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Simultaneous determination of gentisic, salicyluric and salicylic acid in human plasma using solid-phase extraction, liquid chromatography and electrospray ionization mass spectrometry

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Abstract

A method is developed for the simultaneous extraction of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) in human plasma from *Willow Bark* extract, by solid phase extraction (SPE) using Waters Oasis HLB (divinylbenzene–*n*-vinylpyrrolidone copolymer) cartridges. Also, a method is optimized comprising of reversed-phase (RP) high-performance liquid chromatography (HPLC) in connection with electrospray ionization mass spectrometry (ESI-MS), fluorescence detection (FLD) and photo diode array detection (DAD) to identify and quantify GA, SUA and SA in the SPE effluents. An improved sensitivity regarding the lower detection limit (LOD) of <7 ng/ml, the limit of quantitation (LOQ) of 20 ng/ml and short analysis times of <15 min is required. The validated SPE method shows linearity in the range of 9.0–58.2 ng/ml for GA, 9.4–191.5 ng/ml for SUA and 12.8–1101.6 ng/ml for SA. The correlation coefficient values are >0.9994 and 0.99 for fluorescence detection (FLD) and electrospray ionization mass spectrometry (ESI-MS), respectively. The recoveries are from 91.3–102.1% for gentisic acid (GA), 86.8–100.5% for salicyluric acid (SUA) and 75.8–81.4% for salicylic acid (SA) depending on the starting concentrations. RP-LC–ESI-MS/MS studies using collision induced dissociation (CID) confirm that the investigated analytes are not artifacts and facilitate further specific identification in addition to the determination of the parent ion mass even in the presence of co-eluting peaks. The established method is also used to analyze gentisic (GA), salicyluric (SUA) and salicylic acid (SA), not only after intake of *Willow Bark* capsules (Assalix®, BNO 1455) but also as naturally occurring constituents in human plasma after the intake of salicylic acid containing foods.

Keywords: Gentisic acid; Salicyluric acid; Salicylic acid

1. Introduction

The ancient Egyptians have used preparations of Willow Bark for fever, mild rheumatic complaints and pain like mild headache without causing stomach troubles even in the case of a long-lasting therapy. Therefore, *Willow Bark* extracts offer an alternative to the well-known Aspirin® (acetylsalicylic acid, ASA), of which more than 80 billion tablets per year are taken alone in the United States. A principal active ingredient of this is salicin that is metabolized to saligenin [1,2]. The metabolism pathway shown in Fig. 1 is similar to that of Aspirin® [3,4]. In contrast to Aspirin®, whose activity is known, for the therapeutic mechanism of salicin and its salicylate derivatives, several explanations exist [5–7]. One of these theories describes the bonding of salicylic acid (SA) at the active center of cyclooxygenase (COX) and as a result the prostaglandin synthesis is inhibited. The most common technique for the analysis of Aspirin® and its major metabolites in plasma extracts is reversed-phase high-performance liquid chromatography (RP-LC), using either UV-absorbance or fluorescence detection (FLD) [8–10]. Traditionally, liquid–liquid extraction (LLE) is the preferred method for the separation of

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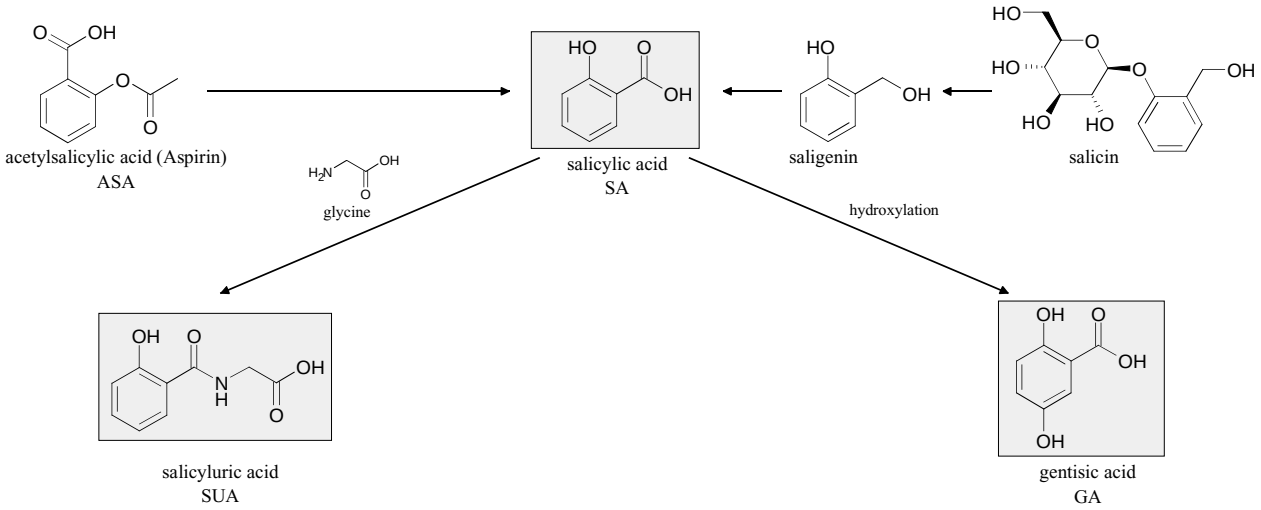

Fig. 1. Metabolites of salicin and acetylsalicylic acid (Aspirin®) found in human plasma.

salicin and its salicylate derivatives from human plasma samples [11–13]; e.g., Klimés et al. [14] described a method of LLE with methylene chloride for the isolation of acetylsalicylic acid, its metabolites, salicylic, gentisic and possibly salicyluric acid from whole blood, isolated erythrocytes and plasma. Separation was performed using reversed-phase chromatography on Separon SGX C18 by applying a mixture of methanol–water (80:100, v/v) as the mobile phase that allowed the determination down to a limit of 20 and 50 ng/ml for SA and GA, respectively. Liu and Smith [15] introduced a method for the direct analysis of salicylic acid, salicyl acyl glucuronide, salicyluric acid and gentisic acid in human plasma and urine after protein precepitation using acetonitrile by RP-LC. The LOD was as low as 200 ng/ml. Shen et al. [16] described the application of ion-pair RP-LC using a mobile phase consisting of methanol, water and TEA for the quantitation of aspirin, salicylic acid, gentisic acid and salicyluric acid in human plasma down to a LOD of 500, 50, 100 and 50 ng/ml, respectively. McMahon and Kelly [17] described a procedure for the determination of Aspirin® and salicylic acid (SA) in human plasma by column-switching liquid chromatography (LC) using on-line solid-phase extraction (SPE), which allowed the determination to a lower limit of detection (LOD) of 40 ng/ml. Hansen et al. [18] described an alternate method of LC, i.e. the non-aqueous capillary electrophoresis (CE) with reversed electroosmotic flow by the use of hexadimethrine bromide, for the separation of acetylsalicylic acid, salicylic acid, salicyluric acid and gentisic acid down to a LOD of 500 ng/ml from plasma. As the concentrations of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) after digestion of *Willow Bark* extracts are one-tenth as compared to that of Aspirin®, some more sensitive preconcentration steps and analytical techniques are required, which can be directly connected to mass spectrometry for further structural identification. This is required especially in the case of complex plasma samples where coelution can appear [19]. Therefore, a highly sensitive and fast analytical procedure was developed which was based on solid-phase extraction (SPE), using Oasis HLB (divinylbenzene–*n*-vinylpyrrolidone copolymer) [20] as stationary phase, followed by RP-LC determination connected to fluorescence detection (FLD), diode array detection (DAD) and mass spectrometry (MS) via an electrospray ionization (ESI) interface. The idea behind the first few steps was deduced from the experiences made by Paterson et al. [21] and Blacklock et al. [22]. Paterson et al. [21] showed the occurrence of gentisic acid (GA) in human plasma extracts by spiking of an "unknown peak" in the LC–UV chromatogram with an aqueous standard. Blacklock et al. [22] compared the salicylic acid (SA) content in serum samples of non-vegetarians and vegetarians not taking aspirin drugs with patients taking Aspirin®. Gentisic (GA) and salicylic acid (SA) is a normal constituent in human plasma because of the fact that salicylic acid (SA) can be found in a large number of foods such as cucumber, melon, cherry, etc. [23]. In this paper, it is shown that the validated method requires decreased sample preparation time, offers higher sensitivity at analysis times <15 min. Furthermore, it is highly suitable for simultaneous qualitative and quantitative analysis of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) in human plasma by RP-LC–DAD, RP-LC–FLD and RP-LC–ESI-MS/MS with high recovery and robustness.

2. Experimental

2.1. Materials and reagents

Acetonitrile (ACN, analytical reagent-grade), methanol (MeOH, analytical reagent-grade) and salicylic acid (SA, analytical reagent-grade) were purchased from Fluka (Buchs, Switzerland), formic acid (analytical reagent-grade), orthophosphoric acid (H_3PO_4, 85% analytical reagent-grade) and potassium dihydrogen phosphate (KH_2PO_4, analytical reagent-grade) from Merck (Darmstadt, Germany), gentisic

(GA) and salicyluric acid (SUA) from Sigma–Aldrich (Deisenhofen, Germany). Bidistillated water was purified by a NanoPure-unit (Barnstead, Boston, MA, USA). Standard stock solutions of GA, SUA and SA were prepared in methanol of concentrations about 11 mg/ml and stored at −18 °C for several weeks. The working solutions were prepared by dilution with bidistillated water to spike human plasma and stored in a refrigerator for 2 days. Human plasma was obtained from the blood bank of the University Hospital (Innsbruck, Austria). Oasis HLB 1cc SPE cartridges were from Waters (Milford, MA, USA). *Willow Bark* capsules (Assalix®, BNO 1455) were provided by Bionorica AG—The Phytoneering Company (Neumarkt/Oberpfalz, Germany).

2.2. Instrumentation

2.2.1. High-performance liquid chromatography (LC)

The earlier LC system consisted of a LC Module I plus (Waters, Milford, MA, USA), to which a fluorescence detector (FLD, Model 474, Waters) with a 16 μl flow cell was connected. The existing system used for RP-PLC consisted of a low-pressure gradient pump (Model 616, Waters), a controller (Model 600S, Waters), a column heater (Model TC 1900, ICI, Welshpool, Australia), a helium degassing system, an autosampler (Model 717 plus, Waters) and a photo diode array detector (DAD, Model 996, Waters) with a 10 mm path length flow cell. Data was recorded on a computer-based data system (Millenium^{32}, Version 3.05.01, Waters). For both systems, a Spherisorb C-8 column (250 mm × 4.6 mm i.d., 5 μm, 120 Å, Sigma–Aldrich, Deisenhofen, Germany) was used. The mobile phase comprised of: (A) 20 mM KH_2PO_4 in 99.8 % bidistillated water/0.2% H_3PO_4 (85 %) (v/v); and (B) ACN; linear gradient: 0 min 88% (A) → 16 min 40% (A) → 18.5 min 88% (A). The flow rate was 1.0 ml/min at a temperature of 25 °C. The volume injected was 20 μl. For fluorescence detection, following wavelengths were used: GA λ_{ex} = 323 nm; λ_{em} = 442 nm; SUA and SA λ_{ex} = 297 nm; λ_{em} = 407 nm.

2.2.2. High-performance liquid chromatography coupled to electrospray ionization quadrupole ion trap mass spectrometry (LC–ESI-MS/MS)

For routine LC–ESI-MS/MS experiments, a low-pressure gradient micropump (Model Rheos 2000, Flux, Karlskoga, Sweden), a degasser (Model DG-301, Phenomenex, Torrance, CA, USA), a microinjector (Model CC00030, Valco, Houston, TX, USA) with a 20 μl internal loop connected to a quadrupole ion trap mass spectrometer (Model LCQ, Finnigan, San Jose, CA, USA) were used. The following parameters were applied in all experiments: negative ion mode; source voltage, 4.5 kV; source current, 80 μA; sheath gas flow rate, 75 (Finnigan units; nitrogen); capillary voltage, −4/−7 V (GA and SA/SUA); temperature of the heated capillary, 185 °C; tube lens offset, −30/−40/−35 V (GA /SUA /SA); first octapole offset, 9/6.5/8 V (GA/SUA/SA); second octapole offset, 7/3.75/4.5 V (GA/SUA/SA); inter octapole lens, 46/28/24 V (GA/SUA/SA). For RP-LC a Prontosil 120-5-C-18-AQ column (250 mm × 2 mm i.d., 5 μm, 120 Å, Bischoff, Leonberg, Germany) was used. The mobile phase consisted of: (A) 99.76% bidistillated water/0.24% acetic acid (v/v); (B) ACN; linear gradient: 0 min 92% (A) → 15 min 22% (A) → 17 min 92% (A). The flow rate was 0.3 ml/min at a temperature of 25 °C. The volume injected was 20 μl. The fragmentation pattern of the three acids was identical with Frauendorf and Herzschuh [24] and Ehring et al. [25]. The found parent and daughter ions of GA, SUA and SA were as: SA: *m/z* = 137 (12%), 93 (100); GA: *m/z*= 153 (3–10), 152 (30–55), 109 (100); SUA: 192 (>2), 193 (5–45), 150 (100), 93 (6).

2.3. Sample preparation

Solid-phase extraction (SPE) of all plasma samples was carried out as follows. Conditioning of the cartridge with 2 ml MeOH and 2 ml eluent A (1000 g bidistillated water, 2.69 g KH_2PO_4 and 2 ml H_3PO_4) by applying gauge pressure. To 1 ml of the plasma containing gentisic (GA), salicyluric (SUA) and salicylic acid (SA) in a glass tube, 20 μl H_3PO_4 was added and vortex mixed for 15 s. *p*-Toluic acid was used as an internal standard (10 μl of a 761 μg/ml MeOH solution were added to the plasma).The mixture was passed through the Oasis HLB 1cc SPE cartridge. The cartridge was washed with 1 ml eluent A and dried with nitrogen for 7 min at a pressure of 1.5 bars. Elution was done with 0.5 ml MeOH and after evaporation under a stream of nitrogen at 50 °C; the residue was dissolved in 0.2 ml eluent A. Finally, an aliquot part was injected into the LC system.

2.4. SPE-method optimization and validation

2.4.1. Calibration curves

Weight least-squares method (weighing factor = concentration^{-2}) was applied to fit the response of the data versus the effective concentration to the equation: height/area (GA and SUA/SA) response = slope × concentration ± intercept. Calibration curves were obtained by injecting spiked plasma standards to achieve the concentrations given in Table 1. Analysis was carried out by RP-LC–FLD.

2.4.2. Precision and recovery

Absolute recoveries for GA, SUA and SA from spiked human plasma were determined at three concentrations for each (GA: 9.1, 27.3, 58.2 ng/ml; SUA: 9.5, 103.9, 191.5 ng/ml; and SA: 12.8, 453.9, 1101.6 ng/ml). Comparison of the peak height was done in the case of SA (due to the slight peak tailing) and comparison of peak area in the case of SUA and GA to that of reference samples based on fluorescence detection. Reference samples were prepared by solid-phase extraction (SPE) of blank plasma and followed by addition to the individual spiking solution to obtain the same concentrations as described earlier. *p*-Toluic acid was used as an

Table 1
Concentrations (ng/ml) of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) for the calibration using aqueous standards or spiked plasma followed by analysis using RP-LC–FLD and RP-LC–ESI-SIM/MS

RP-LC–FLD			RP-LC–ESI-MS					
Spiked plasma			Aqueous standards			Spiked plasma		
GA	SUA	SA	GA	SUA	SA	GA	SUA	SA
9.1	9.5	12.8	29.9	31.0	39.3	49.7	10.3	48.2
18.9	45.9	230.5	59.7	62.0	78.6	98.9	28.7	95.9
28.7	82.3	448.3	89.6	93.1	117.9	147.7	40.8	143.2
38.6	118.7	666.0	119.4	124.1	157.2			
48.4	155.1	888.8	149.3	155.1	196.5			
58.2	191.5	1101.5	179.1	186.1	235.8			

internal standard. For the determination of intra- and inter-day precision, this practice was repeated six times for each concentration in a single day or in the span of 6 days time, respectively.

2.5. Qualitative and quantitative analysis of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) in human plasma using RP-LC–DAD and RP-LC–ESI-MS/MS

Qualitative analysis of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) as the natural constituents of human plasma was performed by solid-phase extraction (SPE) on a 12 ml sample, followed by RP-LC–DAD, and RP-LC–ESI-MS/MS in the selected ion monitoring (SIM) and collision induced dissociation (CID) mode.

Quantitative analysis was performed by RP-LC–FLD using calibration curve obtained by spiked plasma standards with *p*-toluic acid as an internal standard (see Section 2.4.1) in the course of the solid-phase extraction (SPE) validation. Two standardization methods were compared for quantitative analysis based on RP-LC–ESI-SIM/MS. One was the calibration with aqueous standards and the other was calibration using spiked plasma standards (Table 1).

3. Results and discussion

For the optimization and validation of solid-phase extraction (SPE) and reversed-phase LC procedure, we used fluorescence detection (FLD). Further quantitative analyses were carried out by FLD, ESI-SIM/MS. For qualitative analyses also diode array detection (DAD) was applied.

3.1. Optimization and validation of the solid-phase extraction using RP-LC–FLD

In order to increase the efficacy of the solid-phase extraction procedure, acidic solutions with varying amounts of methanol as protein precipitation agents were tested prior to the extraction. During the treatment of 1 ml plasma sample with 3 ml aqueous phosphoric acid (500 ml bidistillated water, 1 ml H_3PO_4), only a partial desorption was observed from endogenous proteins of gentisic (GA), salicyluric (SUA) and salicylic acid (SA), which actually lead to loss in recovery and decreasing limits of detection. Acidification with pure phosphoric acid (20 μl/1 ml plasma) allowed to detect GA, SUA and SA down to a lower limit of detection (LOD) of <7 ng/ml and a limit of quantitation (LOQ) of 20 ng/ml. Elution was performed four times with 0.25 ml methanol to find the minimum amount of methanol required to desorb GA, SUA and SA. In the third effluent no analyte was detected, which meant that quantitative desorption was already obtained by 0.5 ml methanol. Furthermore, it was also tested, especially for salicylic acid (SA), if the drying step caused loss of analytes, because SA is known to sublimate during the evaporation step after liquid–liquid extraction (LLE) [14,26]. This was accomplished by comparing the peak heights and areas of GA, SUA and SA in the effluents created after solid-phase extraction (SPE), with and without a drying step. The loss of analytes with the drying step was smaller than 2% compared to no drying. The drying step enabled faster evaporation without residues from the erythrocyte and from whole blood.

3.1.1. Precision

Precision (repeatability) of the SPE was checked by calculating the intra- and inter-day assay variation of 6 inter-day data sets at three different concentrations for each compound given in Table 2. Precision was defined as the percentage difference between the effective concentration and the mean calculated concentration of GA, SUA and SA in the extracts. In order to ensure the highest possible reliability of results at each concentration, the extraction was repeated six times followed by LC measurements using fluorescence detection. The results reported in Table 2 indicate that the extraction method was reliable within the reported concentration ranges of gentisic, salicyluric and salicylic acid in human blood after taking *Willow Bark* extracts (Assalix®, BNO 1455). Maximum relative standard deviations for intra-day precision were 0.4% for SUA, 5.8% for GA, 2.3% for SA and for inter-day as 2.51 for SUA, 5.46 for GA and 4.48 for GA.

3.1.2. Linearity

Fluorescence detection was used to establish calibration plots of peak height versus concentration for GA, SUA and

Table 2
Intra- and inter-day (6 inter-day data sets) assay precision (repeatability) for gentisic (GA), salicyluric (SUA) and salicylic acid (SA), *n*= 6, of the SPE procedure followed by analysis with RP-LC using fluorescence detection (FLD)

Acid	Effective concentration (ng/ml)	Intra-day				Inter-day			
		Mean calculated concentration (ng/ml)	S.D. (ng/ml)	R.S.D. (%)	Error (%)	Mean calculated concentration (ng/ml)	S.D. (ng/ml)	R.S.D. (%)	Error (%)
Gentisic acid (GA)	9.09	9.65	0.39	4.07	5.80	9.21	0.40	4.18	1.30
	27.27	28.73	0.63	3.76	5.08	28.76	0.64	3.84	5.46
	58.19	57.78	1.06	1.83	0.70	58.91	1.08	1.87	1.24
	9.45	10.24	0.12	1.14	7.72	10.37	1.34	2.56	1.25
Salicyluric acid (SUA)	103.9	105.1	0.54	2.13	1.11	105.4	1.23	3.21	1.40
	191.58	190.7	2.95	1.55	0.40	196.4	3.04	4.25	2.51
	226.9	232.3	4.51	1.94	2.30	237.6	4.71	5.61	4.48
Salicyl acid (SA)	453.9	459.3	4.27	2.76	1.18	463.9	6.93	7.98	2.20
	1101.6	1091	47.99	4.40	0.97	1116	57.88	65.11	1.24

SA by linear regression analysis of the average of six data points per concentration in the range of 9.1–58.2, 9.5–191.5 and 12.8–1101.5 ng/ml for GA, SUA and SA, respectively (Table 1). The variability in these ranges was smaller than 10%. The regression equations were as follows: $y = 322x + 4622$ for gentisic acid, $y = 378x + 5515$ for salicyluric acid and $y = 3258x + 56821$ for salicylic acid using FLD, whereas x is the concentration (ng/ml) and y is the height (mV) or the peak area (mV × s). Values for correlation coefficients were found as 0.9995, 0.9994 and 0.9994. The lower limit of detection (LOD) for GA, SUA and SA was found as 5.05, 4.32, and 6.57 ng/ml, the limit of quantitation (LOQ) as 14.65, 13.54 and 20.21 ng/ml.

3.1.3. Recovery

The mean absolute recoveries for GA at the concentrations of 9.1 and 58.2 ng/ml were 91.3 and 102.1%, for SUA at the concentrations of 9.45 and 191.5 ng/ml were 86.8 and 100.5% and for SA at the concentrations of 12.8 and 1101.6 ng/ml were 81.4 and 75.8%. The lower recoveries of SA are caused by the fact that no quantitative adsorption can be achieved during the solid-phase extraction procedure.

3.2. Optimization of chromatographic parameters for RP-LC using fluorescence detection

For the analysis of GA, SUA and SA in human plasma after SPE, the RP-LC system was optimized to have the shortest possible analysis time with satisfactory selectivity and sensitivity. Different HPLC-columns packed with Si-C18 and Si-C8 particles owing different pore sizes were evaluated. The highest efficiency was achieved with Spherisorb C-8 (250 mm × 4.6 mm i.d., 5 μm, 120 Å) as stationary phase (for chromatographic conditions, see Section 2). This system was used for the validation of established SPE using fluorescence detection (FLD) to avoid interference caused by endogenous analytes. As depicted in Fig. 2, GA, SUA and SA showed peaks at 10.24, 11.78 and 14.88 min on using fluorescence detection after SPE in a spiked human plasma sample.

3.3. Qualitative analysis using RP-LC–DAD and RP-LC–ESI-MS/MS

For the qualitative analysis of GA, SUA and SA as natural constituents of pure human plasma, 12 ml plasma was extracted and analyzed by the optimized SPE and LC procedure. The presence of the three acids was verified by comparison of their recorded UV-spectra from the chromatogram in Fig. 3 to those obtained from aqueous solutions of the analytes using photo diode array detection (DAD). The following UV-maxima for GA and SA in: (a) pure human plasma extracts; (b) spiked human plasma extract (GA 621 ng/ml; SA 592 ng/ml); and (c) an aqueous solution (GA: 62.1 ng/ml; SA: 59.2 ng/ml) were found: GA (nm): (a) 212.6, 233.0, 327.1; (b) 212.6, 236.4, 330.7; (c) 209.1, 237.1, 330.7; SA (nm): (a) 203.1, 240.8, 304.8; (b) 204.4, 238.4, 304.6; (c) 203.3, 234.9, 303.4. In the case of SUA, no UV-spectrum could be achieved because of strong interference with high concentrated other blood compounds. The usefulness of RP-LC separation for a more specific and selective identification of GA, SUA and SA, especially in plasma extracts with co-eluting peaks, was greatly enhanced by MS detection. Coupling of the RP-LC system to MS via an electrospray ionization (ESI) interface

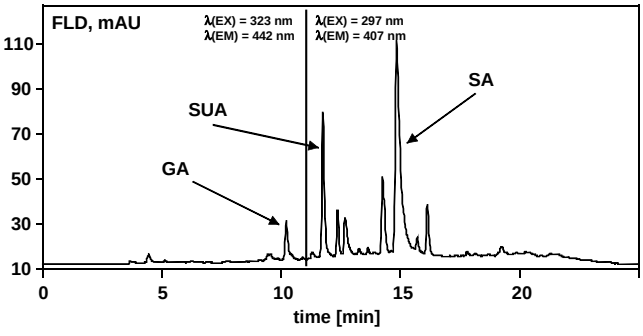

Fig. 2. Reversed-phase LC of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) in spiked human plasma using fluorescence detection. Chromatographic conditions see Section 2; concentrations of the spiked plasma: GA, 37.8 ng/ml; SUA, 127.6 ng/ml and SA, 447.9 ng/ml.

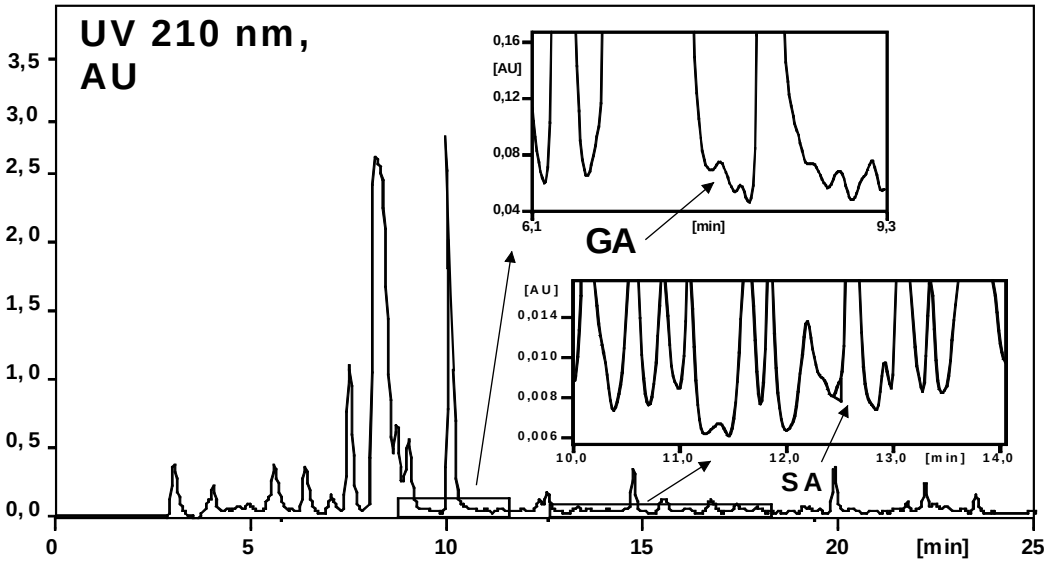

Fig. 3. Reversed-phase LC of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) as natural occurring consituents in human plasma using diode array detection. Chromatographic conditions see Section 2.

allowed, not only the determination of the parent ion mass of GA, SUA and SA in the selected ion monitoring (SIM) mode, but also to record tandem mass spectra using collision induced dissociation (CID), which is depicted in Fig. 4. The yielded fragmentation pattern for GA, SUA and SA in the pure plasma extract, in the extract of spiked plasma and in the aqueous solution are identical. For this reason, it can be affirmed that GA, SUA and SA are natural occurring constituents in human plasma. Furthermore, it was confirmed that GA, SUA and SA were not artifacts. The yielded fragmentation (parent and daughter ions, see Section 2.4.2) of the three acids is identical with those described by Frauendorf and Herzschuh [24] and Ehring et al. [25]. The ESI mass spectra of the analytes in the negative-ion mode are characterized by formation of $[M - H]^-$ and $[M - 2H]^{\bullet -}$ anions in spite of the use of an acidic LC eluent. The most typical fragmentation is characterized by the loss of CO_2.

3.4. Quantitative analysis using RP-LC–FLD and RP-LC–ESI-SIM/MS

The above described optimized methods were used to determine the content of natural occurring GA, SUA and SA in pure human plasma. Therefore, the assembled SPE LC–ESI-MS method was validated using spiked plasma standards. Precision (repeatability) was again checked by calculating the intra- and inter-day assay variation of six data sets at the three concentrations of spiked plasma given in Table 1. Maximum relative standard deviations for intra-day precision were 6.12% for GA, 8.32% for SUA and 3.45% for SA (Table 2). Those for inter-day precision were 7.14% for GA, 2.71% for SUA and 5.68% for SA. Calibration was carried out by the establishment of calibration plots of peak area versus concentrations given in Table 1 with correlation coefficients >0.99 for all the three investigated acids. The

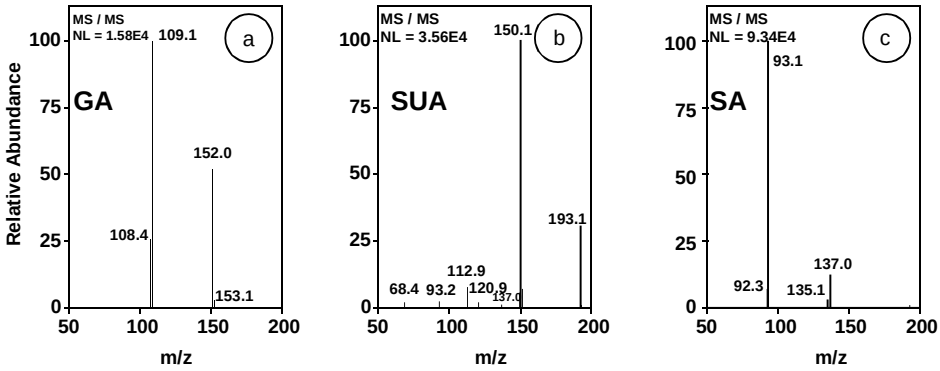

Fig. 4. LC–ESI-MS/MS spectra of: (a) gentisic (GA); (b) salicyluric (SUA); and (c) salicylic acid (SA) as natural occurring constituents in a pure human plasma extract. Stationary phase, Prontosil 120-5-C-18-AQ (250 mm × 2 mm i.d., 5 μm, 120 Å). Chromatographic conditions see Section 2. CID-spectra of parent ions: GA, *m/z*= 153; SUA, *m/z* = 192; SA, *m/z* = 137.

Table 3
Concentration of gentisic (GA), salicyluric (SUA) and salicylic acid (SA) in a pure plasma sample obtained by mass spectrometric (MS) and fluorescence detection (FLD) applying calibration with aqueous standards or spiked plasma

Acid	RP-HPLC-MS/SIM		RP-HPLC-FLD
	Aqueous standards (ng/ml)	Spiked plasma (ng/ml)	Spiked plasma (ng/ml)
Gentisic acid (GA)	6.7	17.6	15.9
Salicyluric acid (SUA)	n.d.	2.2	1.7
Salicylic acid (SA)	13.5	40.5	31.5

lower limit of detection (LOD) and the limit of quantitation (LOQ) for GA, SUA and SA by applying ESI-MS detection was in the same order of magnitude that obtained by fluorescence detection: LOD between 5.12 and 7.12 ng/ml, LOQ between 16.23 and 20.87 ng/ml for GA, SUA and SA. The mean absolute recoveries for GA at the concentrations of 49.7 and 147.7 ng/ml were 92.1 and 103.1%, respectively, and for SUA at the concentrations of 10.3 and 40.8 ng/ml were 87.6 and 100.9% and for SA at the concentrations of 42.8 and 143.2 ng/ml were 86.7 and 89.2%. This validated system was used for the determination of GA, SUA and SA in plasma samples.

Table 3 shows their concentrations obtained as mean of six consecutive determinations, which also depend on the detection method (MS and FLD) used. Compared to earlier published analytical procedures for the analysis of GA, SUA and SA in biological fluids, this new method provides a 100 times increase in sensitivity instead of using LLE [11,12] and a 20 times increase instead of using earlier described SPE procedures [17]. The concentrations achieved with the calibration established upon the injection of spiked plasma samples and the two different detection methods does not differ much except for SA, which can be explained by the stronger tailing of this substance using FLD, caused by interference with coeluting plasma components. Furthermore, we compared this calibration model to a calibration obtained by the injection of aqueous standards. For calibration, the concentrations given in Table 1 were again injected six times. The reason for the significantly lower concentrations determined with the aqueous standard calibration and RP-LC–ESI-SIM/MS detection is due to the ion suppression of analytes in the presence of a variety of "unseen" and undetected matrix components coeluting with the peaks of interest [27]. As a result of this, it can be claimed that quantitative analysis applying aqueous calibration is not suitable for concentration studies of GA, SA and SUA in plasma samples. Finally, Fig. 5a depicts a typical total ion current (TIC) RP-LC–ESI-MS chromatogram of a pure human plasma extract with naturally occurring GA, SUA and SA. Fig. 5b shows the TIC of a spiked plasma extract with concentrations at the LOQ. GA, SUA and SA were tracked in the plasma extract (Fig. 5b) from selected ion traces at *m/z* 151.9–153.9 (GA) (Fig. 5c), *m/z* 192.8–194.8 (SUA) (Fig. 5d) and *m/z* 136.0–138.0 (SA) (Fig. 5e).

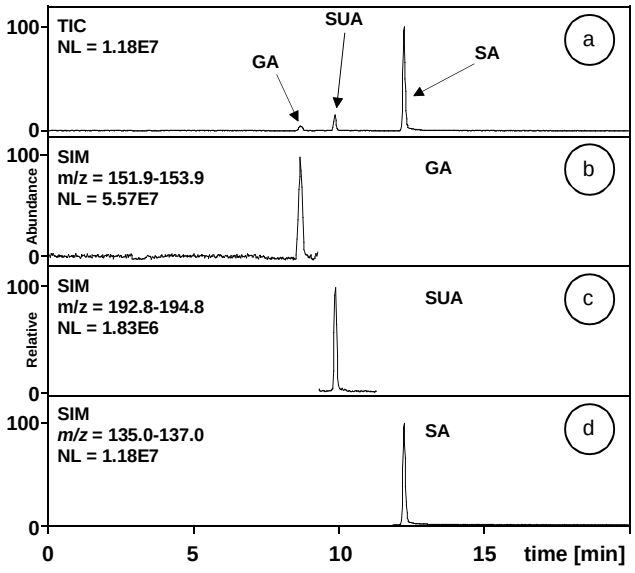

Fig. 5. LC–ESI-MS of GA, SUA and SA in: (a) pure human plasma extract total ion current (TIC); (b) spiked plasma extract (concentrations of the spiked plasma: GA, 28.3 ng/ml; SUA, 28.7 ng/ml; and SA, 25.9 ng/ml; (c)–(e) represent selected ion monitoring (SIM) of GA, SUA, and SA from the TIC in (b). Chromatographic conditions see Section 2.

References

[1] E. Steinegger, H. Hövel, Pharm. Acta Helv. 47 (1972) 222.
[2] R. Pentz, H.G. Busse, R. König, C.-P. Siegers, Dtsch. Apoth. Ztg. 7 (1985) 341.
[3] T.C. Kwong, J. Liq. Chromatogr. 10 (1987) 305.
[4] R.H. Rumble, M.S. Roberts, J. Chromatogr. 225 (1981) 252.
[5] B. Hinz, V. Kraus, A. Pahl, K. Brune, Biochem. Biophys. Res. Commun. 274 (2000) 197.
[6] K. Kun-Yu Wu, Biochem. Pharmacol. 55 (1998) 543.
[7] T.J. Holmes, J.L. Vennerstrom, V. John, J. Biol. Chem. 260 (1985) 14092.
[8] R.J. O'Kruk, M.A. Adams, R.B. Philip, Chromatographia 310 (1984) 343.
[9] S.K. Bakar, S. Niazi, J. Pharm. Sci. 72 (1983) 1020.
[10] U. Reidl, J. Chromatogr. 272 (1983) 325.
[11] D.C. Mays, D.E. Sharp, C.A. Beach, R.A. Kershaw, J.R. Biachine, N. Gerber, J. Chromatogr. 311 (1984) 301.
[12] D. Dadgar, J. Climax, R. Lambe, A. Darragh, J. Chromatogr. 342 (1985) 315.
[13] J.N. Buskin, R.A. Upton, R.L. Williams, Clin. Chem. 28 (1982) 1200.
[14] J. Klimés, J. Sochor, M. Zahradni'cek, J. Sedlácek, J. Chromatogr. 584 (1992) 221.
[15] J.-H. Liu, P.C. Smith, J. Chromatogr. B 675 (1996) 61.
[16] J. Shen, S. Wanwimolruk, C.R. Clark, M.S. Roberts, J. Liq. Chromatogr. 13 (4) (1990) 751.
[17] G.P. McMahon, M.T. Kelly, Anal. Chem. 70 (1998) 409.
[18] S.H. Hansen, M.E. Jensen, I. Bjornsdottir, J. Pharm. Biomed. Anal. 17 (1998) 1155.
[19] B. Schmid, I. Kötter, L. Heide, Eur. J. Clin. Pharmacol. 57 (2001) 387.
[20] C.W. Huck, G.K. Bonn, J. Chromatogr. A 885 (2000) 51.

[21] J.R. Paterson, C. Blacklock, G. Campell, D. Wiles, J.R. Lawrence, J. Clin. Pathol. 51 (1998) 502.
[22] C.J. Blacklock, J.R. Lawrence, D. Wiles, E.A. Malcolm, I.H. Gibson, C.J. Kelly, J.R. Paterson, J. Clin. Pathol. 54 (2001) 553.
[23] A.R. Swain, St.P. Dutton, A. Stewart Truswell, Research 85 (1985) 950.
[24] H. Frauendorf, R. Herzschuh, Eur. Mass Spectrom. 4 (1998) 269.
[25] H. Ehring, M. Karas, F. Hillenkamp, Org. Mass Spectrom. 27 (1992) 472.
[26] L.Y. Lo, A. Bye, J. Liq. Chromatogr. 1818 (1980) 473.
[27] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.