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RESEARCH ARTICLE

Preparation, characterization, and topical delivery of paromomycin ion pairing

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Abstract

Topical chemotherapy with paromomycin (PA) has been used as an alternative for the treatment of cutaneous leishmaniasis; however, poor skin penetration of this drug limits the efficacy of formulations. The objective of this work was to study the ability of the PA free base to form ion pairing with organic acids, as well as evaluate the effect of these compounds on the topical delivery of PA. PA permeation across intact skin was low, while drug penetration into skin from PA ion pairing was the higher than that observed for the PA base. Data obtained on the stripped skin, a damaged skin model, clearly showed that the ion pairing presented a potential to improve PA skin permeation.

Keywords: Cutaneous leishmaniasis, topical treatment, paromomycin, ion pairing, skin penetration

Introduction

Leishmaniasis is a disease with a wide spectrum of clinical manifestations caused by different species of flagellae protozoa belonging to the Leishmania genus[1]. There are two main clinical manifestations of leishmaniasis: cutaneous and visceral. Cutaneous leishmaniasis (CL) most commonly appears first as a localized papule, which then evolves into an ulcer upon the loss of the epidermis, resulting in a great impairment of the skin barrier. Parenteral administration of pentavalent antimony organic compounds (20 mg Sb^{5+}/kg/day for up to 30 days) remains as the first choice therapy for all leishmaniasis forms. However, resistance and high frequency of side effects (anorexy, myalgias, arthralgias, chemical pancreatitis, leucopenia, cardiotoxicity, etc.) are still relevant problems associated with this treatment[2,3].

Over the past few decades, major emphasis has been given to the development of alternative therapies, including the identification of formulations for both oral and topical treatment of CL. Topical treatment represents an interesting alternative, offering various advantages in comparison with the parenteral administration: easy administration, lower adverse reaction incidence, and an attractive cost-benefit ratio.

Skin condition represent an important consideration in the topical treatment of CL. Formulations can be applied either to thickened lesions, or, more commonly, to open lesions (ulcers), in which the barrier properties of the epidermis have been completely lost[4]. In addition, the topical treatment of CL can last for a period of up to 20–30 days[5]. Therefore, re-epithelization of open lesions can be observed, at least partially, during therapy, while the renewal of the epidermis contributes to a decrease in drug penetration due to barrier repair.

Paromomycin (PA), an aminoglycoside antibiotic, has a broad spectrum of activity, including activity against protozoa, such as *Leishmania spp*[6]. In addition, it is the most commonly studied drug for the topical treatment of CL[4]. Two topical formulations were evaluated for the treatment of CL, both of which containing PA, formulated as an ointment. However, these PA ointments possess problems of varying efficacy and toxicity[7–9]. The low-efficacy of these formulations can be related to poor penetration of the PA into the CL lesion. PA is a highly hydrophilic drug

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with a high molecular weight and relatively lipid insoluble. It is well known that hydrophilic drugs usually have poor skin penetration[10]. The formation of ion pairing of oppositely charged molecules is a strategy that has been employed to enhance the skin penetration of drugs[10–13]. PA contains amino groups in its structure (Figure 1) that can be used to form salts with organic acid. These salts, where ions are in close proximity and their charges are masked or shielded by hydrocarbon moieties of the functional groups, display a lower hydrophilicity than do the two ions considered separately[11,14]. In addition, they provide an unusual behavior for an ionic species, such as high solubility in apolar solvents or increased partition toward a lipid phase. Thus, it could be concluded that ion pairing may well provide an interesting alternative for the improved topical delivery of PA.

In this study, the ion pairing of PA with lactic, butyric, benzoic, and cinnamic acids (Figure 2) was prepared and characterized by NMR spectroscopy and thermal analysis. Studies of the solubility of PA free base and its salts were also conducted in various solvents. In addition, the skin permeation of ion pairing was compared with that of the PA free base. *In vitro* permeation and penetration studies were performed on pig ear skin, a relevant model for human skin[15]. As an attempt to mimic the conditions of use, these studies were conducted using two skin models: intact and stripped skin. Although stripped skin is not classified as ulcerated skin, it is a model that has been widely used to simulate a damaged skin barrier[16].

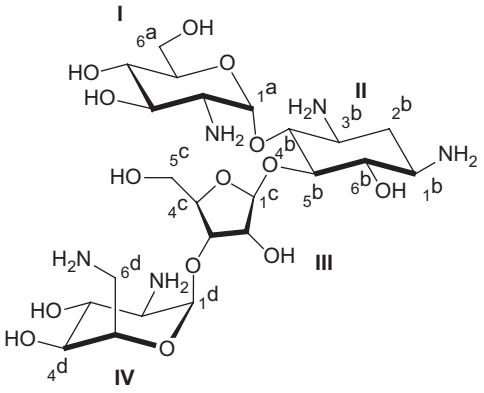

Figure 1. Chemical structure of PA (as base).

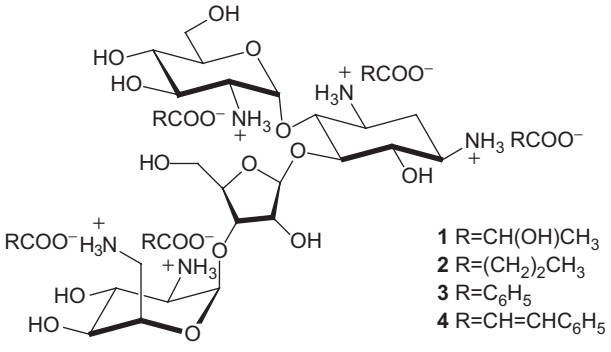

Figure 2. Chemical structure of PA salts.

Methods

Material

PA sulfate, potency of 757 mg/g, was obtained from Antibioticos (Milan, Italy). PA sulfate USP reference standard (potency, 730 mg/g) was used. Benzoic, butyric and lactic acids were obtained from Sigma-Aldrich (St. Louis, MO). Cinnamic acid was synthesized from benzaldehyde and malonic acid by Knoevenagel condensation reaction and used without purification. All other chemicals were of analytical reagent grade. Bacillus subtilis ATCC 6633 was used as the testing organism. The culture mediums used were: antibiotic medium no 1 and antibiotic medium no 5.

NMR spectroscopy

The NMR spectra were recorded on a Bruker *AVANCE* DRX400 instruments, using tetramethylsilane as the internal standard. Chemical shifts are given in δ (ppm) scale and *J* values are given in Hz. Samples were dissolved in deuteromethanol or deuterium oxide.

The structure of PA base and its salts was confirmed by ^{1}H and ^{13}C NMR spectral data, DEPT experiment, and ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC and HMBC experiments.

Thermal analysis

The simultaneous thermogravimetry (TG), derivative thermogravimetry (DTG) and differential thermal analysis (DTA) curves were performed with a Shimadzu DTG60 apparatus using about 2.0–3.0 mg of samples packed in alumina crucibles. Samples were heated at 10°C min^{-1} from room temperature to 650°C under a dynamic nitrogen flow of 100 ml·min^{-1}.

Preparation of PA free base

The PA free base was prepared from PA sulfate, using ion-exchange resin Amberlite IRA-402 (OH), as previously described[17]. Amberlite IRA-402 resin (70 ml) was transferred to a 100-ml beaker. Distilled water was added in sufficient amount to cover the resin. After decantation, the supernatant was discarded. The same procedure was repeated five times. Then, the washed resin was placed in a glass column 2.5 cm in diameter and 30 cm in length, and 50 ml of water. A 1.0 ml solution of 0.5 M PA sulfate was loaded onto the column. The PA was eluted from the column with 300 ml of water at a flow rate of 1.0 ml/min, and 10 fractions of 30 ml were collected. The pH of each fraction was measured. The fractions having a pH 9.0 were pooled, lyophilized and stored in desiccator.

^{1}HNMR (400 MHz, D_2O): **Ring I** δ 2.70–2.63 (m, H-2a), 3.19–3.26 (m, H-4a), 3.57–3.63 (m, H-3a), 3.32–3.41, 3.77–3.84 (H-6a and H-6a'), 3.86–3.90 (m, H-5a), 5.29–5.31 (m, H-1a); **Ring II** δ 1.14 (q, H-2b, *J* = 13.0 Hz), 1.91 (m, H-2b'), 2.63–2.70 (m, H-1b or H-3b), 2.78–2.86 (m, H-3b or H-1b), 3.22 (t, H-4b or H-6b, *J* = 9.8 Hz), 3.32–3.41 (m, H-6b or H-4b), 3.59–3.63 (m, H-5b); **Ring III** δ 3.65–3.71 (m, H-5c), 3.77–3.84 (m, H-5c), 4.09 (m, H-4c), 4.23 (m, H-2c), 4.35–4.38 (m, H-3c), 5.29–5.31 (m, H-1c); **Ring IV**

δ 2.78–2.86 (m, H-6d), 2.93–2.99 (m, H-2d and H-6d), 3.50–3.63 (m, H-4d), 3.86–3.90 (m, H-5d), 3.96–3.97 (m, H-3d), 4.91 (m, H-1d).

Microbiological assay

The PA concentration was determined by a validated agar-diffusion inhibition assay of the growth of *Bacillus subtilis* (ATCC 6633), as previously described[18]. Briefly, a standard curve was obtained with the following PA concentrations: 0.2, 0.4, 0.8, 1.2, and 3.2 μg/mL. Limits of detection and quantification were 0.15 and 0.2 μg/mL, respectively. Three separate calibration curves were obtained on different days. Statistical analyses were performed significance test of regression, intercept and slope of the straight line.

Synthesis of PA salts

PA base (one equivalent) and lactic, butyric, benzoic or cinnamic acids (five equivalents) were dissolved in methanol and the mixture was stirred at room temperature for 16 h. The solvent was removed in a rotatory evaporator. The salts were obtained with quantitative yield.

PA lactate (1)

^{1}HNMR (400 MHz, CD_3OD): **Ring I** δ 3.26–3.23 (m, H-2a), 3.79–3.60 (m, H-6a), 3.95–3.79 (m, H-6a′, H-4a and H-5a), 4.36–4.32 (m, H-3a), 5.56 (m, H-1a); **Ring II** δ 1.69 (m, H-2b), 2.33 (m, H-2b′), 3.26–3.10 (m, H-1b and H-3b), 3.58 (m, H-4b or H-6b), 3.95–3.79 (m, H-6b or H-4b and H-5b); **Ring III** δ 3.79–3.60 (m, H-5c), 3.95–3.79 (m, H-5c′), 4.17 (m, H-4c), 4.36–4.32 (m, H-2c), 4.53 (m, H-3c), 5.35 (m, H-1c); **Ring IV** δ 3.26–3.23 (m, H-6d), 3.42–3.32 (m, H-6d′ and H-2d), 3.79–3.60 (m, H-4d), 4.17 (m, H-3d), 4.36–4.32 (m, H-5d), 5.25 (m, H-1d); **Lactate** δ 1.34 (d, C*H*$_3$, *J* = 6.8 Hz), 4.04 (m, C*H*OH).

PA butyrate (2)

^{1}HNMR (400 MHz, CD_3OD): **Ring I** δ 3.12 (dd, H-2a, *J* = 13.2 and 3.6 Hz), 3.35–3.25 (m, H-4a), 3.69–3.62 (m, H-5a), 3.84–3.62 (m, H-6a), 3.92–3.86 (m, H-6a′), 4.22 (m, H-3a), 5.47 (d, H-1a, *J* = 3.6 Hz); **Ring II** δ 1.49 (q, H-2b, *J* = 12.8 Hz), 2.21–2.16 (m, H-2b′), 2.98–3.02 (m, H-1b and H-3b), 3.45 (t, H-4b or H-6b, *J* = 9.2 Hz), 3.53 (t, H-6b or H-4b, *J* = 9.2 Hz), 3.69–3.62 (m, H-5b); **Ring III** δ 3.84–3.62 (m, H-5c), 3.92–3.86 (m, H-5c), 4.13–4.11 (m, H-4c), 4.30–4.28 (m, H-2c), 4.50–4.47 (m, H-3c), 5.34 (d, H-1c, *J* = 2.4 Hz); **Ring IV** δ 3.20 (dd, H-6d, *J* = 13.2 and 3.6 Hz), 3.35–3.25 (m, H-6d′ and H-2d), 3.69–3.62 (m, H-4d), 3.84–3.62 (m, H-5d), 5.19 (m, H-1d); **Butyrate** δ 0.95 (t, C*H*$_3$, J = 7.6 Hz), 1.62 (sx, C*H*$_2$CH$_3$, J = 7.6 Hz), 2.19 (t, C*H*$_2$COOR, J = 7.6 Hz).

PA benzoate (3)

^{1}HNMR (400 MHz, CD_3OD): **Ring I** δ 3.28–3.19 (m, H-2a), 3.39–3.32 (m, H-4a), 3.66–3.59 (m, H-6a), 3.88–3.76 (m, H-5a), 3.95–3.90 (m, H-6a′), 4.29–4.25 (m, H-3a), 5.52 (d, H-1a, *J* = 3.6 Hz); **Ring II** δ 1.60 (q, H-2b, *J* = 13,2 Hz), 2.27 (dt, H-2b′, *J* = 13,2 and 4,8 Hz), 3.08–3.02 (m, H-1b and H-3b), 3.54 (t, H-4b or H-6b, *J* = 9.6 Hz), 3.66–3.59 (m, H-5b and H-4b or H-6b); **Ring III** δ 3.88–3.76 (m, H-5c and H-5c′), 4.17–4.11 (m, H-4c), 4.33 (dd, H-2c, *J* = 5,2 and 3.6 Hz), 4.52 (dd, H-3c, *J* = 6.8 and 5.2 Hz), 5.36 (d, H-1c, *J* = 3.6 Hz); **Ring IV** δ 3.28–3.19 (m, H-6d), 3.39–3.32 (m, H-6d′), 3.42–3.40 (m, H-2d), 3.70–3.68 (m, H-4d), 3.88–3.76 (m, H-5d), 4.20 (m, H-3d), 5.26 (d, H-1d, *J* = 1.2 Hz); **Benzoate** δ 7.38 (m, 2 x H-*meta*), 7.44 (m, H-*para*), 7.96 (m, 2 x H-*orto*).

PA cinnamate (4)

^{1}HNMR (400 MHz, CD_3OD): **Ring I** δ 3.27–3.18 (m, H-2a), 3.41–3.34 (m, H-4a), 3.87–3.62 (m, H-6a), 3.95–3.88 (m, H-6a′ and H-5a), 4.29 (m, H-3a), 5.51 (d, H-1a, *J* = 3.2 Hz); **Ring II** δ 1.58 (q, H-2b, *J* = 12.8 Hz), 2.27–2.23 (m, H-2b′), 3.07–3.03 (m, H-1b and H-3b), 3.53 (t, H-4b or H-6b, *J* = 9.2 Hz), 3.70–3.62 (m, H-6b or H-4b and H-5b); **Ring III** δ 3.87–3.62 (m, H-5c), 3.95–3.88 (m, H-5c′), 4.19 (m, H-4c), 4.35 (m, H-2c), 4.55–4.51 (m, H-3c), 5.37 (sl, H-1c); **Ring IV** δ 3.27–3.18 (m, H-6d), 3.41–3.34 (m, H-6d′ and H-2d), 3.87–3.62 (m, H-4d), 4.19 (m, H-3d), 4.29 (m, H-5d), 5.28 (sl, H-1d); **Cinnamate** δ 6.50 (d, C*H*COOR, *J* = 16 Hz), 7.38–7.31 (m, 2 x H-*meta* and H-*para*), 7.48 (d, C*H*C$_6$H$_5$, J = 16 Hz), 7.34–7.52 (m, 2 x H-*orto*).

Determination of solubility and partition coefficient

In this study, it was not possible to evaluate the influence of the ion pairing on the partition coefficient of PA as the drug concentration in octanol was below the detection limit of the method of analysis. Thus, to investigate the influence of the ion pairing on drug lipophilic properties, the solubility of PA sulfate, PA free base and its salts was determined in three different solvents, namely water, methanol and ethanol.

An excess of these compounds (sulfate, base and salts) was added to the formation of a suspension of the drug. These suspensions were kept under agitation for 24 h at 25°C, in absence of light and in nitrogen atmosphere in order to avoid the PA oxidation. At the end of 24 h, the suspensions were filtered through a 0.45 μm Millex filter HV (Millipore, Billerica, MA) to eliminate the insoluble drug and diluted in phosphate buffer. The PA concentration in the filtrate was determined by microbiological assay.

In vitro skin permeation and penetration

In vitro skin permeation and penetration of PA and its ion-pairs were determined using a Franz diffusion cell (membrane surface area of 1.77 cm^2 and a receptor fluid volume of 6.7 mL) with full-thickness skin (~1 mm) excised from the ears of pigs as previously described[19]. The skin sections were kept horizontally dividing the cell into two compartments: the donor and the receptor. Sink conditions were obtained with phosphate buffered saline (PBS) (pH 7.4), containing 0.01% $HgCl_2$ as a preservative. The receptor phase, continuously stirred with a small magnetic bar, was maintained at 37 ± 0.5°C. The skin was

allowed to equilibrate with the environment for 1 h. After this period, the receptor fluid was completely removed, and this compartment was filled with PBS without preservative to avoid interference with the microbiological assay. The interference of $HgCl_2$ was completely eliminated after having rinsed the receptor chamber three times.

Permeation experiments were conducted in intact and stripped skin. The donor compartment was left open and a relevant clinical dose (100 µL containing 1% PA w/w in ethanol) was applied. Sampling was performed at 2, 4, 6, and 8 h through the total removal of the receptor fluid. The PA concentration was determined by microbiological assay.

Skin penetration experiments were conducted with intact skin. Removal of excess formulation was carried out as previously described[18]. The skin was removed from the Franz cells for PA extraction and, after 2 h of contact with 4 mL of 0.1 M phosphate buffer (pH 8.0), the fragments were vortex mixed for 5 min. PA concentration was determined by microbiological assay. Previous experiments, performed immediately after application of the preparations, were conducted to validate extraction procedure. The PA recovery from skin was of 72 ± 4.7% ($n = 3$)

Considering that parasites in CL are located mainly in the dermis, the amount of PA permeated across and into skin was considered as an indicator of topical delivery.

Preparation of stripped skin

The SC was removed from the skin by stripping it 30 times with adhesive tape (Book Tape no 845 Scotch, 3M, EUA) as previously described[20]. The tapes were pressed on the skin using a roll and removed with one quick movement. SC removal was confirmed by histological analysis.

Statistical analysis

Student's t-test was used to evaluate differences between means in studies of skin permeation. Differences were considered statistically significant when the P value was <0.05.

Results and discussion

PA free base

The PA base was obtained from PA sulfate with a yield of 70%. The PA free base was characterized by NMR spectroscopy. Comparison of ^{1}H NMR spectra of the PA free base and PA sulfate showed significant chemical shift differences for the protons near amine nitrogen atoms (Table 1). All signals of the PA free base protons, as compared to those of the PA sulfate, showed upfield shifts, an observation indicating that PA is fully deprotonated. The ^{1}H and ^{13}C NMR signals assignment was based on homonuclear and heteronuclear 2D experiments (COSY, HMQC, and HMBC).

Microbiological assays

The microbiological activity of the PA free base was similar to PA sulfate. Statistical analysis of the standard curve obtained for the PA free base ($y = 18.8459 + 8.5211x$; $r = 0.9832$) and another for PA sulfate ($y = 18.641 + 8.3554x$; $r = 0.9926$) revealed similar data.

Characterization of PA salts

NMR spectroscopy

The ^{1}H NMR measurements were important to obtain evidence for the formation of ion pairing between the PA free base and organic acids due to chemical shift changes to protons near the ionized groups. The signals of the protons on carbons adjacent to nitrogen, as compared to the free base amine, are recognized by their downfield position in the salt.

Initially, the chemical shifts of PA free base protons were compared with those of the salts. The smaller difference between the chemical shifts of the PA free base and PA salt protons reflects a greater charge neutralization and, therefore, offers more evidence toward the formation of ion pairing[10]. Comparison between chemical shifts of the protons near nitrogen atoms of PA free base amines and its salts are indicated in Table 2.

From results shown in Table 2, it is evident that smaller downfield shifts can be observed for PA butyrate (**2**) proton signals as compared to those of the others salts. In contrast, larger downfield shifts are observed for PA lactate (**1**) protons. The degree of charge neutralization was higher in the following sequence: PA lactate (**1**) <PA benzoate (**3**) ≅PA cinnamate (**4**) <PA butyrate (**2**).

The structural characterization of PA salts was also based on the comparison among the chemical shifts of the acids in their protonated forms, sodium salts, and PA salts (Table 3).

The chemical shifts of protonated acids (RCOOH) were considered as values for complete protonation, while the chemical shifts of sodium salts ($RCOO^-Na^+$) were considered as values for complete ionization. Intermediate chemical shift values reflect the degree of charge neutralization resulting from the interaction between the carboxylate anion and the nitrogen cation in the PA salts.

Table 1. Comparison of ^{1}H NMR data of PA free base and PA sulfate.

		Chemical shifts (δ)*	
Assignment		PA free base	PA sulfate
Ring I	H-2a	2.63–2.70	3.31–3.36
Ring II	H-1b	2.63–2.70 or 2.78–2.86	3.23–3.32 or 3.37–3.40
	H-2b axial	1.14	1.76
	H-2b equatorial	1.91	2.37
	H-3b	2.78–2.86 or 2.63–2.70	3.37–3.40 or 3.23–3.32
Ring IV	H-2d	2.93–2.99	3.54
	H-6d	2.78–2.86	3.31–3.36
	H-6d'	2.93–2.95	3.31–3.36

*Samples were dissolved in D_2O.

Table 2. Comparison of ^{1}H NMR data of PA free base and its salts.

Assignment		Chemical shifts (δ)*				
		PA free base	PA lactate	PA butyrate	PA benzoate	PA cinnamate
Ring I	H-2a	2.52	3.23–3.26	3.12	3.19–3.28	3.18–3.27
Ring II	H-1b	2.52 or 2.64–2.69	3.10–3.26	2.98–3.02	3.02–3.08	3.03–3.07
	H-2b axial	1.08	1.69	1.49	1.60	1.58
	H-2b equatorial	1.85	2.33	2.16–2.21	2.27	2.23–2.27
	H-3b	2.64–2.69 or 2.52	3.10–3.26	2.98–3.02	3.02–3.08	3.03–3.07
Ring IV	H-2d	2.86	3.32–3.42	3.25–3.35	3.40–3.42	3.34–3.41
	H-6d	2.85–2.91	3.23–3.26	3.20	3.19–3.28	3.18–3.27
	H-6d'	2.85–2.91	3.32–3.42	3.25–3.35	3.32–3.39	3.34–3.41

*Samples were dissolved in CD_3OD.

Table 3. ^{1}H NMR chemical shifts of protons of organic acids and their salts.

	Chemical shifts (δ)[a]											
	$CH_3CH(OH)CO_2R$			$CH_3(CH2)_2CO_2R$			$C_6H_5CO_2R$			$C_6H_5CH=CHCO_2R$		
Assignment	R=H	R=PA	R=Na	R=H	R=PA	R=Na	R=H	R=PA	R=Na	R=H	R=PA	R=Na[b]
CH_3	1.38	1.34	1.30	0.96	0.95	0.82	—	—	—	—	—	—
CH(OH)	4.22	4.04	4.05	—	—	—	—	—	—	—	—	—
CH_2CH_3	—	—	—	1.62	1.62	1.50	—	—	—	—	—	—
CH_2CO_2R	—	—	—	2.26	2.19	2.03	—	—	—	—	—	—
H-*orto*	—	—	—	—	—	—	8.03	7.96	7.82	7.57	7.53	—
H-*meta*	—	—	—	—	—	—	7.46	7.38	7.24	7.39	7.35	—
H-*para*	—	—	—	—	—	—	7.58	7.44	7.24	7.39	7.35	—
C_6H_5CH	—	—	—	—	—	—	—	—	—	7.67	7.48	—
$CHCO_2R$	—	—	—	—	—	—	—	—	—	6.47	6.50	—

[a]Samples were dissolved in CD_3OD.
[b]Sample insoluble in CD_3OD.

The chemical shift changes observed for proton signals from PA butyrate (**2**) and benzoate (**3**) are smaller than those for the corresponding sodium salts, as compared to the protonated acid (Table 3). These results indicate that (**2**) and (**3**) are in the form of ion pairing due to the greater charge neutralization. On the other hand, the comparison of the chemical shift of the proton adjacent to the carboxylate anion of the PA lactate (**1**) (4.04 ppm) and sodium lactate (4.05 ppm) showed no significant change, which indicates a higher ionization of (**1**). NMR characterization of the PA cinnamate (**4**) was performed by comparing it only with the spectra of the cinnamic acid, due to the fact that sodium cinnamate was insoluble in CD_3OD. The chemical shifts of the protons of (**4**) and cinnamic acid are very similar (difference ≤0.04 ppm), except for the chemical shift of proton attached to carbon adjacent to the aromatic ring (difference 0.19 ppm). These values indicate that considerable charge neutralization occurs in (**2**), (**3**), and (**4**). Less charge neutralization occurs in (**1**).

Thermal analysis

The thermal behavior of the PA free base and salts was evaluated by simultaneous DTA–TG–DTG. None of the compounds studied were found to be undergoing a true melting process. Instead, they decomposed endothermically. Significant differences between DTA-TG curves of salts and the PA free base could be observed (Figure 3). In the TG-DTG curves of the salts, decomposition peaks <220°C may well be due to the loss of counterions. Decomposition temperatures of the compounds can be used as primary parameters of their thermal stability. Thus, the PA free base decomposes at higher temperatures than do their salts, decreasing their stability in the sequence PA base > PA cinnamate > PA benzoate > PA lactate > PA butyrate. The endothermic event observed for all compounds between 50 and 70°C can be associated with the elimination of water.

Determination of solubility and partition coefficient

First, the influence of ion pairing on the octanol/water partition coefficient of PA was investigated. Our data showed that ion paring produces a negligible influence on this parameter (data not shown). Next, the solubility of PA and its salts was evaluated using solvents with different polarities (water, methanol, and ethanol) (Table 4). The formation of PA organic salts tends to result in a significant decrease in solubility in water. In contrast, the solubility of the PA free base and PA sulfate in methanol and ethanol was lower than that observed for ion pairing. It is important to note that PA benzoate solubility in ethanol, for example, was 40 times higher than that observed for the PA free base. These results suggest that the PA lipophilic properties increased slightly when in the form of organic salts.

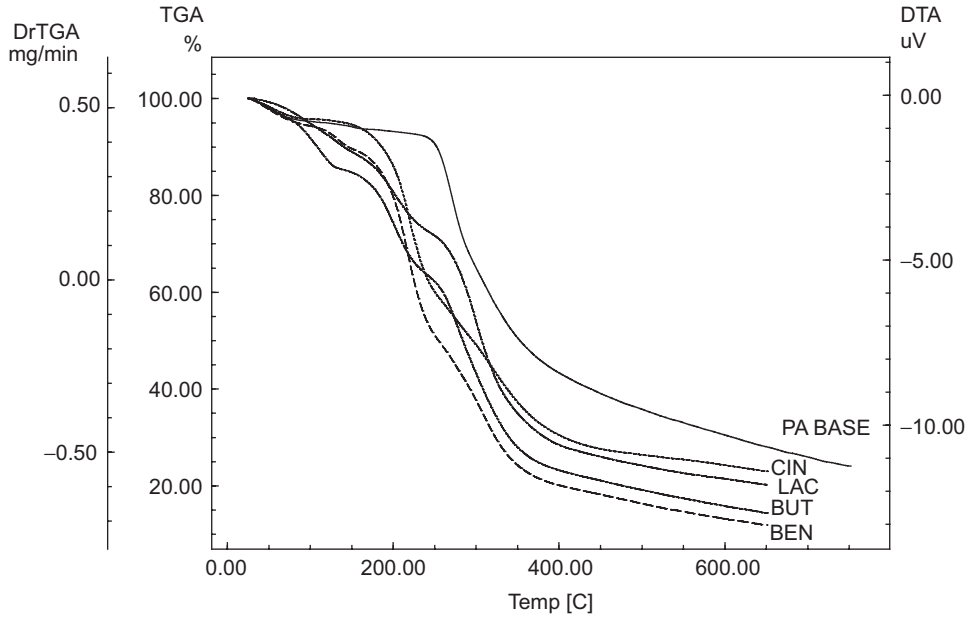

Figure 3. TG curves of PA base and salts.

Table 4. Solubility (mg/ml) of PA and PA salt's in different solvents.

	Solubility (mg/ml) ± SD*		
PA salt's	Water	Methanol	Ethanol
PA sulfate	>1000	<0.01	<0.005
PA free base	>1000	8.3 ± 0.2	1.4 ± 0.1
PA lactate (**1**)	26.4 ± 2.4	110.6 ± 3.2	10.3 ± 0.7
PA butyrate (**2**)	25.5 ± 1.0	110.4 ± 4.3	12.4 ± 0.4
PA benzoate (**3**)	34.8 ± 1.3	280.5 ± 21.5	57.9 ± 2.7
PA cinnamate (**4**)	14.2 ± 0.9	113.8 ± 4.0	34.8 ± 1.3

*Values represent the mean ± SD ($n = 3$).

In vitro skin permeation and penetration

In vitro skin permeation experiments were conducted with intact and stripped skin. Permeation of PA and its salts across intact skin after 8 h were low. In all cases, the amount of permeated PA was found to be below the detection limit of the analytical method (Table 5). These data can be attributed to hydrophilic properties as well as to the molecular mass of the PA free base and their salts. The formation of ion pairing has been described as a strategy to increase the lipophilic properties of drugs, which can favor skin permeation[10–13]. Megwa et al. showed that secondary, tertiary, and quaternary amines increased the *in vitro* permeation of salicylate across human skin, with the largest increase observed when applying triethylamine, a tertiary amine. These effects can be attributed to an increased partition coefficient[12]. Our data showed that the partition coefficient of ion pairing between PA and organic acids was not increased when comparison to the PA free base. This finding may well explain our data of permeation.

In contrast, the PA skin penetration from ion pairing was generally higher than that observed from the PA free base (Table 5). Penetration of PA lactate and benzoate into the skin (epidermis plus dermis) was approximately 2.6 and 1.6 times higher, respectively, than that observed for the PA free base. These differences were also found to be statistically significant ($P < 0.05$). In contrast, there was no significant difference in the skin penetration from PA cinnamate, PA butyrate, and the PA free base.

Table 5. *In vitro* skin permeation[a] and penetration of PA as free base and salts (% dose applied) across intact and stripped skin after 8 h.

	Intact skin		Stripped skin[c]
Compounds	Permeation	Penetration	Permeation
PA base	ND[b]	0.30 ± 0.02	10 ± 1
PA lactate (**1**)	ND	0.77 ± 0.04	11 ± 7
PA butyrate (**2**)	ND	0.32 ± 0.04	33 ± 14
PA benzoate (**3**)	ND	0.49 ± 0.02	22 ± 14
PA cinnamate (**4**)	ND	0.37 ± 0.09	17 ± 9

[a]Values represent the mean ± SD ($n = 4$); the receptor liquid was collected after 8 h.
[b]No detected.
[c]SC was removed by stripping 30 times the skin with adhesive tape (Book Tape no 845, Scoth[3M]); penetration studies were not conducted with stripped skin.

Data concerning PA permeation across stripped skin, a model of damaged skin, are also shown in Table 5. As expected, PA permeation across stripped skin was higher than that observed in the intact skin, which was observed for all compounds studied. It is noteworthy that, in spite of the complete removal of the SC, the PA free base permeation across the stripped porcine skin after 8 h was still low. These data are consistent with our previous findings which showed that permeation of PA sulfate across stripped skin was low[21]. However, it is possible to observe a strong tendency of salts, as compared to the PA free base, to increase drug permeation. Permeation of the PA butyrate was significantly higher than that observed for PA free base. PA permeation from salts benzoate, lactate and cinnamate was also higher than that observed

for the free base. However, these differences were not statistically significant. PA butyrate resulted in the highest skin permeation, while PA lactate showed the lowest permeation. It is also possible to speculate that our data of penetration into skin, as well as those observed for permeation across stripped skin, can be attributed to different solubility properties among the PA free base and their ion pairing with organic acids. On the other hand, these findings are in agreement with those obtained from NMR spectral data: PA butyrate presents the largest degree of ion-pair formation, whereas the lowest degree was observed between PA and lactic acid.

Conclusion

In summary, the formation of PA salts results in changes to the ^{1}H and ^{13}C NMR spectra. These spectral shifts reflect changes in the molecular environment, which may be due to hydrophobic or electronic interactions, including ion-pair formation among the molecules. In addition, PA salts exhibited a thermal profile similar to each other but different from that of the PA free base. Finally, our data showed that ion pairing has little influence on PA permeation across intact skin. This finding can be attributed to the low partition coefficient of these compounds. In contrast, data obtained on the stripped skin clearly showed that the ion pairing presented a potential to improve PA skin permeation. This is particularly relevant for the topical treatment of CL, especially when considering that the formulations are more frequently applied to damaged skin than to normal skin.

Declaration of interest

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