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Gas chromatographic–tandem mass spectrometric determination of acetylsalicylic acid in human plasma after oral administration of low-dose aspirin and guaimesal

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Abstract

A fully validated gas chromatographic–tandem mass spectrometric (GC–MS–MS) method is described for the accurate determination of acetylsalicylic acid (ASA) in human plasma after a single low-dose oral administration of aspirin or guaimesal, an ASA releasing prodrug. ASA and the newly prepared *O*-[$^2H_3$]-acetylsalicylic acid (d3-ASA) used as internal standard were determined in 100-μl aliquots of plasma by extractive pentafluorobenzyl (PFB) esterification using PFB bromide and tetrabutylammoniumhydrogen sulphate as the esterifying and ion-pairing agent, respectively, and by GC–MS–MS analysis in the negative-ion chemical ionization mode. The overall relative standard deviations were below 8% for ASA levels in the range 0–1 μg/ml plasma. Mean accuracy was 3.8% for ASA levels within the range 0–100 ng/ml. The limit of quantitation of the method was determined as 200 pg/ml ASA at an accuracy of 5.5% and a precision of 15.2%. The limit of detection was determined as 546 amol of ASA at a signal-to-noise ratio of 10:1. © 1998 Elsevier Science B.V.

Keywords: Acetylsalicylic acid; Aspirin; Guaimesal

1. Introduction

Aspirin (acetylsalicylic acid, ASA) is worldwide one of the most frequently used antiinflammatory drugs. Its antiinflammatory and antithrombotic actions result from the irreversible inhibition of the cyclooxygenase. This enzyme catalyzes the formation of thromboxane and prostacyclin which have opposite effects on aggregation and vasodilation [1]. It has been suggested that extremely low doses of aspirin (<100 mg) would selectively inhibit formation of thromboxane [2]. In fact, recently it has been shown that intravenous low-dose aspirin rapidly and selectively inhibits thromboxane formation and thus platelet aggregation while it maintains systemic cyclooxygenase activity [3]. In vivo ASA is deesterified to salicylic acid (SA) which is the major circulating metabolite [4]. Low-dose aspirin treatment and its substantial 'first-pass' effect limit its systemic bioavailability. This represents a challenge for analytical methods in quantitating ASA in plasma and urine. Stable isotope dilution utilizing deuterated

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ASA and SA and gas chromatography–mass spectrometry (GC–MS) has been demonstrated to be excellently applicable to pharmacokinetic studies with low-dose aspirin [5]. This technique solves problems resulting from insensitivity and unspecificity. Pedersen and FitzGerald have described the synthesis of tetradeuterated ASA from SA by catalytical exchange followed by *O*-acetylation [5]. In the present paper we describe a simple method for the synthesis of a ^{2}H-labeled ASA, e.g. *O*-[$^{2}H_3$]-acetylsalicylic acid (d3-ASA), in high isotopic purity and yield starting from commercially available SA and [$^{2}H_6$]-acetic anhydride. Utilizing a previously described extractive esterification procedure [5], we developed and validated a method allowing highly sensitive and specific quantitation of ASA in human plasma by GC–MS–MS. We demonstrate here that this method is excellently applicable to determine reliably ASA in plasma after oral administration of aspirin in low doses or of orally administered guaimesal, e.g. 2-methyl-2-[2-(methoxy)phenoxy]-4*H*-1,3-benzodioxin-4-one (code name MR 693) (see [6] and references therein), which is an ASA-releasing compound.

2. Experimental

2.1. Chemicals and reagents

Tetrabutylammoniumhydrogen sulphate (TBAHS) was obtained from Merck-Schuhardt (Hohenbrunn/Munich, Germany). Pentafluorobenzyl (PFB) bromide and [$^{2}H_6$]-acetic anhydride (99%+at ^{2}H) were obtained from Aldrich (Steinheim, Germany). *N,N*-Diisopropylethylamine, ASA and SA were bought from Sigma Chemie (Munich, Germany). [Carboxyl-^{14}C]-acetylsalicylic acid (55.8 Ci/mol) was obtained from Dupont NEN Products (Boston, MA, USA). Acetonitrile gradient grade and all other chemicals were obtained from Merck (Darmstadt, Germany).

2.2. Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC analyses were performed on an LKB solvent delivery system model 2150 coupled with a variable UV–VIS LKB detector model 2151 (Bromma, Sweden) and a Shimadzu integrator model C-R3A (Kyoto, Japan). The stationary phase consisted of a column (250×4.6 mm I.D.) packed with Nucleosil 100-5C_{18}, 5 μm particles size, from Bischoff (Solingen, Germany). Underivatized unlabeled and labeled ASA were analyzed isocratically using acetonitrile–water (30:70, v/v), as the mobile phase; the pH was adjusted to 2.5 with *o*-phosphoric acid. The flow-rate was 1.0 ml/min and the eluent was detected at 236 nm. A similar RP-HPLC system has recently been reported for ASA and SA [7]. For RP-HPLC analysis of PFB esters the same system was used with the exception that the mobile phase was acetonitrile–water (50:50, v/v), pH 2.5. In this RP-HPLC system the PFB derivatives of ASA and SA were eluted at 30.7±0.1 and 56.1±0.3 min (mean±S.D., $n=3$), respectively.

2.3. Extractive PFB esterification and derivatization procedures

The extractive PFB esterification procedure for the analysis of ASA and SA in plasma and aqueous buffered solutions was substantially the same as originally described by Pedersen and FitzGerlad [5]. Briefly, a 100-μl aliquot of plasma was treated with 500 μl of −20°C cool acetone, the internal standard d3-ASA was added and the suspension was allowed to stand for 15 min at −20°C. Following centrifugation (5 min, 2000 *g*) the supernatant was transferred into 1.5-ml vials and acetone removed under a stream of nitrogen. The residue was dissolved in 100 μl of distilled water and treated with 400 μl of a 5 m*M* solution of TBAHS in 10 m*M* phosphate buffer, pH 6.5, followed by 10 μl of PFB bromide and 400 μl of dichloromethane. After mixing by vortexing for 6 min the samples were centrifuged (5 min, 2000 *g*), the upper aqueous phase was removed and the solvents of the organic phase were evaporated to dryness under nitrogen. 200-μl aliquots of distilled water and 600-μl aliquots of hexane were added and the mixture was vortexed for 1 min. Following centrifugation (5 min, 2000 *g*) the hexane phase was transferred into conus reactivials, hexane was removed under nitrogen and the residue was diluted with 20- to 100-μl aliquots of isooctane.

The PFB esters of reference unlabeled and labeled ASA and SA were prepared as follows: aliquots of

stock solutions of these compounds in dry acetonitrile were brought to dryness under a stream of nitrogen, the residue was dissolved in dry acetonitrile (100 μl) and treated further with *N,N*-diisopropylethylamine (10 μl) and a 30 wt.% solution of PFB bromide in acetonitrile (10 μl). The resulting mixture was allowed to stand at 30°C for 60 min. For GC–MS analysis, solvent and reagent excess were removed under nitrogen and the residue was reconstituted in isooctane. Methylesters of unlabeled and labeled ASA were prepared using a freshly prepared etheral solution of diazomethane.

The recovery rate of ASA after the extractive PFB esterification step was determined as follows: ASA (1 μg/ml) was incubated in 50 m*M* phosphate buffer (pH 7.4). After immediate extractive PFB esterification with five 100-μl aliquots exactly as described for plasma samples centrifugation was performed (5 min, 2000 *g*). The aqueous phases were decanted and centrifuged again. 200-μl aliquots of these solutions and a 200-μl aliquot of a freshly prepared ASA solution (1 μg/ml) in 10 m*M* phosphate buffer, pH 6.5, containing 5 m*M* of TBAHS were analyzed by RP-HPLC. In the aqueous phase ASA was found at a concentration of 0.23±0.05 μg/ml (mean±S.D.) which corresponds to a mean recovery rate of about 77%. The recovery rate was found to depend on the amount of PFB bromide used and on the time of vortexing (data not shown). The recovery rate of ASA at lower concentrations was determined by extractive PFB esterification of a 42 ng/ml solution of [^{14}C]-acetylsalicylic acid in 50 m*M* phosphate buffer (pH 7.4). The recovery rate was determined to be 53±6% (mean±S.D., n=3). RP-HPC analysis of the dichloromethane phases resulted in elution of 98.2±0.5% of the radioactivity injected as a single peak with the retention time of synthetic ASA-PFB. No radioactivity was found to elute with the retention time of SA or of its PFB derivative.

2.4. Gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry

GC–MS in the negative-ion chemical ionization (NICI) and electron impact (EI) mode was performed on a Hewlett-Packard MS Engine 5989A interfaced with a gas chromatograph HP 5890 series II (Waldbronn, Germany). A fused-silica capillary column DB-1701 (15 m×0.25 mm I.D., 0.25 μm film thickness) from J&W Scientific (Rancho Cordova, CA, USA) was used. Helium (25 kPa) and methane (200 Pa) were used as a carrier and reagent gas, respectively. Interface, injector, ion source and quadrupole were kept at 280°C, 250°C, 225°C and 120°C. Electron energy and electron current were set to 230 eV and 300 μA, respectively. The column was held at 100°C for 2 min, then increased to 300°C at a rate of 25°C/min.

GC–MS–MS analyses were performed on a Finnigan MAT TSQ 45 triple stage quadrupole mass spectrometer equipped with a Finnigan gas chromatograph model 9611 (San Jose, CA, USA). A fused-silica capillary column DB5-MS (30 m×0.25 mm I.D., 0.25 μm film thickness) from J&W Scientific (Rancho Cordova, CA, USA) was used. Helium was the carrier gas at a pressure of 55 kPa. For NICI methane was used as reagent gas at a pressure of 65 Pa. Argon was applied for collisionally activated dissociation (CAD) at a pressure of 0.2 Pa, a collision and electron energy of 14 eV and 90 eV, respectively, and an emission current of 200 μA. Injector, interface and ion source were kept at 280°C, 290°C and 140°C, respectively. The column was held at 80°C for 2 min, then increased to 320°C at a rate of 25°C/min. Aliquots of 1–2 μl were injected in the splitless mode both in GC–MS and GC–MS–MS.

2.5. Synthesis of O-[$^2H_3$]-acetylsalicylic acid (d3-ASA)

In a 100-ml glass flask, 3 g (21.7 mmol) of SA and 2.8 ml of [$^2H_6$]-acetic anhydride were placed. The suspension was treated with 50 μl of concentrated sulphuric acid. The reaction mixture was immediately heated in a water bath at 50°C until a clear solution was obtained. The reaction mixture was immediately cooled in a icewater bath and then allowed to stand at room temperature. The clear liquid was treated with further 200 μl of [$^2H_6$]-acetic anhydride under gentle shaking while a solid white material precipitated. 50 ml of ice-water was added, the suspension was filtered and the white crystals were washed with 20 ml of −80°C cool diisopropylether. After drying at room temperature, 3.58 g of white needles was obtained. Subsequent analyses showed a single compound in this prepara-

tion with the identity of *O*-[$^2H_3$]-acetylsalicylic acid (see Section 3). The yield was calculated to be 90%. Stock solutions of d3-ASA were prepared in dry acetonitrile and stored at −20°C.

2.6. ^1H NMR analysis of O-[$^2H_3$]-acetylsalicylic acid

^1H NMR spectra of ASA and d3-ASA diluted in $CDCl_3$/TMS were obtained on a Bruker WH-270 Spectrometer at a proton resonance frequency of 270 MHz.

2.7. Oral administration of low-dose aspirin and guaimesal

The volunteers participated in the following studies have not received for the last 2 weeks aspirin or other ASA-containing drugs.

Four healthy volunteers (three males, one female; body weight: 70±10; age: 25±3; both mean±S.D.) received orally a 100-mg tablet of aspirin (Ratiopharm, Ulm, Germany). After 10, 20, 45 and 60 min of each administration, 2-ml blood was taken from antecubital veins using citrate for anticoagulation. Plasma was generated by centrifugation at 2°C (5 min, 1800 *g*). To three 100-μl aliquots of each plasma sample d3-ASA was added to achieve a final concentration of 500 ng/ml. Further treatment was performed as described above.

Also, a healthy volunteer (92 kg body weight, 38 years old) received orally one 500-mg capsule guaimesal (L. Manetti and H. Roberts, Florence, Italy). 1 h prior to, 3 h and 24 h after oral administration venous blood was taken and treated as described above for measurement of ASA in plasma.

3. Results

3.1. Synthesis and spectrometric analysis of O-[$^2H_3$]-acetylsalicylic acid

RP-HPLC analysis of freshly prepared ^2H-labeled ASA resulted in the elution of a single peak with the retention time of commercially available unlabeled ASA (8.25±0.06, mean±S.D., n=10). Aqueous solutions of unlabeled and ^2H-labeled ASA showed additionally a minor peak with the retention time of commercially available SA (11.7±0.1 min, mean±S.D., n=10) from hydrolysis of d3-ASA and ASA, respectively. Solid d3-ASA remained unchanged for at least 6 months when stored at −20°C as found by RP-HPLC.

The GC–MS EI mass spectrum of the methyl ester of ^2H-labeled ASA showing a weak mass fragment at m/z 197 ($M^{+\cdot}$, 2%) and prominent ions at m/z 166 ($C_6H_4(CO)OCOCD_3$, 6%), 153 ($C_6H_4(COOCH_3)OD$, 79%), 122 ($C_6H_4(CO)OD$, 25%), 120 ($C_6H_4(CO)O$, 100%), 92 (C_6H_4O, 29%) and 46 (CD_3CO, 47%) unequivocally identifies this material as *O*-[$^2H_3$]-acetylsalicylic acid (d3-ASA). In the GC–MS EI mass spectrum of the methyl ester of unlabeled ASA the following mass fragments were observed: a weak mass fragment at m/z 194 ($M^{+\cdot}$, 2%) and prominent ions at m/z 163 ($C_6H_4(CO)OCOCH_3$, 10%), 152 ($C_6H_4(COOCH_3)OH$, 88%), 120 ($C_6H_4(CO)O$, 100%), 92 (C_6H_4O, 45%) and 43 (CH_3CO, 45%). The retention times of the methyl ester derivatives of unlabeled and ^2H-labeled ASA were practically identical. No peak was observed at the retention time of the methyl ester of SA suggesting that the freshly prepared solution of d3-ASA in acetonitrile did not contain SA.

Fig. 1 shows GC–MS NICI mass spectra of the PFB esters of unlabeled and d3-ASA. The most intense mass fragments observed were m/z 179 and m/z 182 which correspond the $[M-PFB]^-$ ions of the unlabeled and d3-ASA PFB derivatives, respectively. In the mass spectrum of d3-ASA PFB derivative a very weak mass fragment at m/z 179 was observed which indicates the presence of trace amounts of unlabeled ASA in the synthesized d3-ASA. The retention times of the PFB ester derivatives of ASA and d3-ASA were practically identical. Selected ion monitoring at m/z 179 and m/z 182 of the PFB ester of d3-ASA showed that the d3-ASA preparation contained (mean ± S.D., n=3) 99.2±0.2% of *O*-[$^2H_3$]-acetylsalicylic acid and only 0.8±0.3% of unlabeled ASA.

GC–MS–MS spectra of the PBF ester derivatives of ASA and d3-ASA were derived by CAD of the parent ions, e.g. $[M-PFB]^-$, at m/z 179 and m/z 182, respectively. The most intense daughter ions were observed at m/z 137 and m/z 138, respectively.

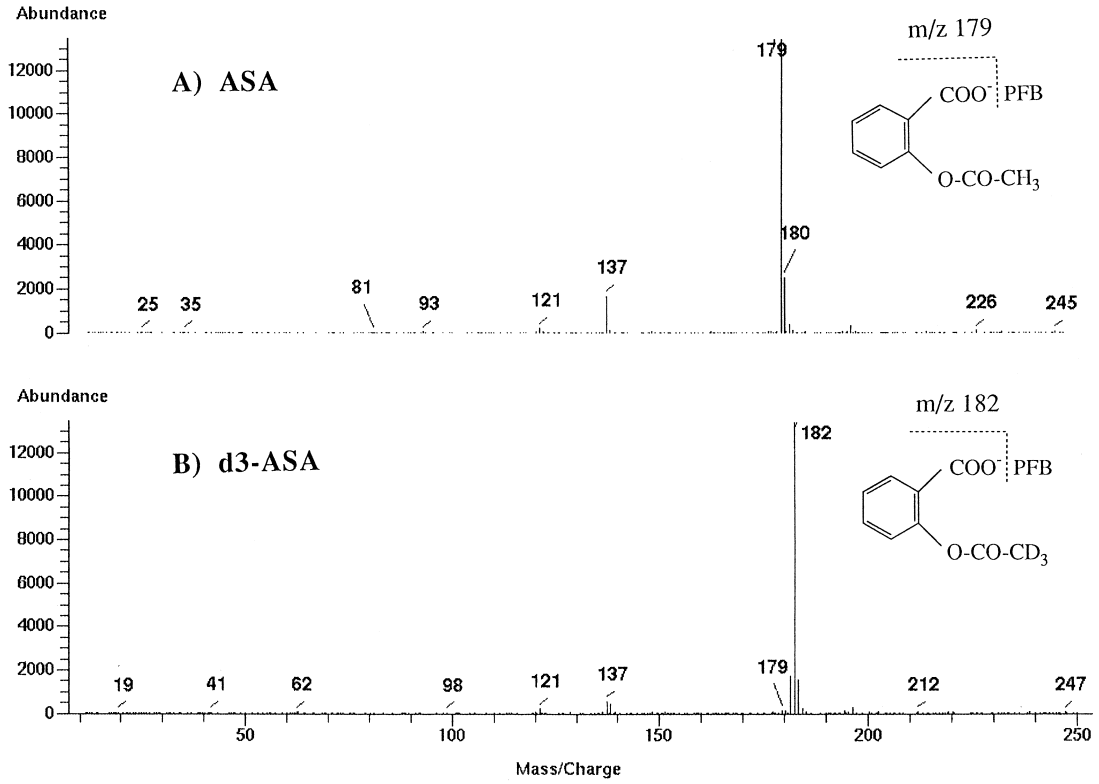

Fig. 1. GC–MS NICI mass spectra of the PFB esters of unlabeled (A, upper panel) and ^{2}H-labeled (B, lower panel) ASA.

These ions correspond to the carboxylate or phenolate anions of unlabeled and labeled SA, respectively. Less intense daughter ions (intensity of 60%) were observed at m/z 93 ($C_6H_5O^-$) and m/z 94 ($C_6H_4DO^-$), respectively. Weak daughter ions (intensity of 10%) were also observed at m/z 59 and m/z 62 due to CH_3COO^- and CD_3COO^-, respectively. For quantitative determinations by GC–MS–MS in the selected reaction monitoring (SRM) mode the daughter ions at m/z 137 for ASA and m/z 138 for d3-ASA were chosen. Mixtures containing each 5 ng of d3-ASA and 0, 0.5, 1.0, 2.5, 3.75 and 5 ng of ASA from accurately weighed amounts of d3-ASA and ASA were derivatized and analyzed by SRM of m/z 137 and 138. Linear regression analysis of the ratio m/z 137 to m/z 138 (y) vs. the ratio of the amount of ASA to the amount of d3-ASA (x) resulted in the following regression equation: $y = 0.0017 + 0.9917x$ ($r^2 = 0.999$). Mixtures containing equal amounts (1, 2, 3, 4, 5 ng) of ASA and d3-ASA were derivatized and analyzed by SRM of m/z 137 and 138. This ratio was determined as 0.999 ± 0.034 at a R.S.D. of 3.4%. These results show that the response factor between the ratio of the peak areas of m/z 137 to 138 and the ratio of the amounts of ASA to d3-ASA is very close to unity. The concentration of ASA in spiked and unspiked plasma samples was therefore calculated by multiplying the ratio m/z 137 to 138 measured with the respective concentration of d3-ASA used corrected by the factor 0.992 which takes into consideration the isotopic purity of d3-ASA.

The data from mass spectrometric analyses were verified by ^{1}H NMR spectroscopy. The acetyl group of non-deuterated ASA gave rise to a singulet at 2.34 ppm. d3-ASA instead showed the typical 1:2:3:2:1 quintet of a CHD_2 group at 2.31 ppm with a relative intensity of 0.08 protons. This signal was regularly found as a result of incomplete deuteration in [$^{2}H_6$]-acetic anhydride. In other respects the spectra of

ASA and d3-ASA were practically identical. No impurities could be detected in the d3-ASA preparation.

3.2. Quantitative determination of acetylsalicylic acid in human plasma

Linearity was checked by spiking 0.1-ml aliquots taken from a pooled plasma sample each with 50 ng of d3-ASA and various amounts of ASA (e.g. 0, 10, 20, 50 and 100 ng) in duplicate. After extractive PFB esterification the samples were analyzed by GC–MS–MS. Linear relationships was observed between the ratio m/z 137 to 138 (y) and the concentration (in ng/ml) of ASA (x) added to plasma $y=0.057+0.00195x$ ($r^2=0.996$). The value for the mean R.S.D. was 5.8%.

Instrumental precision was checked by five-fold analysis by GC–MS–MS (m/z 137 and 138) of a 100-μl aliquot of a plasma sample spiked with 50 ng of d3-ASA and 100 ng of ASA. The ratio of m/z 137 to 138 was measured as 2.056±0.028 (mean±S.D.) at a R.S.D. of 1.9%.

The accuracy of the method was assessed within the concentration range 0–100 ng/ml by analysing in tetraplicate 100-μl aliquots of a pooled plasma sample each spiked with 20 ng/ml of d3-ASA and 0, 5, 10, 20, 50, 75 and 100 ng/ml of ASA. In the unspiked plasma sample ASA was measured at a concentration of about 1 ng/ml. The identity of ASA in this plasma sample was shown by GC–MS–MS that followed RP-HPLC analysis of a 200-μl aliquot of plasma ultrafiltrate (cut-off 20 kDa) of the basal plasma sample spiked only with 20 ng/ml of d3-ASA, extraction of the RP-HPLC fraction eluted with the retention time of ASA, derivatization by PFB bromide and GC–MS–MS analysis at m/z 137/138, 93/94 and 59/62. The data from accuracy experiments given in Table 1 and the excellent correlation between the concentration of ASA measured by GC–MS–MS (y) and the concentration of ASA added to plasma (x) ($y=0.787+0.997x$, $r^2=1.000$), indicate the high accuracy and precision of the method and the quantitative relative recovery for ASA.

Intra-assay variability was investigated by spiking four 0.1-ml aliquots of a plasma pool with (a) 50 ng of d3-ASA and 100 ng of ASA, and (b) 0.5 ng of d3-ASA and 1 ng of ASA and by analysing in the SRM mode. The ratio m/z 137 to 138 was determined to be (mean ± S.D.) 2.056±0.077 in (a) and 1.988±0.109 in (b) at a R.S.D. of 3.8% and 5.5%, respectively. The inter-assay variability was tested similarly on four consecutive days. The ratio m/z 137 to 138 was determined to be (mean±S.D.) 1.998±0.146 in experiment (a) and 1.996±0.189 in experiment (b) and at a R.S.D. of 7.3% and 9.45%, respectively.

The limit of detection was determined by injecting onto the column the PFB ester derivative of d3-ASA in amounts equivalent to 100, 10, 1 and 0.1 pg of d3-ASA in the GC–MS–MS instrument. High linearity between amount injected and peak height measured was observed ($r^2=0.998$). The lowest

Table 1
Accuracy and precision of the method for the determination of acetylsalicylic acid (ASA) in human plasma by GC–MS–MS using d3-ASA as the internal standard (20 ng/ml)

ASA added (ng/ml)	ASA found (mean±S.D., $n=4$) (ng/ml)	R.S.D. (%)	Accuracy (%)
0	1.16±0.13	10.8	Not Applicable
5	5.72±0.36	6.31	8.9
10	10.4±0.47	4.49	7.8
20	20.6±1.53	7.41	2.8
50	51.5±2.63	5.11	0.7
75	74.4±2.49	3.36	2.4
100	101.1±5.85	5.78	0.1
Mean±S.D.		6.18±2.43	3.78±3.69

Accuracy was calculated after subtraction of the basal ASA value.

amount injected, e.g. 0.1 pg equivalent to 546 amol of d3-ASA, was detected at a signal-to-noise ratio of 10:1 by GC–MS–MS.

The limit of quantitation of the method was determined by analysing 100-μl plasma samples spiked each with 5 ng/ml of d3-ASA and 0, 100 and 200 pg/ml of ASA in hexaplicate and with 0.5, 1, 5 and 10 ng/ml of ASA in duplicate. The plasma used in this experiment was found to be free of ASA. A linear relationship ($r^2=0.985$) was obtained between the ratio m/z 137 over 138 and the concentration of ASA added. The lowest ASA concentration that was accurately measured was 200 pg/ml. In the plasma sample spiked with 200 pg/ml we measured a concentration of 189±29 pg/ml (mean±S.D.). The precision (R.S.D.) and the accuracy of the measurement of this sample were 15.2% and 5.5%, respectively.

The specificity of the method was established as follows. Plasma was obtained from six healthy volunteers who have not received any aspirin for the last two weeks. 0.1-ml aliquots of plasma samples were spiked with 20 ng of d3-ASA. One set of samples was analyzed without addition of ASA in duplicate. The second set of samples was spiked each with 100 ng of ASA in duplicate. GC–MS–MS analysis of the samples spiked only with d3-ASA showed ASA in a mean concentration of 0.18 pg/ml at a mean R.S.D. of 19.9%. This concentration is almost exclusively caused by ASA present in d3-ASA as isotopic impurity. In the samples spiked with 100 ng/ml a mean ASA concentration of 95.2 ng/ml at a mean R.S.D. of 6.5% was measured.

Quality control was carried out as follows: 0.1-ml aliquots of a pooled plasma proved to be free of ASA were spiked in duplicate each with 5 ng/ml of d3-ASA and with 1, 5 and 50 ng/ml of ASA. A set of five runs was performed. The concentrations of ASA measured in these samples were determined as (mean±S.D., each $n=10$) 1.08±0.14, 5.19±0.37 and 52.35±3.46 ng/ml with a R.S.D. of 13%, 7.2% and 6.6%, respectively.

3.3. Oral administration of low-dose aspirin and guaimesal

Fig. 2 shows a GC–MS–MS chromatogram from the analysis of a plasma sample obtained 10 min after oral administration of a 100-mg aspirin tablet by a healthy volunteer. In this chromatogram no other peaks except for aspirin-derived ASA and the internal standard d3-ASA are present. The dependency of the plasma concentration of aspirin-derived ASA on the time which follows the oral administration of a 100-mg aspirin tablet is shown in Fig. 3. Although the volunteers have not received the same doses per kg body weight, Fig. 3 suggests that peak maximum concentrations of ASA were achieved 20–45 min after oral administration. This result is in agreement with a previous similar pharmacokinetic study on enteric-uncoated aspirin in man [7].

We found by GC–MS–MS that oral administration of a guaimesal capsule containing 500 mg of guaimesal by a healthy volunteer resulted in the formation of 765 ng/ml of ASA 3 h after administration. ASA was not present in the plasma sample before and 24 h after administration of guaimesal. To our knowledge no data are currently available on pharmacokinetic studies with guaimesal.

4. Discussion

Aspirin (acetylsalicylic acid) is the best investigated drug inhibiting platelet aggregation. In low doses aspirin inhibits efficiently platelet aggregation and thromboxane formation but it inhibits only weakly prostacyclin synthesis [2,3,8]. In the last years several clinical attempts have been made to optimize the selectivity of aspirin treatment. They include the use of very low daily doses, the use of enteric-coated, slow-release oral formulations of aspirin [9–12] or single intravenous administration of aspirin in low doses in clinical situations associated with an acute platelet activation such as thrombolysis, angioplasty or acute myocardial infarction [13].

It is known that aspirin undergoes a substantial ‘first-pass’ effect [14] which may limit systemic bioavailability following oral administration of low doses. Aspirin is rapidly deesterified to salicylic acid in vivo [4]. Of the many published assay methods for aspirin in biological fluids very few enabled detection of aspirin at concentrations below 100 ng/ml [5,15–17]. Previously described assays lack the requisite sensitivity and specificity to characterize

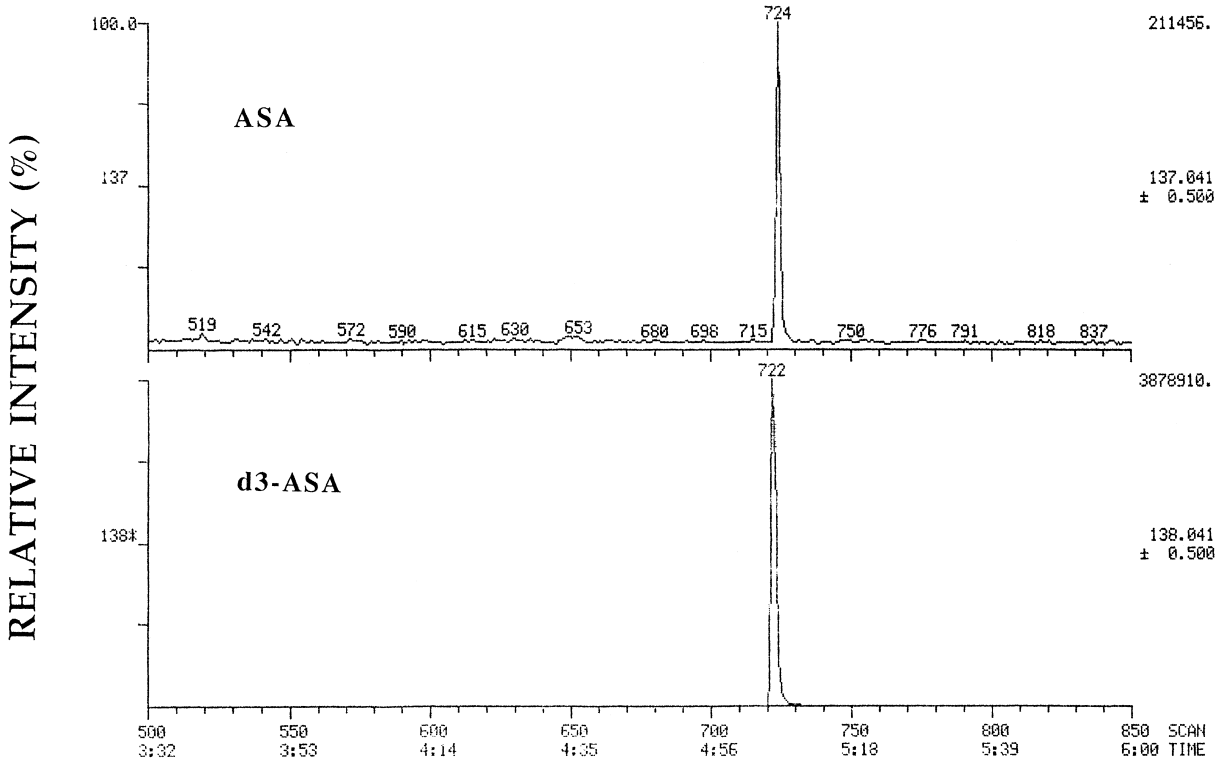

Fig. 2. Partial GC–MS–MS chromatogram from analysis of ASA in a plasma sample of a healthy volunteer who received orally a 100-mg aspirin tablet. Blood was taken 10 min after administration. The concentration of d3-ASA added to the plasma sample was 500 ng/ml. The concentration of ASA in this plasma sample was determined to be 20±2.6 ng/ml (mean±S.D., $n=3$). See also Fig. 3.

aspirin following low doses (≤100 mg). Pedersen and FitzGerald have demonstrated that GC–MS is superior to analytical methods using HPLC and, thus, better applicable to studies of low-dose aspirin [5]. Because no stable-isotope labeled ASA was available they synthesized ^{2}H-labeled aspirin from SA by catalytic exchange and subsequent acetylation. Since this time no other reports appeared on the measurement of ASA in human plasma by GC–MS by using ^{2}H or other stable-isotope labeled ASA analogs.

In the present study we report a very easy, rapid and efficient method for the synthesis of ^{2}H-labeled ASA, e.g. *O*-[$^{2}H_3$]-acetylsalicylic acid (d3-ASA), at high isotopic purity and at high yield at the low gram-scale. We started from unlabeled salicylic acid and performed *O*-acetylation with commercially available [$^{2}H_6$]-acetic anhydride. Since the label stays in the acetylic group synthesis of ^{2}H-labeled SA by hydrolysis of *O*-[$^{2}H_3$]-acetylsalicylic acid is not possible. This is not disadvantageous because SA is not of major interest and SA, which appears in plasma at much higher concentrations, can easily be detected by others techniques. However, we found that ASA is suitable as the external standard for the quantitation of SA in the same sample by GC–MS. A standard curve was generated by analysing mixtures of varying amounts of d3-ASA and SA (range 0–1 μg/ml). The following regression equation was obtained from linear regression analysis between the peak area ratio (y) of m/z 137 ([M-PFB]$^-$ for the PFB ester of SA) to m/z 182 ([M-PFB]$^-$ for the PFB ester of d3-ASA) and the amount ratio (x) of SA to d3-ASS: $y=0.150+0.886x$ ($r^2=0.991$).

Pedersen and FitzGerald [5] applied in their assay extractive esterification of ASA benzyl bromide and tetrahexylammonium hydroxide. Our results confirm the use of extractive esterification for reliable determination of aspirin in plasma. Furthermore, we

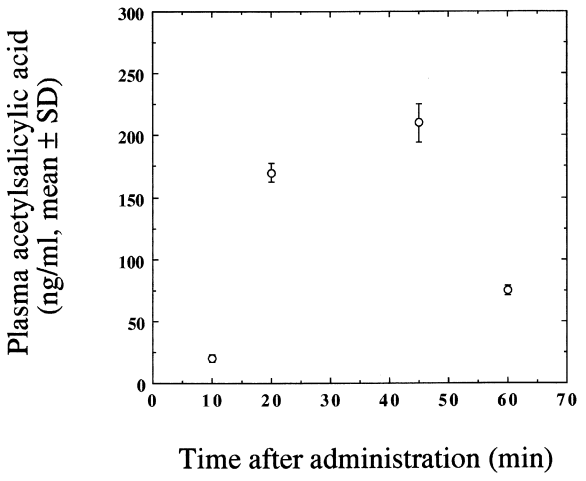

Fig. 3. Concentration profile of ASA in human plasma following oral administration (times zero) each of a 100-mg aspirin tablet to four healthy volunteers. At the indicated times blood was taken, three 100-μl aliquots of the resulting plasma samples were spiked each with 50 ng of d3-ASA, samples were treated and analysed by GC–MS–MS as described in Section 2.

improved this assay method, e.g. we lowered the detection limit by approximately two orders of magnitude, by introducing the stronger electron-capturing agent PFB bromide instead of benzyl bromide and NICI instead of EI ionization.

Our results demonstrate that d3-ASA is excellently suited for the quantitative determination of ASA in human plasma after oral administration of aspirin in low dose or after oral administration of ASA prodrugs such as guaimesal. The lowest ASA concentration measured in the plasma of a volunteer 10 min after oral administration of a 100-mg aspirin tablet was determined as 20 ng/ml at a signal-to-noise ratio of about 850:1. We could accurately and precisely measure d3-ASA in only 100-μl aliquots of human plasma at an added concentration of 0.2 ng/ml. This limit of quantitation for ASA of our method is about 10 times lower than that of the most efficient HPLC assay published to date [18] and was achieved with only 1/10th of plasma volume. Furthermore, the detection of low-ASA plasma concentrations by HPLC may be not accurate due to co-eluting unknown interfering compounds. The high specificity of GC–MS–MS practically excludes such interferences and allows accurate quantification of low concentrations of ASA in human plasma as they occur after oral low-dose aspirin using enteric-coated or enteric-uncoated formulations and after transdermal application of aspirin [19].

We have shown in this work that d3-ASA is a useful internal standard for the GC–MS–MS determination of ASA in plasma in a wide concentration range. Nevertheless, the choice of suited concentrations for d3-ASA added to plasma is of decisive importance because d3-ASA contains about 0.8% of unlabeled ASA. High d3-ASA concentrations would simulate elevated ASA concentrations and would increase the limit of quantitation. Appropriate choice of the concentration of the internal standard d3-ASA should permit accurate quantification of ASA in human plasma by the method described here in clinical studies on the administration of aspirin and aspirin-prodrugs in different formulations and doses.

5. Conclusions

Starting from commercially available chemicals, *O*-[$^2H_3$]-acetylsalicylic acid was synthesized in one step in high isotopic purity and yield. It was demonstrated that *O*-[$^2H_3$]-acetylsalicylic acid is a reliable internal standard for the accurate quantitative determination of ASA in human plasma after oral administration of low-dose aspirin as well as of guaimesal, an ASA-releasing drug. Extractive PFB esterification and GC–MS–MS analysis in the NICI mode is a reliable technique for rapid, accurate and sensitive determination of ASA in human plasma in pharmacokinetic studies in man with oral low-dose aspirin. *O*-[$^2H_3$]-Acetylsalicylic acid may also be used as an external standard for the quantitative determination of SA by GC–MS.

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