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Determination of acetylsalicylic acid and salicylic acid in skin and plasma by high-performance liquid chromatography

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Abstract

This study describes a HPLC method to determine the concentrations of acetylsalicylic acid (ASA) and salicylic acid (SA) in human stratum corneum and in plasma. The stratum corneum layers for ASA/SA analysis were removed from three patients with postherpetic hyperalgesia treated with topical and oral aspirin. Blood samples were also collected from the same patients. Tape strippings were placed in acetonitrile and sonicated for 15 min. After centrifuging, aliquots of the supernatant were injected into the chromatograph. ASA and SA from plasma samples were extracted on Isolute C_8 columns. Due to interfering peaks in the tape samples, HPLC conditions were slightly different for tape and plasma samples. ASA and SA were separated on a LiChrospher 100 RP-18 column at 1 ml/min using a water–phosphate buffer (pH 2.5)–acetonitrile mobile phase (35:40:25, v/v/v). A linear response to quantities of ASA from 0.1 to 100 $\mu g/cm^2$ and of SA from 0.1 to 5 $\mu g/cm^2$ in tape and to quantities of ASA 0.1 to 2 μg/ml and 1 to 50 μg/ml was obtained and the recovery from tape and plasma samples was over 98%. The method is sensitive (0.1 $\mu g/cm^2$) and specific enough to allow the determination of the drugs in the skin not only after topical but also after oral administration. A good sensitivity was also obtained in plasma (0.1 μg/ml) allowing study of the kinetics of ASA and SA in plasma after oral administration. Concentrations of ASA after topical administration were 100–200 times higher than after oral administration. Plasma levels of ASA and SA after oral administration were similar to those previously found. No ASA or SA were detected in plasma after topical ASA administration. © 1998 Elsevier Science B.V.

Keywords: Pharmacokinetics; Acetylsalicylic acid; Salicylic acid

1. Introduction

The use of topically administered acetylsalicylic acid (ASA) in various solvents has been recently proposed as treatment for pain relief in skin infections [1,2]. To determine whether the analgesic effect is related to the concentrations of the drug in the skin or in plasma, i.e. if the analgesic effect was due to a local or systemic mechanism [3], a method to measure the concentrations of ASA in the skin had to be developed. To date there is only one study in which ASA has been measured in skin but, as is the case with other drugs, a radiolabeled compound was used [4].

A number of methods have been described for the extraction of antinflammatory drugs ASA and SA from plasma [5,6] involving the use of liquid–liquid extraction with organic solvents. The use of solid

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adsorbents as an alternative method of extraction is becoming increasingly popular [7,8]. These adsorbents offer the advantage of avoiding emulsion formation and reducing the volume of solvent required for an efficient extraction. Solid-phase extraction methods are also simple and far less time consuming than liquid–liquid extractions.

This study describes an HPLC method to determine the concentrations of ASA and SA in human stratum corneum using the stripping method and direct injection as well as in plasma using solid-phase extraction.

2. Experimental

2.1. Chemicals and reagents

ASA, SA, Piroxicam (PIR, tape internal standard, I.S.) and Phenobarbital (PHE, plasma I.S.) were acquired from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were HPLC-grade and the other reagents and solvents were of analytical grade (Merck, Darmstadt, Germany).

Tape strippings were performed using 5 cm of 3M adhesive tape (Scotch Magic, 3M) in a ribbon of 2-cm width.

Isolute liquid–solid extraction columns, packed with 100 mg of C_8-bonded 40 μm silica with average pore size of 60 Å and with 1 ml reservoir (International Sorbent Technology, Mid Glamorgan, UK) were used to process the plasma samples.

Stock solutions of drugs (1 mg/ml SA, PHE, and 5 mg/ml ASA, PIR) in acetonitrile were prepared weekly and stored at 4°C protected from light. Phosphate buffer was 200 m*M* potassium dihydrogenphosphate adjusted to pH 2.5 with phosphoric acid. Standard working solutions were prepared daily immediately before use.

2.2. Biological samples

The stratum corneum layers for ASA/SA analysis were removed from two patients with postherpetic hyperalgesia which was treated with topical and oral aspirin. An aspirin–diethyl ether (ADE) mixture was daubed onto the painful hyperaesthetic cutaneous area. A mean dose of 750 mg of ASA was crushed to a fine powder; 20 ml of diethyl ether were then added and stirred to a uniform cloudiness. A 10 cm^2 surface of the skin was delimited by an open circular cell that was fixed to the skin using silicone glue to prevent chemical loss. The same areas served as reference areas for the systemic ASA experiment.

After 30 min of topical contact, or 60 min after oral administration, and after washing with water, the stratum corneum of the treated areas was removed by five successive tape strippings with pieces of adhesive tape (5×2 cm). A second series of strippings was also performed on the same day on each subject in the contralateral area for protein determination. Tapes were frozen and kept at −25°C until analysis within 1 month.

On different days, the same subject was given a single oral dose of 500 mg of ASA. Blood samples were drawn before and 20, 40, 60, 90, 120 and 240 min after either oral or topical ASA administration. After centrifuging, plasma samples were stored frozen at −25°C until analysis within 1 month.

At the beginning of the study, three series of standard tape and plasma samples spiked with low, medium and high concentrations of ASA and SA and with the I.S. (quality control samples) were frozen at −25°C and then one of each concentration analyzed with each run of the unknown samples.

2.3. Extraction of ASA and SA from tape samples

Tapes were placed in a 15-ml glass tube containing 3 ml of acetonitrile and 500 μg of the I.S. (PIR) and sonicated for 15 min. After centrifuging for 10 min at 1500 *g*, 50 μl of the supernatant were injected into the chromatograph.

Tape strippings obtained in normal subjects without drug treatment were used to prepare standard samples by applying known amounts of ASA (1–1000 μg) and SA (1–50 μg) to the adhesive side to produce concentrations of 0.1–100 μg/cm^2 and 0.1–5 μg/cm^2 of tape. After drying, the standard samples were processed as above.

Calibration curves were calculated by linear regression analysis of the ratios of the area of the peak of ASA or SA and those of I.S. versus the ASA and SA concentrations in the standard samples. These curves were used to calculate the concentrations of ASA and SA in unknown samples.

2.4. Extraction of ASA and SA from plasma samples

A 200-μg amount of PHE (I.S.) and 1 ml of 2 *M* HCl were added to 1 ml of plasma. After vortexing for 1 min and centrifuging for 10 min at 1500 *g* the sample was applied to an Isolute C_8 column placed on a Vac-Elut apparatus connected to a vacuum. Before applying a plasma sample, columns were washed, with the vacuum on, with two column volumes of methanol, followed by one volume of 0.1 *M* HCl. The vacuum was then turned off and, without allowing the column to dry, the sample was added to the column and the vacuum turned on. The columns were then washed with five volumes of 2 *M* HCl. The columns were left to dry for 15 min and then the vacuum was turned off. A 6-ml conical centrifuge tube was placed in the VacElut rack under each column. With the vacuum off, 500 μl×2 of methanol–1% NH_4OH–acetonitrile (50:30:20, v/v) were added to each column and the vacuum turned on until all the eluate was collected, and then turned off. A volume of 50–100 μl of the combined eluates were injected into the chromatograph.

Standard plasma samples were prepared by spiking 1 ml of drug-free plasma with known amounts of ASA (0.1–2 μg/ml) and SA (1–50 μg/ml) and 200 μg of I.S. The samples were analyzed according to the procedure described above.

Calibration curves were calculated by linear regression analysis of the ratios of the area of the peak of ASA or SA and those of I.S. versus the ASA and SA concentrations in the standard samples. These curves were used to calculate the concentrations of ASA and SA in unknown samples.

2.5. Liquid chromatography

HPLC analysis was carried out using a Shimadzu (Kyoto, Japan) LC-6A HPLC pump, a SPD-10A UV detector, a SIL-6A autosampler and a SCL-6A CR4-A controller integrator. Wavelength was set at 234 nm.

Separation of ASA and SA was achieved on a LiChrospher 100 RP-18, 250×4 mm I.D., 5 μm (Merck), with a 2-cm pre-column filled with the same material. Elution of the compounds was performed at 1 ml/min using water–phosphate buffer (pH 2.5)–acetonitrile (35:40:25, v/v) as a mobile phase. In these conditions the retention times of ASA, SA and I.S. were about 6.8, 8.7 and 21.6 min (tape I.S.) and 16 min (plasma I.S.).

2.6. Accuracy and precision

Accuracy and precision were evaluated with values obtained following analyses of five standard samples replicated on the same day (intra-day accuracy and precision) and following daily analyses of three quality control standard samples at three different concentrations (inter-day accuracy and precision). Accuracy was calculated as percentage of the measured versus the known concentrations. Precision was determined as the coefficient of variation (C.V.), i.e. the ratio between the mean of the found concentrations and its S.D.

2.7. Recovery

The absolute recovery of ASA and SA from tape and plasma samples was determined by comparing the areas of the peaks of standards not extracted with those of standards extracted according to the procedure described above.

2.8. Protein analysis in the tape strips

A 0.5-ml volume of 0.1 *M* phosphate buffer (pH 7.4) was added to each tape strip in a glass tube and the stratum corneum was eluted from the tape by vortexing for 2 min and sonicating for 10 min. The supernatant was transferred to a fresh tube, the cells destroyed with a potter and then the proteins assayed according to the Lowry method [9].

3. Results

3.1. Determination of ASA and SA in the tape samples

Whereas no interfering peaks near the retention times of ASA, SA and I.S. were present in the chromatograms of blank or basal (time 0) plasma samples, in the blank of tape samples there was a peak interfering with phenobarbital (the I.S. used for

plasma) and therefore a different I.S. (piroxicam) had to be used. Representative chromatograms are shown in Fig. 1 (tape sample).

3.1.1. Linearity

Five standard tape samples between 0.1–5 μg/cm^2 (SA) and 0.1–100 μg/cm^2 (ASA) were analyzed in triplicate to determine the linearity of the assay. The ratios of the areas of the peaks of ASA and SA versus I.S. were linearly related to the drug concentrations within the range of concentrations studied.

3.1.2. Accuracy and precision

Mean intra-day accuracy for the assay of the compounds had a mean accuracy of 98.8 and 99.3% with a precision of 2.3 and 1.8%. Inter-day assay of ASA and SA had a mean accuracy of 100.2 and 98.8% with a mean precision (C.V.) of 2.8 and 1.5%. These results, therefore, validate the calibration curves used for each set of samples.

Experimental results on the quality control samples kept at −25°C (see inter-day accuracy above) guarantee the stability of the samples under our conditions.

3.1.3. Sensitivity and recovery

The lower limit of detection of both ASA and SA (signal three times that of the blank) was 0.05 μg/cm^2 and the lower limit of quantitation (C.V.≤7%) of ASA was 0.1 μg/cm^2 and of SA 1 μg/cm^2. The absolute recovery of ASA and SA, determined by comparing the areas of the peaks of standards not extracted with those of standards extracted according

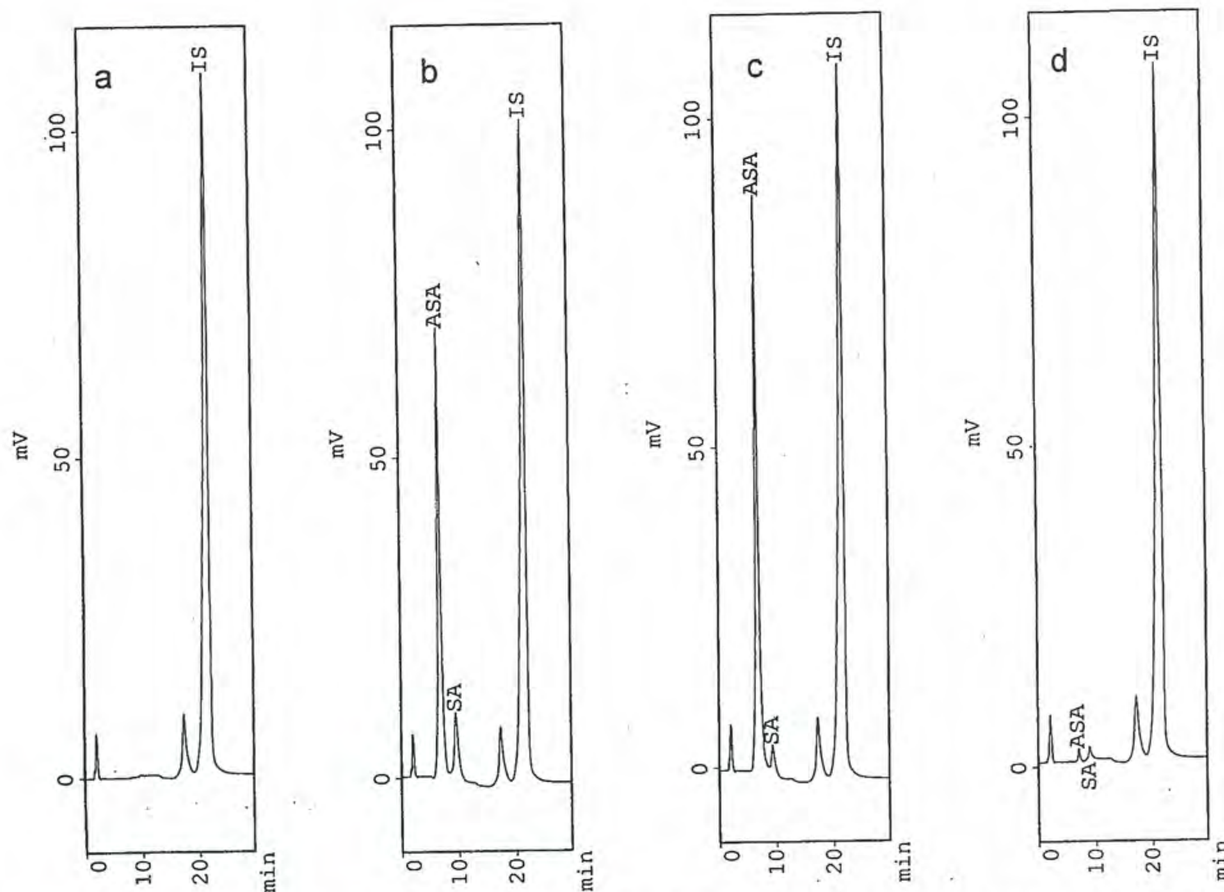

Fig. 1. (a) Tape blank; (b) tape standard, ASA 400 μg/cm^2, SA 40 μg/cm^2; (c) tape sample after topical administration, ASA 520.2 μg/cm^2, SA 5.5 μg/cm^2; (d) tape sample after oral administration, ASA 0.8 μg/cm^2, SA 1.3 μg/cm^2.

to the procedure described above, was higher than 98%.

3.1.4. ASA and SA in the human stratum corneum

Mean protein content in the stratum corneum of the three patients was rather similar: 8.5, 7.9, 9.1 μg/cm^2. Therefore, ASA and SA concentrations in the tapes are reported per cm^2. Concentrations of ASA and SA in the stratum corneum are shown in Table 1. Concentrations of ASA after topical administration were much higher than after oral administration (100–200 times).

3.2. Determination of ASA and SA in plasma

3.2.1. Linearity

Five standard plasma samples between 1 and 50 μg/ml (SA) and 0.1 and 2 μg/ml (ASA) were analyzed in triplicate to determine the linearity of the assay. The ratios of the areas of the peaks of ASA and SA versus I.S. were linearly related to the drug concentrations within the range of concentrations studied. Fig. 2

3.2.2. Accuracy and precision

Mean intra-day accuracy for the assay of the compounds in plasma was 101.3 and 99.7% for ASA and SA, and mean precision (C.V.) was 4.3 and 3.3%. Inter-day assay of ASA and SA in plasma had a mean accuracy of 98.1 and 99.5% and a mean precision (C.V.) of 3.8 and 1.7%. These results, therefore, validate the calibration curves used for each set of samples.

Experimental results on the quality control samples kept at −25°C (see inter-day accuracy above) guarantee the stability of the samples under our conditions.

3.2.3. Sensitivity and recovery

The lower limit of detection of both ASA and SA (signal three times that of the blank) was 0.05 μg/ml and the lower limit of quantitation (C.V.≤7%) of ASA was 0.1 μg/ml, and of SA 0.1 μg/ml.

The absolute recovery of ASA and SA, determined by comparing the areas of the peaks of standards not extracted with those of standards extracted according to the procedure described above, was higher than 98%.

3.2.4. ASA and SA in human plasma

Peak concentrations of ASA and SA in plasma are shown in Table 1. Plasma levels of ASA after oral administration had peak concentrations of 1 mg/l and a rapid decay (half-life of elimination 0.8 h). On the other hand, mean SA concentrations were much higher at the beginning (mean 10 μg/ml) and continuously increased thereafter to about 25 μg/ml at 240 min. No ASA or SA was present in plasma after topical application of ADE.

4. Conclusions

The use of topically administered drugs is becoming a widespread system to overcome toleration problems with certain drugs. Thus, noninvasive methods to measure the concentration of drugs at the site of administration are important, particularly with nonradioactive techniques.

This method using noninvasive tape-stripping sampling and HPLC chromatography appears to be very suitable for the accurate determination of aspirin and its main metabolite, salicylic acid, within the human stratum corneum. The large amounts of

Table 1
Concentrations of ASA and SA in the stratum corneum and plasma (peak concentrations) after topical (750 mg) or oral (500 mg) administration of ASA

Subject	Tape (μg/cm^2)				Plasma (μg/ml)			
	Topical		Oral		Topical		Oral	
	ASA	SA	ASA	SA	ASA	SA	ASA	SA
1	680.5	3.5	1.9	2.1	<0.1	<0.1	1.2	22.4
2	520.2	5.5	0.8	1.3	<0.1	<0.1	1.5	29.8
3	388.6	2.2	1.0	1.7	<0.1	<0.1	1.1	24.8

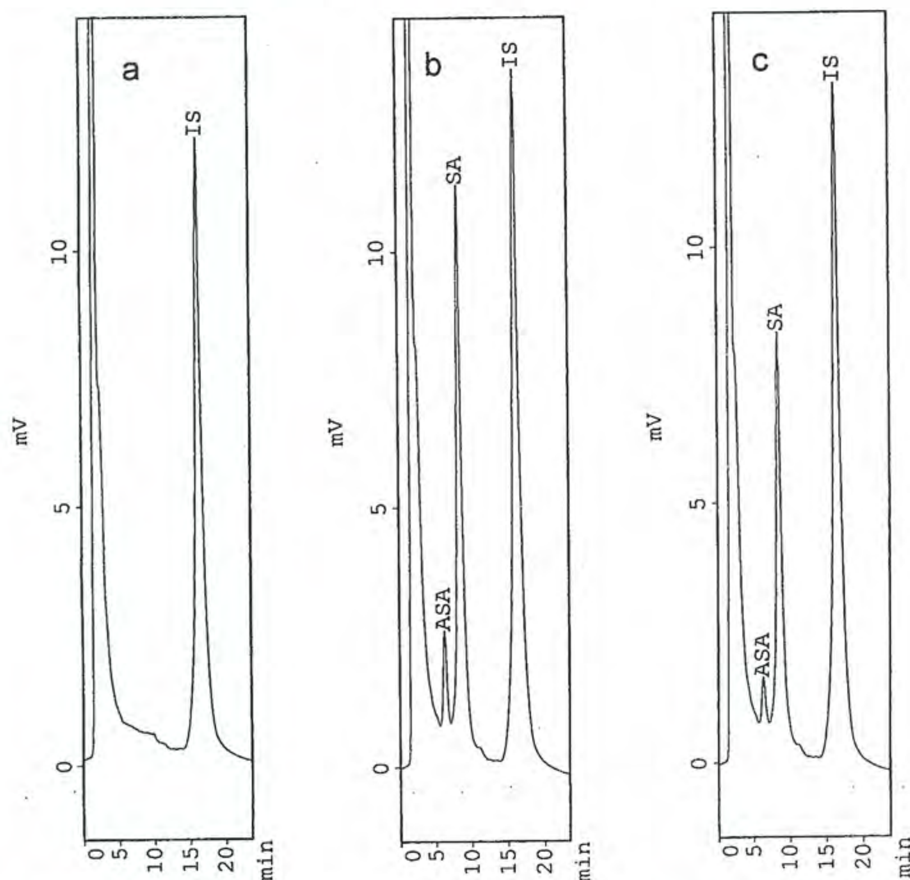

Fig. 2. (a) Plasma blank; (b) plasma standard, ASA 1 μg/ml, SA 10 μg/ml; (c) plasma sample, ASA 0.4 μg/ml, SA 8.6 μg/ml.

substance present after topical administration (about 100–200 times those found after oral administration) makes the analytical procedure, in this case, rather simple. Our method, however, is sensitive and specific enough to allow the determination of the drugs in the skin not only after topical but also after oral administration i.e. when the concentrations of the drugs are rather low.

The simultaneous determination of the drugs in plasma may allow determination of whether the drug has an extensive percutaneous absorption. This was not so in our case because the levels of ASA and SA in plasma after topical application were below the sensitivity of the method. This is not surprising because, as previously indicated by Rougier et al. [4], the percutaneous absorption of aspirin after single topical administration is slow and very low, about 2% of the drug administered and can only be detected by measuring the drug excreted in urine over a period of several days. The described method of extraction of ASA and SA from plasma samples whilst has a very good recovery of the compounds has the advantage of giving cleaner extracts than liquid–liquid extraction, lipid material being retained on the column. The problem of emulsion formation is also avoided. Overall, the extraction is efficient and rapid, allowing the extraction of several samples on the same day.

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