formation of hydroxyl radicals is in the acidic range, near pH 3 [21]. In the presence of salicylic acid complex formation between iron ions and salicylic acid may also influence the quality and the quantity of the reaction products [22-24].

To identify and quantify the largest number of possible oxidative products of salicylic acid under the investigated conditions, an HPLC-UV-Vis coupled with an HPLC-MS method was developed. Earlier, identification and quantitation of the hydroxylated derivatives has been accomplished by GC [25-27] and RP-HPLC methods [13, 28-32]. None of the published methods can be applied, however, for separation of the parent salicylic acid and all the previously known salicylic acid oxidation products.

In our present experiments Fenton-oxidation of salicylic acid was carried out in an acidic medium (pH 3.0) with two reagent (molar) ratios: (1) salicylic acid: iron(II): hydrogen peroxide 1:3:1, and (2) salicylic acid: iron(II): hydrogen peroxide 1:0.3:1. Under the first condition large excess of iron(II) was used, which can act as a hydroxyl radical scavenger in the absence of organic substrates [33]. The latter conditions (iron(II): hydrogen peroxide appr. 1:3) is like that one reported to be the most effective oxidative conditions [34]. Under such conditions all possible oxidative derivative of salicylic acid is expected to be formed.

In the *in vivo* experiments, we aimed to investigate how the experimental diabetes affects the intestinal elimination and metabolism of salicylic acid. To receive information about the possible role of hyperglycemia-induced ROS formation in biotransformation of phenolic xenobiotics, jejunal perfusion of streptozotocin (STZ) -treated animals with sodium salicylate was performed and its elimination and oxidative metabolism was studied to compare the results with those obtained in the non STZ-treated rats [16].

2. Materials and methods

2.1. Chemicals

2,3-Dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) standards were purchased from Sigma-Aldrich (Budapest, Hungary). Salicylic acid (SA), 2,4-dihydroxybenzoic acid (2,4-DHBA)and 4-nitrophenol (4-NP) standards were obtained from Fluka (Budapest, Hungary).Sodium sulfate, potassium chloride, magnesium sulfate, calcium chloride dihydrate, glucose, mannitol and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Spektrum-3D Kft. (Debrecen, Hungary). HPLC-grade methanol and *tert*-butyl methyl ether were obtained from VWR International Kft. (Debrecen,

Hungary). HPLC-grade formic acid was purchased from Sigma-Aldrich (Budapest, Hungary). Iron(II)sulfateheptahydrate, catechol (CAT) and 2,6-dihydroxybenzoic acid (2,6-DHBA) were purchased from Reanal (Budapest, Hungary). Sulfuric acid and concentrated hydrogenperoxide (30 % v/v) were purchased from Molar Chemicals (Budapest, Hungary). Distilled water was purified in the Department of Pharmaceutical Chemistry, University of Pécs by use of a Purelab Option Q7 Water System.

2.2. Fenton incubations

In order to examine the outcome of the Fenton reaction with different iron (II) ion concentrations and with different incubation time intervals 1.00 mL samples were prepared. Each incubate contained 700 µL of sulfuric acid (pH 3.0), 100 µL of 10 mM salicylic acid solution, 100 µL of 30 mM or 3 mM iron(II) solution and 100 µL of 10 mM hydrogen peroxide solution. The components were mixed in the respective order and the reaction mixtures were placed in a 37°C water bath. The samples were analyzed after „0", 1, 5, 10 and 30 minutes of incubation. Before analysis the incubates were concentrated with liquid-liquid extraction.

2.3. Quantum chemical calculations

Quantum Chemical calculations were performed using Chem 3D Ultra 10.0 (Cambridge Scientific Computing, Inc.) molecular modeling and analysis software product. Mulliken atomic charges were calculated by means of the MOPAC interface on the MM2-minimized ground state structures. Mulliken atomic charge data are summarized in **Table 1**.

2.4. Animal experiments

Diabetic male Wistar rats (n=5; average bodyweight: 234.7 g; average blood glucose concentration: 23.1 mmol/l) were treated according to the experimental protocol of Bojcsev et al. [35].The experimental diabetes was induced by 65 mg/kg intravenous streptozotocin injection pretreatment one week before the intestinal perfusion. The animals were anesthesized by 1.2 g/kg intraperitoneal urethane injection, then a 10 cm long jejunal segment was isolated and cannulated. The intestinal perfusion was carried out by a peristaltic pump (flow rate: 13 ml/min) with 250 µM sodium-salicylate dissolved in isotonic perfusion medium. During the 90 minute time-course of the perfusion, samples with the volume of 250 µl were collected in previously determined time points. The chromatographic analysis of the perfusion samples was performed immediately after the sample collection and sample preparation.

Animals were kept according to standard protocols controlled by institutional regulations, which adhere to the Act of Animal Care issued by the Hungarian Ministry of Economy and Environmental Protection which is based on appropriate EU regulations.

2.5. Sample preparation method

Both the *in vitro* and the *in vivo* samples were concentrated and purified by the following liquid-liquid extraction method.Before extraction, 10 µL of 1 mg/mL 4-nitrophenol solution as an internal standard was added to the samples. Afterwards, the samples were acidified with 10 µL of 2 M sulfuric acid and vortex mixed for 30 seconds. Then 500 µL of tert-butyl methyl ether was added and vortex mixed for 30 seconds. The upper phase was separated and 300 µL of formic acid solution (pH 2.6) was added in order to purify it. After 30 seconds of vortex mixing the purified upper phase was separated and was evaporated under nitrogen. The dry residue was dissolved in 50 µL chromatographic mobile phase (pH 2.6 formic acid/methanol; 77/23; v/v).

3. Instrumentation and chromatographic conditions

3.1. Agilent 1100 HPLC system

Analysis of the extracted incubates and identification of the formed products was performed by an Agilent 1100 HPLC system.
The high performance liquid chromatography system was equipped with a quaternary HPLC pump (G1311A), a degasser (G1379A), an autosampler (G1313A), a thermostated column compartment (G1316A) and a diode-array detector (G1315B). Data were recorded and evaluated by Agilent ChemStation software (Rev.B.03.02-SR2).

Chromatographic separation of the hydroxylated products was performed through a reversed-phase chromatographic column (Poroshell 120 EC-C18, 4.6 mm x 150 mm, 2.7 µm particle size). The mobile phase consisted of formic acid solution (pH 2.6) and methanol (77:23, v/v). Runtime was 30 min. The isocratic separation was performed at 0.600 mL/min flow rate at 45 °C temperature. The injected volume was 5 µL. Detection was performed at 237 nm.

3.2. Jasco HPLC/Waters MS system

The integrated high performance liquid chromatography system (Jasco) was equipped with an intelligent HPLC pump (PU-980), a degasser, a manual injector (RHEODYNE 7725i) with a 5 µL loop, a column oven and a diode-array detector (MD-2010). Data were recorded and evaluated by ChromNav software (ver. No. 1.21). Jasco LC-system is connected with a Waters Xevo TQ-S Mass Detector. MS data were recorded and evaluated by MassLynx V4.1 software.
Separation of compounds was performed on a 4.6 mm x 150 mm, 2.7 µm particle size, PowerShell 120 EC-C18 analytical column. The mobile phase consisted of formic acid solution (pH 2.6) and methanol (77/23, v/v). Chromatography was performed at 45 °C, the flow rate was 0.6 mL/min. The injected volume was 5 µL. MS measurement was performed in negative ion mode. The ESI source was operated with a spray voltage of 3.00 kV, cone voltage was 30 V. Desolvation gas was delivered at 500 L/hour and temperatured at 500 °C. Cone gas was delivered at 150 L/hour. Collision gas flow was 0.13 mL/min. Full-range mass spectra (80–400 m/z) were collected under the optimal conditions. During daughter scan measurement (20-420 m/z) of the selected parent ion the collision energy was 20 eV.

3.3. Preparation of working standard solutions and HPLC-MS stock solutions

10 mM (0.0138 g/10.0 mL) stock solution of SA was prepared in distilled water. 10 mM hydrogen peroxide solution was obtained by dilution of 11.3 µL concentrated hydrogen peroxide (30 % v/v) with distilled water to 10.0 mL. 30 mM (0.0834 g/10.0 mL) iron(II) sulfate stock solution was prepared in sulfuric acid (pH 3.0). The 3 mM iron(II) sulfate solution was prepared by tenfold dilution of 1.0 mL of the 30 M stock solution with sulfuric acid. For analytical method development 1 mg/ml acetonitrile solutions were prepared from CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA, 2,6-DHBA, SA and 4-nitrophenol. The working standard solutions were always prepared freshly before the experiments; the stock solutions were stable at least for 1 month, stored at -8°C.

4. Validation of the chromatographic method

4.1. Linearity

Linearity was studied by preparing standard acetonitrile solutions of CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA, 2,6-DHBA and SA at seven different concentration points between 0.5 µg/ml and 50 µg/ml (0.5; 0.75; 1; 5; 10; 25; 50 µg/mL). Data were obtained from three parallel injections of two independent weighings (6 injections altogether) applied at each concentration level. Calibration curves were created by plotting the theoretical concentrations against the peak areas. Linearity was determined by least-squares regression. Linearity data are summarized in **Table 2**.

4.2. System suitability

System suitability data were extracted from chromatograms of the standard solutions of CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA, 2,6-DHBA and SA, 10 µg/mLeach. Results were obtained from five parallel injections. System suitability data are summarized in **Table 3**.

4.3. Precision

Precision was studied by investigating repeatability and intermediate precision. Repeatability was determined by measuring intra-day data of 3 parallel injections of 3 parallel dilutions of 2 independent weighings at three different concentration levels (1; 10; 50 µg/mL). Intermediate precision was determined by measuring inter-day (by injection of the samples over three consecutive days) data of 3 parallel injections of 3 dilutions from two weighings at three different concentration levels (1; 10; 50 µg/mL).Evaluation was based on relative standard deviation (RSD %). Data for precision were summarized in **Tables 4 and 5**.

4.5. Determination of LOD and LOQ

Limit of detection (LOD) was determined experimentally, and taken as the concentration producing a detector signal that could be clearly distinguished from the baseline noise (3 times baseline noise). The limit of quantification (LOQ) taken as the concentration that produced a detector signal tentimes greater than the baseline noise. The LOQ values for 2,3-DHBA, 2,4-

DHBA, 2,5-DHBA, 2,6-DHBA and SA was 0.5 µg/mL and for CAT, were 1.0 µg/mL. The LOD values for for 2,3-DHBA, 2,4-DHBA, 2,5-DHBA, 2,6-DHBA and SA was 1.0 µg/mL and for CAT, were 5.0 µg/mL.

4.6. Specificity

Specificity was defined as the ability of the method to differentiate and quantify the analytes in the presence of endogenous constituents of the perfusate sample. **Figure 6.** depicts the representative HPLC chromatograms of the 0 minute perfusate containing only the isotonic perfusion medium, 250 µM sodium salicylate and the internal standard, 4-NP (**Figure 6a**) and the perfusate generated in rat small intestine luminal perfusion experiments at the 45th minute of the perfusion period (**Figure 6b**). There are no endogenous peaks in the regions of the retention times of CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA, 2,6-DHBA, SA and 4-NP.

4.7. Precision of liquid–liquid extraction

Precision of liquid-liquid extraction was also studied by preparing standard solutions of CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA and SA at five different concentrations ranging from 500 to 10000 ng/mL (500, 750, 1000, 5000 and 10000 ng/mL) using the chromatographic mobile phase (pH 2.6 formic acid/methanol; 77/23; v/v) as solvent. Each solution had a known concentration (10 µg/mL) of 4-NP as internal standard. Data were obtained from three parallel injections of two independent weighings applied at each concentration level. Calibration curves were generated by plotting the theoretical concentrations against the peak areas related to the internal standard. Linearity was determined by least-squares regression calculated from six injections. The method was linear with respect to each analyte over the examined range, where y is the ratios of the peak areas of the examined compound and the internal standard and x is the concentration of the examined compound expressed in ng/mL (CAT: $y = 0.00001x - 0.00039$, $r^2 = 0.9978$; 2,3-DHBA: $y = 0.00003x - 0.00188$, $r^2 = 0.9992$; 2,4-DHBA: $y = 0.00005x + 0.00248$, $r^2 = 0.9999$; 2,5-DHBA: $y = 0.00005x + 0.00005$, $r^2 = 0.9995$; SA: $y = 0.00006x - 0.00570$, $r^2 = 0.9996$).

4.8. Recovery of liquid–liquid extraction

Dilution series of CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA and SA using pH 3.0 sulfuric acid as solvent were prepared at three levels of concentration (1000, 500 and 100 ng/mL). Each solution had a known concentration (10 µg/mL) of 4-NP as internal standard. Application of the above liquid–liquid extraction ('Extraction method') five parallel extractions were performed at each concentration level. After dissolution of the dry residues, correlation between the concentration of the standards and the measured peak areas related to that of the internal standard was determined. The 1000 ng/ml samples resulted in 47.95%, 84.6%, 51.14%, 54.35% and 53.99 % mean recoveries for CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA and SA, respectively. Similar data of the 500 ng/ml samples were 50.84%, 78.75%, 58.7%, 61.14% and 54.0% for CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA and SA, respectively. Reliable direct recovery determination at the lowest investigated concentration level (100 ng/mL) was not possible,

however, regarding the linearity data and the RSD% values (see **Table 6**), the extraction was reproducible at that concentration level as well.

5. Results

The validated HPLC-DAD method was applied for monitoring the jejunal elimination of salicylic acid in samples generated in rat small intestine luminal perfusion experiments. **Figure 5**shows the amount of salicylic acid which is continuously decreased from the 15th minutes until the end of the perfusion experiment.

When hydroxyl radicals react with SA, it may follow one of the three paths: (1) hydrogen atom abstraction, (2) addition to the aromatic ring and (3) radical substitution [36]. All the previously published primary Fenton-oxidation products of SA (CAT, 2,3-DHBA, 2,4-DHBA and 2,5-DHBA) can be considered as a result of hydrogen atom abstraction or addition reactions of the hydroxyl radicals and the aromatic ring.

The developed HPLC method was suitable for separation of SA, CAT, 2,3-DHBA, 2,4-DHBA, 2,5-DHBA and 2,6-DHBA, all the possible primary oxidation products of Fenton reaction of SA. (**Figure 1)** The products in the incubates were identified based on their chromatographic retention times (see **Table 3**) and their structure was verified by means of mass spectrometric measurements. The Fenton oxidation of SA under the investigated conditions resulted in formation of 2,3-DHBA, 2,4-DHBA, 2,5-DHBA and CAT. 2,6-DHBA could not be found in the samples. (**Figures 2a and 2b**)

After evaluation of the chromatograms it could be ascertained that 2,3-DHBA and 2,5-DHBA were formed in the biggest amount as monohydroxylated derivatives of SA. In smaller amounts CAT and 2,4-DHBA could also be identified in the incubates. At t^R= 4.876 min a peak appeared, which was not identical to either of the standards used in the present analytical method. MS measurements identified the compound as trihydroxybenzoic acid with 169 *m/z* (**Figures 3a and 3b**).

For determination of relative amount of the products, relative ratios of the peak areas to that of the internal standard was calculated. To assign, how iron(II) concentration and incubation time affected the product distribution, the relative amounts of SA and its hydroxylated products was plotted as a function of the incubation time. The product distributions in the different sets of experiments can be seen in **Figure 4a and 4b**.

In the samples containing 1 mM SA, 3 mM iron(II) and 1 mM hydrogen peroxide, the amount of SA only slightly changed during the incubation time. The amount of 2,5-DHBA continuously decreased while concentration of 2,3-DHBA remained unchanged during the time of the experiment.

In the incubates containing 1 mM SA, 0.3 mM iron(II) and 1 mM hydrogen peroxide, the initial concentration of the unreacted SA was twice as high over the first fifth minutes as

in those with the 3 mM iron(II) concentration. At the later sampling times the amount of SA was comparable to those measured in the samples using 3 mM iron(II). The area of 2,5-DHBA showed a similar kinetic pattern to that one observed in the experiment using 3 mM iron(II); it continuously decreased during the incubation time. The amount of 2,3-DHBA increased until the fifth minute of incubation, after that, a "steady-state" concentration could be observed with no significant change.

In vivo intestinal elimination and oxidative metabolism of SA was investigated by the newly developed HPLC-UV-Vis method applying the same experimental protocol as used earlier with non-hyperglycaemic rats [16]. Based on the HPLC-UV-Vis studies, continuous decrease of the amount of SA could be detected in the small intestinal perfusates from the 15th minute of the experiment. The time course of the intestinal elimination of the compound in the hyperglycaemic animals (**Figure 5**) showed no significant difference in comparison to that one observed in the non-STZ-treated rats [10]. Besides the parent compound (and the internal standard) two more peaks corresponding to more polar compounds with t^R= 6.45 and 7.84 min retention times appeared in the chromatograms (**Figure 6b**). By means of mass spectrometric analysis they could be identified as 2,5-DHBA and 2,3-DHBA, respectively.

6. Discussion

Our experimental results support the earlier observations that in *in vitro* Fenton reaction of SA 2,3-DHBA and 2,5-DHBA are produced in the largest amount as primary monohydroxylated products [30]. It was also noticed that 2,3-DHBA was formed in the bigger amount. This observation is in accordance with the calculated atomic charges showing the C3 and C5 atoms of SA possessing the two highest electron densities. (**Table 1**.) The result also strengthens the earlier observations that increased amount of 2,3-DHBA is a diagnostic value of non-enzymatic hydroxylation of SA [5,7]. Furthermore, formation of trihydroxybenzoic acid was detected and identified as a result of further oxidation of the DHBAs (**Figure 3a and 3b**). This latter observation draw attention to further oxidative transformation of the primary dihydroxybenzoic acid metabolites. Further oxidative transformations are supported by the electron densities calculated for the four isomeric DHBAs. (**Table 1**) Such transformations can occur under physiological conditions (e.g., at the sites of inflammation) and can be important from both chemical and biological points of views [37].

For evaluation of the results, it is important to note that hydrogen peroxide was added to the mixture of SA and iron(II) ions. Under such conditions it can be assumed that in the samples containing 0.3 mM iron(II) most of the iron(II) ions are complexed wit SA [38]. On the contrary, the greater proportion of the iron(II) ions are in non-complexed form in the samples with 3 mM iron(II) concentration.

Non-complexed iron(II) ions are readily oxidized to iron(III) which form very stable complex with SA. (The violet color of the reaction mixtures that could be observed after mixing the reactants are in agreement with this reaction.) Since non-complexed iron(II) ions react with hydroxyl radicals in a very fast reaction ($Fe^{2+} + \cdot OH = OH^- + Fe^{3+}$) [39] this reactivity can be the reason why the amount of SA only slightly changed during the incubation time in the samples containing 1 mM SA and 3 mM iron(II). 2,3-DHBA is a powerful iron-chelating molecule [40,41] while 2,5-DHBA has been reported a powerful radical scavenger [40]. This latter activity can be the reason for its decreasing concentration in the incubations.

In the case of incubations containing 1 mM SA, 0.3 mM iron(II) and 1 mM hydrogen peroxide, most of the iron(II) ions are complexed with SA [38]. Under such conditions the initial salicylate concentrations were found to be much higher than those measured in the incubations containing 3 mM iron(II). This observation is in accordance with the low Fenton-type reactivity of salicylate-complexed iron(II) ions [38]. Change in 2,3-DHBA and 2,5-DHBA concentrations showed similar patterns to those observed in the case of experiments using 3 mM iron(II).

Furthermore, it is reasonable to suppose thatin the incubations containing 0.3 mM iron(II) less (if any) non-complexediron(III) ions -formed in the Fenton oxidation - could participate in the redox cycle which is the driving force of the reaction and responsible for the continuous production of the oxidizingintermediates[42]. The amount of the reaction products also supports this presumption because in the incubate with 3 mM iron(II) the reaction yielded significantly more oxidizedproducts than in that with 0.3 mM iron(II).

The validated HPLC-UV-Vis method was applied for monitoring the jejunal elimination of SA in samples generated in rat small intestine luminal perfusion experiments. **Figure 5** shows the amount of salicylic acid which is continuously decreased from the 15th minutes until the end of the perfusion experiment. Although the timecourse of elimination of SA is very much the same as that observed in similar experiment wit non-hyperglycaemic rats, the amount of eliminated salicylate (appr. 80 % of the initial concentration) was found to be much higher than that (appr. 60%) found in the non STZ-treated rats [10]. Recent studies revealed that ASA causes small intestinal mucosal injury at a considerably higher rate than previously believed. The first step of NSAID-induced enteropathy is the increase of small intestinal epithelial cell permeability [43]. It has also been reported that ASA also increases intestinal epithelial cell permeability as well as conventional NSAIDs in human studies [44]. Based on these literature data and the observed increased elimination of SA from the small intestine, it is reasonable to presume that SA, like ASA, can also increase permeability of epithelial cells. Level of SA in the perfusate, however, is the net result of the kinetics of twodirectional transport of the drug between the lumen and the epithelial cells [45].

HPLC-UV-Vis measurements indicated that the absorbed salicylate can be metabolized in the proximal jejunal loop and its two hydroxylated metabolites (2,5-DHB and 2,3-DHB) appeared in the intestinal lumen (**Figure 6b**).

In the small intestine of untreated rats CYP1A1, 2B1, 2C6, 2C11, 2D1-4 and 3A1 were found to be expressed [46]. Among the CYP isoforms reported to metabolize SA, however, only the CYP3A1 (homologue of the human CYP3A4) expressed in the rat small intestine, which showed catalyzing almost exclusive formation of 2,5-DHBA [47]. Accordingly, the increased amount of hydroxylated metabolites – 2,3-DHBA, in particular - are formed in non-CYP catalyzed, hydroxyl radical initiated hydroxylation reaction. This finding indicates that non-enzymatic hydroxylation plays an important role in the biotransformation of phenolic xenobiotics.

One of the major source of ROS formation in diabetic patients are NADPH oxidases of the Nox family [48]. Nox1 is particularly highly expressed in the epithelial cells of the gastrointestinal tract [49]. Another source of ROS could be the increased myeloperoxidase activity of the intestinal mucosa caused by the local inflammation caused by the relatively high SA level [10]. As the consequence of the hydroxyl radical scavenger effect of SA, its aromatic hydroxylation can occur at the site of formation of the reactive oxygene species [50]

Our earlier results indicated low levels of 2,3- and 2,5-DHBA in the same experiments performed with non STZ-treated rats. In those experiments small amounts of 2,3- and 2,5-DHBAs could be detected and identified by HPLC-MS. The levels of the formed compounds did not reach the LOQ of the analytical method (0,5 μg/mL and 0.75 μg/mL, respectively) [16]. The respective LOQ of the present method is 1 μg/ml for both DHBAs and the quantitated amounts of the compounds in the 90 minute perfusate are 1.076 μmol/L and 0.242 μmol/L , respectively. Accordingly, hyperglycaemia increased the amount of hydroxyl radical mediated intestinal biotransformation of salicylic acid.

7. Summary

Non-enzymatic *in vitro* and *in vivo* oxidation of SA was investigated by a newly developed HPLC-UV-Vis method. The method was used to analyze product composition of oxidation products of SA (CAT and isomeric dihydroxybenzoic acids) under different experimental conditions. It was found that the primary hydroxylated metabolites of SA can be further metabolized under the Fenton's conditions. *In vivo* experimental results with hyperglycaemic rats also indicated non-enzyme catalyzed hydroxylation of salicylate in the small intestine of hypertglycaemic rats. The results give further evidence for involvement of *in vivo* hydroxyl radical mediated biotransformation of salicylates at the sites where high hydroxyl radical level could orrur.

8. Conflict of interest

The authors confirm that this article content has no conflict of interest.

9. Acknowledgements

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10. Figure captions:

Figure 1: HPLC-UV-Vis chromatogram (λ=237 nm) of the system suitability solution containing catechol (CAT), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,4-dihydroxybenzoic acid (2,4-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 2,6-dihydroxybenzoic acid (2,6-DHBA), salicylic acid (SA) (25 μg/mL for each compound) and 4-nitrophenol as an internal standard (10 μg/mL)

Figure 2a: HPLC-UV-Vis chromatogram (λ=237 nm) of the 10 min Fenton incubate of salicylic acid with 3 mM iron(II)

Abbreviations: SA: salicylic acid; 2,3-DHBA: 2,3-dihydroxybenzoic acid; 2,4-DHBA: 2,4-dihydroxybenzoic acid; 2,5-DHBA: 2,5-dihydroxybenzoic acid; CAT: catechol

Figure 2b: HPLC-UV-Vis chromatogram (λ=237 nm) of the 10 min Fenton incubate of salicylic acid with 0.3 mM iron(II)

Abbreviations: SA: salicylic acid; 2,3-DHBA: 2,3-dihydroxybenzoic acid; 2,4-DHBA: 2,4-dihydroxybenzoic acid; 2,5-DHBA: 2,5-dihydroxybenzoic acid; CAT: catechol

Figure 3a: The mass spectrum belonging to the peak at t=4.876 min.

Figure 3b: The daughters of the m/z 169 parent ion at t=4.876 min.

Figure 4a: Product distribution in Fenton incubations with 3 mM iron(II) ions

Figure 4b: Product distribution in Fenton incubations with 0.3 mM iron(II) ions

Figure 5: Intestinal elimination of sodium salicylate in diabetic rat during the course of a 90-minute intestinal perfusion. The results were obtained from 5 independent measurements.

Figure 6a: HPLC-UV-Vis chromatogram (λ=237 nm) of the 0 min intestinal perfusion sample of diabetic rat with 250 μM sodium salicylate**.**

Figure 6b: HPLC-UV-Vis chromatogram (λ=237 nm) of the 45 min intestinal perfusion sample of diabetic rat with 250 μM sodium salicylate.

Abbreviations: SA: salicylic acid; 2,3-DHBA: 2,3-dihydroxybenzoic acid; 2,5-DHBA: 2,5-dihydroxybenzoic acid, 4-NP: 4-nitrophenol (IS).

Table 1: Mulliken atomic charges

Table 2: Linearity data

Table 3: System suitability data

Table 4: Data for intra-day precision

Table 5: Data for inter-day precision

Table 6: Data for precision of liquid-liquid extraction

11. References

(1) Essman W.B., Wollman S.B., 1990: Free Radicals, Central Nervous System Processes and Brain Functions, in Das D.K., Essman W.B. (eds): *Oxygen Radicals: Systemic Events and Disease Processes*. Karger, Basel, Chapter 7, pp 172-192. DOI: 10.1159/000418008

(2) Favier A.E., 1995: How to demonstrate the occurrence of an oxidative stress in human?, in Favier A.E., Cadet J., Kalyanaraman B., Fontecave M., Pierre J.-L. (eds): *Analysis of Free Radicals in Biological Systems*; Birkhauser, Basel, pp. 99-117. DOI: 10.1007/978-3-0348-9074-8_8

(3) Sies H. (ed), 1997: Antioxidants in Disease Mechanisms and Therapy. Adv. Pharmacol. Vol. 38. Academic Press, San Diego.

(4) Ziech D., Franco R., Georgakilas A.G., Georgakila S., Malamou-Mitsi V., Schoneveld O., Pappa A., Panayiotidis M.I., 2010: The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development, *Chem-Biol Interact* 188, 334–339. 10.1016/j.cbi.2010.07.010

(5) Ghiselli A., Laurenti O., de Mattia G., Maiani G. and Ferro-Luzzi A.,1992: Salicylate Hydroxylation as an Early Marker of In Vivo Oxydative Stress in Diabetic Patients, *Free Rad Biol Med* 13, 621-626. DOI: 10.1016/0891-5849(92)90036-g

(6) Hiller K.O., Hodd P.L., Willson R.L., 1983: Antiinflammatory drugs: protection of a bacterial virus as an in vitro biological measure of free radical activity. *Chem Biol Interact* 47, 293-305. DOI: 10.1016/0009-2797(83)90165-5

(7) Halliwell B., Kaur H., Ingelman-Sunndberg M., 1991: Hydroxylation of salicylate as an assay for hydroxyl radicals: A cautionary note. *Free Rad Biol Med*, 10, 439-441. DOI: 10.1016/0891-5849(91)90052-5

(8) Schrör K., 2009: Acetylsalicylic acid, Wiley-VCH, Weinheim

(9) Mays D.C., Sharp D.E., Beach C.A., Kershaw R.A., Bianchine J.R., Gerber N., 1984: Improved method for the determination of aspirin and its metabolites in biological fluids by high performance liquid chromatography: applications to human and animal studies, *J Chromatogr* 311, 301-309. DOI: 10.1016/s0378-4347(00)84723-4

(10) Hutt A.J., Caldwell J., Smith R.L., 1986: The metabolism of aspirin in man: a population study, *Xenobiotica*, 16, 239-249 DOI: 10.3109/00498258609043527

(11) Reidl U., 1983: Determination of acetylsalicylic acid and metabolites in biological fluids by high-performance liquid chromatography, *J Chromatogr B* 272, 325-331. DOI:

(12) Grootveld M., Halliwell B., 1986: Aromatic hydroxylation as a potential measure of hydroxyl-radical formation *in vivo*, *Biochem J* 237, 499-504.

(13) Grootveld M., Halliwell B., 1988: 2,3-Dihydroxybenzoic acid is a product of human aspirin metabolism,*Biochem Pharmacol 37,* 271-280. DOI: 10.1016/0006-2952(88)90729-0

(14) Coudray C., Talla M., Martin S., Fatome M., Favier A., 1995: High performance liquid chromatography-electrochemical determination of salicylate hydroxylation products as an in vivo marker of oxidative stress, *Anal Biochem* 227, 101-111.

(15) Ingelman-Sundberg M., Kaur H., Terelius Y., Person J.-O., Halliwell B., 1991: Hydroxylation of salicylate by microsomal fractions and cytochrome P-450, *Biochem J* 276: 753-757.

(16) Kuzma M., Nyúl E., Mayer M., Fischer E., Perjési P., 2016: HPLC analysis of in vivo intestinal absorption and oxidative metabolism of salycilic acid in the rat, *Biomed Chrom* 30, 2044-2052. DOI: 10.1002/bmc.3783

(17) Ullah A., Khan A., Ismail Khan I., 2016: Diabetes mellitus and oxidative stress—A concise review, *Saudi Pharm J* 24, 547-553. DOI: 10.1016/j.jsps.2015.03.013

(18) Neyens E., Baeyens J., 2003: A review of classic Fenton's peroxidation as an advenced oxidation technique, *J.Hazard Materials* 98, 33–50. DOI: 10.1016/s0304-3894(02)00282-0

(19) (a) Kremer M.L., 1999: Mechanism of the Fenton reaction. Evidence for a new intermediate, *Phys Chem Chem Phys* 1, 3595-3605. DOI: 10.1039/a903915e (b) Quian S.Y., Buettner G.R., 1999: Iron and dioxygen chemistry is an important route to initiation of biological free radical oxidations: An electron paramagnetic resonace spin trapping study, *Free Rad Biol Med* 26, 1447–1456. DOI: 10.1016/S0891-5849(99)00002-7

(20) Nichelaa A.D., Donadellia J.A., Carama B.F., Haddoub M., Rodriguez Nietoa F.J., Oliveros E., García Einschlag F.S., 2015: Iron cycling during the autocatalytic decomposition of benzoic acid derivatives by Fenton-like and photo-Fenton techniques, *Appl Catal B: Environ*170-171, 312–321. DOI: 10.1016/j.apcatb.2015.01.028

(21) Hickey W.J., Arnold S.M., Harris R.F., 1995: Degradation of atrazine by Fenton's reagent: condition optimisation and product quantification, *Environ Sci Technol* 29, 2083–2089. DOI: 10.1021/es00008a030

(22) Oktavia B., Lim L.W., Takeguchi T., 2008: Simultaneous determination of Fe(III) and Fe(II) ions via complexation with salicylic acid and 1,10-phenanthroline in microcolumn ion chromatography, *Anal Sci* 24, 1487-1492. DOI: 10.2116/analsci.24.1487

(23) Waquar D., Hussein W., 2011: Kinetics and mechanism of reduction of Fe(III) salicylate complex by hydroquinone, *Pak J Pharm Sci* 24, 31-35.

(24) Barbusiński K., Filipek K., 2001: Use of Fenton's reagent for removal of pesticides from industrial wastewater, *Polish J Environ Stud* 10, 207-212.

(25) Richmond R., B. Halliwell B., Chauhan J., Darbre A., 1981: Superoxide-dependent formation of hydroxyl radicals: Detection of hydroxyl radicals by the hydroxylation of aromatic compounds, *Anal Biochem* 118, 328-335. DOI: 10.1016/0003-2697(81)90590-x

(26) Luo X., Lehotay D.C., 1997: Determination of hydroxyl radicals using salicylate as a trapping agent by gas chromatography-mass spectrometry, *Clin Biochem* 30, 41–46. DOI: 10.1016/s0009-9120(96)00137-3

(27) Nguyen V., Bonds D.V., Prokai L., 2008:Measurement of hydroxyl-radical formation in the rat striatum by in vivo microdialysis and GC-MS, *Chromatographia* 68(Suppl 1), s57-s62. DOI: 10.1365/s10337-008-0703-6

(28) Bakar S.K., Niazi S., 1983: High performance liquid chromatographic determination of aspirin and its metabolites in plasma and urine, *J Pharm Sci* 72, 1020-1023. DOI: 10.1002/jps.2600720913

(29) O'Kruk R.J., Adams M.A., Philip R.B., 1984: Rapid and sensitive determination of acetylsalicylic acid and its metabolites using reversed-phase high-performance liquid chromatography, *J Chromatogr B* 310, 343-352. DOI: 10.1016/0378-4347(84)80099-7

(30) Coudray C., Mangournet C., Bouhadjeb S., Faure H., Favier A., 1996:Rapid high-performance liquid chromatographic assay for salicylic acid in plasma without solvent extraction.*J Chrom Sci* 34, 166-173. DOI:10.1093/chromsci/34.4.166

(31) Yamamoto E., Takakuwa S., Kato T., Asakawa N., 2007: Sensitive determination of aspirin and its metabolites in plasma by LC-UV using on-line solid-phase extraction with methylcellulose-immobilized anion-exchange restricted access media, *J Chromatogr B* 846, 132-138. DOI: 10.1016/j.jchromb.2006.08.037

(32) Bektaşoğlu B., Özyürek M., Güçlü K., Apak R., 2008: Hydroxyl radical detection with a salicylate probe using modified CUPRAC spectrophotometry and HPLC, *Talanta* 77, 90-97. DOI: 10.1016/j.talanta.2008.05.043

(33) Panchompoo J., Aldous L., Kabeshov M., Pilgrim B.S., Donohoeb T.J., Compton R.G., 2012: A green approach to Fenton chemistry: mono-hydroxylation of salicylic acid in aqueous medium by the electrogeneration of Fenton's reagent, *New J. Chem* 36, 1265–1272. DOI: 10.1039/c2nj21007j

(34) Walling C., 1975: Fenton's reagent revisited, *Acc Chem Res* 8, 125-131. DOI: 10.1021/ar50088a003

(35) Bojcsev S., Almási A., Simon H., Perjési P., Fischer E., 2013: Investigation of drug metabolism in various segments of small intestine in the rat, *Acta Physiol. Hung*. 100, 115-123. DOI: 10.1556/APhysiol.99.2012.002

(36) Grant R.D., Rizzardo E., Solomon, D.H. (1985): Reactions of hydroxyl radicals with polymerizable olefins *J. Chem. Soc.-Perkin Trans. II*. 1985, 379-384. DOI:10.1039/P29850000379

(37) Prousek J., 2007: Fenton chemistry in biology and medicine *Pure Appl Chem* 79, 2325–2338. DOI:10.1351/pac200779122325

(38) Cheng I.F., Zhao C.P., Amolins A., Galazka, M., Doneski L., 1996: A hypothesis for thein vivo antioxidant action of salicyclic acid. *Biometals*, 9, 285–290. DOI: 10.1007/bf00817929

(39) Barbusinski K., 2009: Fenton reaction controversy concerning the chemistry, *Ecol. Chem. Engin. S* 16, 347-358.

(40) Graziano J.H., Grady R.W., Cerami A.,1974:The identification of 2,3-dihroxybenzoic acid as a potentially useful iron-chelating drug.*J Pharmacol Exp Ther*190, 570–575.

(41) Exner M., Hermann M., Hofbauer R., Kapiotis S., Speiser W., Held I., Seelos C., Gmeiner B.M.K., 2000: The salicylate metabolite gentisic acid, but not the parent drug inhibits glucose autoxidation-mediated atherogenic modification of low density lipoprotein. *FEBS Letters* 470, 47-50. DOI: 10.1016/s0014-5793(00)01289-8.

(42) Nichela D.A., Donadelli J.A., Caram B.F., Haddou M., Rodriguez Nieto F.J., Oliveros E., García Einschlag F.S., 2015: Iron cycling during the autocatalytic decomposition of benzoic acid derivatives by Fenton-like and photo-Fenton techniques, *Appl Catal B: Environ.* 170-171, 312–321. DOI: 10.1016/j.apcatb.2015.01.028

(43) Mitschke D., Reichel A., Fricker G., Moenning U., 2008: Characterization of cytochrome P450 protein expression along the entire length of the intestine of male and female rats, *Drug Metabol Dispos* 36, 1039-1045. DOI: 10.1124/dmd.107.019687

(44) Bojic M., Sedgeman C., Nagy L.D., Guengerich F.P., (2015): Aromatic Hydroxylation of Salicylic Acid and Aspirin by Human Cytochromes P450, *Eur J Pharm Sci*, 73, 49-56. DOI:10.1016/j.ejps.2015.03.015

(45) Fukui A., Naito Y., Handa O., Kugai M., Tsuji T., Yoriki H., Qin Y., Adachi S., Higashimura Y., Mizushima K., Kamada K., Katada K., Uchiyama K., Ishikawa T., Takagi T., Yagi N., Kokura S., Yoshikawa T., 2012: Acetyl salicylic acid induces damage to intestinal epithelial cells by oxidation-related modifications of ZO-1, *Am J Physiol Gastrointest Liver Physiol* 303, G927-36. DOI: 10.1152/ajpgi.00236.2012.

(46) Smecuol E., Pinto Sanchez M.I., Suarez A., Argonz J.E., Sugai E., Vazquez H., Litwin N., Piazuelo E., Meddings J.B., Bai J.C., Lanas A., 2009: Low-dose aspirin affects the small bowel mucosa: results of a pilot study with a multidimensional assessment,*Clin Gastroenterol Hepatol*7, 524-5299. DOI: 10.1016/j.cgh.2008.12.019.

(47) Liu Y., Hunt C.A., 2005: Studies of intestinal drug transport using an in silicoepithelio-mimetic device,*Biosystems* 82, 154-167.DOI: 10.1016/j.biosystems.2005.06.008

(48) Brandes R.P., Weissmann N., Schröder K., 2010: NADPH oxidases in cardiovascular disease, Free Rad Biol Med 49, 687-706 DOI: 10.1016/j.freeradbiomed.2010.04.030

(49) Brandes R.P., Weissmann N., Schröder K., 2014: Nox family NADPH oxidases: Molecular mechanisms of activation, Free Rad. Biol. Med. 76, 208-226 DOI: 10.1016/ j.freeradbiomed.2014.07.046

(50) Halliwell B and Grootveld M. The measurement of free radical reactions in humans. Some thoughts for future experimentation. FEBS Lett. 1987; 213(1):9-14. DOI: 10.1016/0014-5793(87)81455-2

Table1. Mulliken atomic charges

Compound	C1	C2	C3	C4	C5	C6
SA	- 0.3004	0.4262	- 0.2437	- 0.2084	- 0.2580	- 0.1573
2,3-DHBA	- 0.2823	0.3693	0.3791	- 0.2117	- 0.2484	- 0.1755
2,4-DHBA	- 0.3114	0.4395	- 0.2943	0.4125	- 0.2497	- 0.1545
2,5-DHBA	- 0.2920	0.4135	- 0.2329	- 0.2610	0.3713	- 0.1704
2,6-DHBA	- 0.3446	0.4446	- 0.2611	- 0.2003	- 0.2592	0.4622

SA: salicylic acid
2,5-DHB: 2,5-dihydroxybenzoic acid
2,6-DHB: 2,6-dihydroxybenzoic acid
2,4-DHB: 2,4-dihydroxybenzoic acid
2,3-DHB: 2,3-dihydroxybenzoic acid

Table 2. Linearity data.

Compound	Regression equation	R^2
CAT	y=0.0032x-0.5131	0.9998
2,5-DHB	y=0.017x-4.4739	0.9996
2,6-DHB	y = 14.158x – 6.9466	0.9993
2,4-DHB	y=0.0168x-4.6572	0.9996
2,3-DHB	y=0.0114x-5.9960	0.9992
SA	y=0.0204x-7.1844	0.9997

CAT: catechol
2,5-DHBA: 2,5-dihydroxybenzoic acid
2,6-DHBA: 2,6-dihydroxybenzoic acid
2,4-DHBA: 2,4-dihydroxybenzoic acid
2,3-DHBA: 2,3-dihydroxybenzoic acid
SA: salicylic acid

Table 3. System suitability data.

Standard	t^R	A	A^{sym}	R	N
CAT	6,410	124,535	0,87		23542
2,5-DHBA	6,652	779,779	0,89	1,42	23977
2,6-DHBA	7,143	548,893	0,33	1,93	7186
2,4-DHBA	8,815	540,52	0,91	5,99	25475
2,3-DHBA	9,567	502,857	0,96	3,29	26497
SA	25,944	734,257	0,99	11,89	21826

t^R: retention time
A: peak area
A^{sym}: peak symmetry
R: resolution
N: number of theoretical plates
A: average peak area
CAT: catechol
2,5-DHBA: 2,5-dihydroxybenzoic acid
2,6-DHBA: 2,6-dihydroxybenzoic acid
2,4-DHBA: 2,4-dihydroxybenzoic acid
2,3-DHBA: 2,3-dihydroxybenzoic acid
SA: salicylic acid

Table 4. Data for intra-day precision

Concentration (µg/ml)	Measurement	A(2,5-DHBA)	A(2,6-DHBA)	A(2,4-DHBA)	A(2,3-DHBA)	A(CAT)	A(SA)
50	1	897.343	820.864	847.282	890.861	157.867	1107.669
	2	898.666	802.553	867.546	863.364	158.409	1112.370
average*		898.004	811.709	857.414	877.113	158.138	1110.020
RSD%**		0.766	1.595	1.826	2.427	1.157	0.586
10	1	162.752	150.160	170.655	156.590	31.382	213.199
	2	156.363	156.363	170.520	139.351	31.908	201.298
average*		159.557	153.262	170.588	147.971	31.645	207.248
RSD%**		2.831	2.862	1.361	6.468	2.605	3.194
1	1	14.402	14.335	18.653	9.905	3.526	19.729
	2	14.384	13.999	20.699	8.956	3.766	20.280
average*		14.393	14.167	19.676	9.430	3.646	20.004
RSD%**		3.997	1.678	6.300	6.847	4.215	2.686

*calculated from 9 parallel injections
**calculated from 9 parallel injections
A: average peak area
CAT: catechol
2,5-DHBA: 2,5-dihydroxybenzoic acid
2,6-DHBA: 2,6-dihydroxybenzoic acid
2,4-DHBA: 2,4-dihydroxybenzoic acid
2,3-DHBA: 2,3-dihydroxybenzoic acid
SA: salicylic acid

Table 5. Data for inter-day precision

Concentration (µg/ml)	Day Nº	A(2,5-DHBA)	A(2,6-DHBA)	A(2,4-DHBA)	A(2,3-DHBA)	A(CAT)	A(SA)
50	1	923.180	802.497	832.608	894.127	159.443	1119.334
	2	898.004	805.542	857.414	877.113	158.138	1110.020
	3	1075.637	816.846	945.144	966.843	181.963	1243.851
average*		973.708	808.295	882.968	916.283	167.590	1164.139
RSD%**		8.685	0.935	5.929	4.955	7.264	5.585
10	1	148.650	130.047	136.417	144.012	26.130	183.542
	2	159.557	144.891	170.588	147.971	31.645	207.248
	3	207.972	169.531	192.693	185.501	37.367	253.062
average*		173.058	148.156	166.217	159.519	31.688	214.717
RSD%**		16.315	13.461	15.362	13.428	15.968	14.871
1	1	16.926	15.299	15.320	15.950	2.971	21.653
	2	14.393	14.167	19.676	14.167	3.646	20.004
	3	20.352	16.769	19.183	17.210	3.789	25.753
average*		17.559	15.412	18.002	14.743	3.460	22.795
RSD%**		16.004	8.465	13.831	24.132	12.576	12.545

*calculated from 18 parallel injections

**calculated from 18 parallel injections

A: average peak area
CAT: catechol

2,5-DHBA: 2,5-dihydroxybenzoic acid

2,6-DHBA: 2,6-dihydroxybenzoic acid

2,4-DHBA: 2,4-dihydroxybenzoic acid

2,3-DHBA: 2,3-dihydroxybenzoic acid

SA: salicylic acid

Table 6. Precision of liquid–liquid extraction.

Concentration (ng/mL)	CAT	2,3-DHBA	2,4-DHBA	2,5-DHBA	SA
1000	1.098 (*RSD%)	3.185 (*RSD%)	2.275 (*RSD%)	1.386 (*RSD%)	1.250 (*RSD%)
500	3.116 (*RSD%)	2.230 (*RSD%)	1.395 (*RSD%)	4.851 (*RSD%)	4.017 (*RSD%)
100	4.578 (*RSD%)	3.756 (*RSD%)	2.439 (*RSD%)	2.575 (*RSD%)	1.232 (*RSD%)
Equation	y=0.00002x +0.00100	y=0.00011x +0.00020	y=0.00013x +0.00100	y=0.00013x-0.0014	y=0.00013x +0.00227
r^2	0.9897	0.9999	1.0000	0.9999	0.9998

CAT: catechol
2,3-DHBA: 2,3-dihydroxybenzoic acid
2,4-DHBA: 2,4-dihydroxybenzoic acid
2,5-DHBA: 2,5-dihydroxybenzoic acid
SA: salicylic acid
*RSD%: relative standard deviation calculated from ratios of the peak areas of the examined compound and the internal standard (from three parallel extractions)