ELSEVIER

Journal of Chromatography B, 707 (1998) 322–327

JOURNAL OF CHROMATOGRAPHY B

Short communication

Determination of aspirin and salicylic acid in transdermal perfusates

Gillian P. McMahon[a,*], Shane J. O'Connor[b], Desmond J. Fitzgerald[b], Sylvie le Roy[b], Mary T. Kelly[a]

[a]*Department of Chemistry, The Royal College of Surgeons in Ireland, St. Stephen's Green, Dublin 2, Ireland*
[b]*Department of Clinical Pharmacology, The Royal College of Surgeons in Ireland, St. Stephen's Green, Dublin 2, Ireland*

Received 5 May 1997; received in revised form 14 November 1997; accepted 14 November 1997

Abstract

A high-performance liquid chromatographic (HPLC) method has been developed for the simultaneous determination of aspirin and salicylic acid in transdermal perfusates. The compounds were separated on a C_8 Nucleosil column (5 μm, 250×4.6 mm) using a mobile phase containing a mixture of water–acetonitrile–orthophosphoric acid (650:350:2, v/v/v) and a flow-rate of 1 ml/min. The transdermal samples were in phosphate-buffered saline (PBS) and could be injected directly onto the HPLC system. The method was reproducible with inter-day R.S.D. values of no greater than 3.46 and 2.60% for aspirin and salicylic acid, respectively. The method was linear over the concentration range 0.2–5.0 μg/ml and had a limit of detection of 0.05 μg/ml for both compounds. For certain samples, it was necessary to ensure that no transmembrane leakage of the aspirin prodrugs had occurred. In these cases, a gradient was introduced by increasing the acetonitrile content of the mobile phase after the salicylic acid had eluted. The method has been applied to the determination of aspirin and salicylic acid in PBS following in vitro application of the compounds to mouse skin samples.

Keywords: Aspirin; Salicylic acid

1. Introduction

Aspirin (ASA) is an antipyretic and analgesic agent and is the oldest known prescription drug. In the UK, about 25000 people die per year from acute myocardial infarction, other heart disease or stroke, events that are largely due to platelet activation and thrombosis [1]. A regime of long term, low-dose ASA can reduce the occurrence of, and risk of death from cardiovascular events [2,3]. ASA is usually given orally but there are some problems associated with this approach such as gastrointestinal bleeding and peptic ulceration [4,5]. Hence, the skin has been investigated as a route for continuous low-dose ASA administration. ASA itself is polar and can cross the skin without difficulty, but it is rapidly hydrolysed to salicylic acid (SAL) during transport. A 1993 study showed that ASA applied directly to the skin surface selectively inhibited the activity of cyclooxygenase in platelets [6]. ASA in a transdermal patch, at a lower dose, was also found to induce marked suppression of platelet cyclooxygenase, but the bioavailability of ASA was calculated to be only 20% [7]. Since ASA is rapidly hydrolysed to SAL, studies have focused on the synthesis of stable aspirin analogues and derivatives, the idea being that these molecules would traverse the skin efficiently, yielding ASA subcutaneously. The structure and polarity

*Corresponding author.

0378-4347/98/$19.00
PII S0378-4347(97)00580-X

of the derivative are major determinants of transdermal permeability. The compounds investigated in this study were esters or anhydrides which were predicted to release the more polar ASA by their hydrolysis within the skin. While much HPLC methodology has been reported in the literature which can determine both ASA and SAL, most of the samples are in biological fluids (such as plasma and whole blood) [8–10], food [11,12] or tablet formulations [13,14]. Many of these involve protein precipitation and/or solvent extraction. However, this study required analysis of the compounds of interest in a different matrix and direct injection of the samples. It was also necessary to have a method which could be readily adapted to ensure that no transdermal leakage of intact prodrugs occurred. This paper reports the development and implementation of such a HPLC method for the analysis of ASA and SAL in transdermal perfusates.

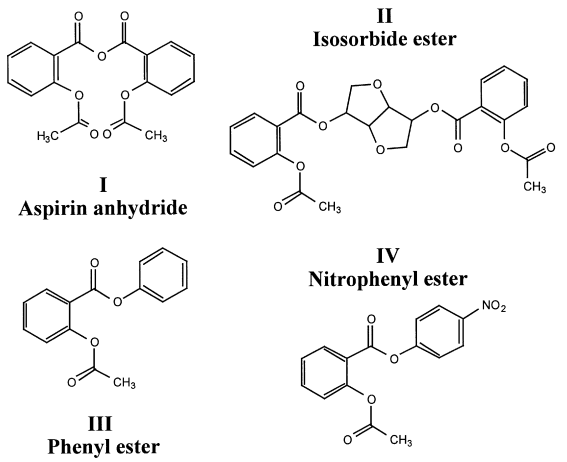

Fig. 1. Structures and names of the four aspirin prodrugs.

2. Experimental

2.1. Equipment

The high-performance liquid chromatograph was equipped with Waters (Millford, MA, USA) Models 501 single-piston and 510 dual-piston pumps, a Waters Model 486 tunable absorbance detector, a Waters Model 680 gradient controller and a Waters Model 746 data module. The Rheodyne injection port (Cotati, CA, USA) was fitted with a 20 μl loop. A Hypersil C_8 guard column (30 μm, 10×4.0 mm) was fitted prior to the Nucleosil C_8 analytical column (5 μm, 250×4.6 mm). The flow-rate of the eluent was 1 ml/min, UV detection was at 225 nm, attenuation was set to 32, chart speed was 0.5 cm/min and all measurements were made at ambient temperature.

2.2. Reagents and chemicals

Aspirin was supplied by Sigma Chemical Co. (Dorset, UK) and salicylic acid by BDH (Poole, UK). The four aspirin derivatives (**I**, **II**, **III** and **IV**) were prepared in-house (see Fig. 1). Acetonitrile and water were both HPLC grade and were purchased from Labscan (Dublin, Ireland). Analytical grade orthophosphoric acid (OPA) and polyethylene glycol (M_r 6000) came from BDH. Phosphate-buffered saline (PBS) sachets were obtained from Sigma Diagnostics (St. Louis, MO, USA). High purity ethanol (>99.8%) was purchased from Merck (Darmstadt, Germany).

2.3. Mobile phase

Eluent A was a mixture of water–acetonitrile–OPA (650:350:2, v/v/v). The aqueous component of eluent A had a pH of 2.0 and the mixture was pH 2.5* when the acetonitrile was added (ASA is most stable at this pH value) [15]. Eluent B was a mixture of acetonitrile–OPA (1000:2, v/v). Both eluents were filtered (0.45 μm filter) under vacuum and sonicated for 20 to 30 min to remove dissolved gases. The solvent program used was: time 0 to 6.5 min – 100% A, time 10.5 to 19.0 min – 70% A/30% B, time 22.0 to 28.0 min – 100% A, employing a linear gradient ramp.

2.4. Standard preparation

Stock solutions of ASA, SAL, **I**, **II**, **III** and **IV** were prepared by dissolving each of the compounds in acetonitrile to a concentration of 1 mg/ml. Working standards were prepared on a daily basis by dilution of the stock solutions with PBS. The ASA

and SAL standards were mixed together for the validation procedure and had final concentrations of 0.2, 0.5, 1.0, 2.0 and 5.0 μg/ml. The standard solutions of the derivatives were prepared individually in PBS down to a concentration of 2 μg/ml as required.

2.5. Sample preparation

The in vitro transdermal experiments were performed on fresh sha/sha mouse skins directly in contact with a 22 ml Franz cell reservoir of PBS at physiological pH and 37°C. Twenty mg of the prodrug being examined was diluted in 250 μl ethanol, mixed with 250 μl of 15% poly(ethylene glycol) (PEG) and topically applied to the 5 cm^2 area of skin. Aliquots were taken from the buffer vessel at 0, 2, 4 and 6 h, injected directly onto the HPLC system and analysed by comparison with external standards of the same matrix composition. Blank skin perfusates containing only formulation components, i.e. ethanol and PEG were run in parallel with the drug perfusates at all times to eliminate any formulation effects. At the end of the transdermal experiment for each prodrug, a sample from the top of the skin was taken by dissolving the remaining drug in 1 ml of acetonitrile. A 1 in 1000 dilution with PBS was usually required for these samples prior to analysis.

3. Results and discussion

3.1. Optimisation of protocol

The derivatives shown in Fig. 1 were selected for this study because they were found to inhibit platelet cyclooxygenase in vitro. Because of their hydrophobicity, special formulations have to be used in order to apply them to the skin. A number of possible formulations were tried and tested before deciding on 250 μl ethanol, mixed with 250 μl 15% PEG. When this was used for dissolution of the compounds, there was no interference from the formulation on the HPLC.

3.2. Validation of the method

3.2.1. Calibration

Calibration was based on regression analysis of concentration versus peak areas (which were found to correlate better than peak heights with concentration). Calibration curves were linear in the concentration range necessary for the detection of the compounds in the PBS buffer, i.e. 0.2 to 5.0 μg/ml. Since the release of ASA and/or SAL was the most important criterion and since none of the parent drugs permeated the skin intact, full validation was carried out only on the analysis of ASA and SAL in the PBS.

3.2.2. Precision

Precision was defined in terms of the inter-day variability (reproducibility) and intra-day variability (repeatability). Inter-assay reproducibility was assessed in four replicate runs on four consecutive days, covering the concentration range 0.2 to 5.0 μg/ml. Intra-assay repeatability was determined in quadruplicate on day two of the study in the same concentration range. The precision of the method was described by the mean relative standard deviation (R.S.D.) of the recovered amounts, determined by interpolation of the peak areas on the regression lines. Precision data are presented in Table 1, and

Table 1
Precision and accuracy data[a]

Amount added (μg/ml)	Amount found (μg/ml)		R.S.D. (%)		Accuracy (%)	
	ASA	SAL	ASA	SAL	ASA	SAL
Inter-assay (reproducibility)						
0.200	0.194	0.212	3.46	2.60	3.00	6.00
0.500	0.499	0.503	2.20	1.24	0.20	0.60
1.000	1.004	0.994	1.18	0.89	0.40	0.60
2.000	2.008	1.979	0.62	1.67	0.40	1.05
5.000	4.997	5.006	0.11	0.12	0.06	0.12
Intra-assay (repeatability)						
0.200	0.199	0.215	1.67	2.18	0.50	7.50
0.500	0.498	0.498	0.99	3.28	0.40	0.40
1.000	1.004	0.990	2.24	2.14	0.40	1.00
2.000	2.002	1.993	1.72	2.26	0.10	0.35
5.000	4.999	5.005	0.83	2.71	0.02	0.10

[a] $n=4$.

they demonstrate that the reproducibility (mean R.S.D.=1.51 and 1.30% for ASA and SAL, respectively) and repeatability (mean R.S.D.=1.49 and 2.51% for ASA and SAL, respectively) are well within accepted values for bioanalyses.

3.2.3. Linearity and accuracy

The correlation coefficient of the regression line for the mean intra-assay values was 0.9999 for both ASA and SAL. Accuracy (presented in Table 1), defined as the percentage difference between the nominal amount and the amount found, gave mean inter-day values of 0.81 and 1.67% for ASA and SAL, respectively, and mean intra-day values of 0.28 and 1.87% for ASA and SAL, respectively.

3.2.4. Limit of detection

The limit of detection was found to be 0.05 μg/ml for both compounds which corresponded to a peak which was three times the standard deviation of the baseline noise.

3.3. Quantitative analyses of ASA and SAL in perfusate samples

The retention times for ASA and SAL were 6.7±0.1 and 9.0±0.3 min, respectively. No ASA was found in the perfusates for prodrugs **II**, **III** or **IV**, but SAL was found in these samples. The presence of SAL in the perfusates would appear to indicate that the prodrugs did in fact break down in the skin, and that the rate of release of ASA was less than the rate of its subsequent hydrolysis to SAL. The poor permeation of the skin was probably due to the preferential solubility of the prodrugs in the formulation as opposed to the skin. Prodrug **I** (an anhydride) was significantly more susceptible to hydrolysis than the ester prodrugs, yielding ASA (and hence SAL) in the perfusate samples. Fig. 2

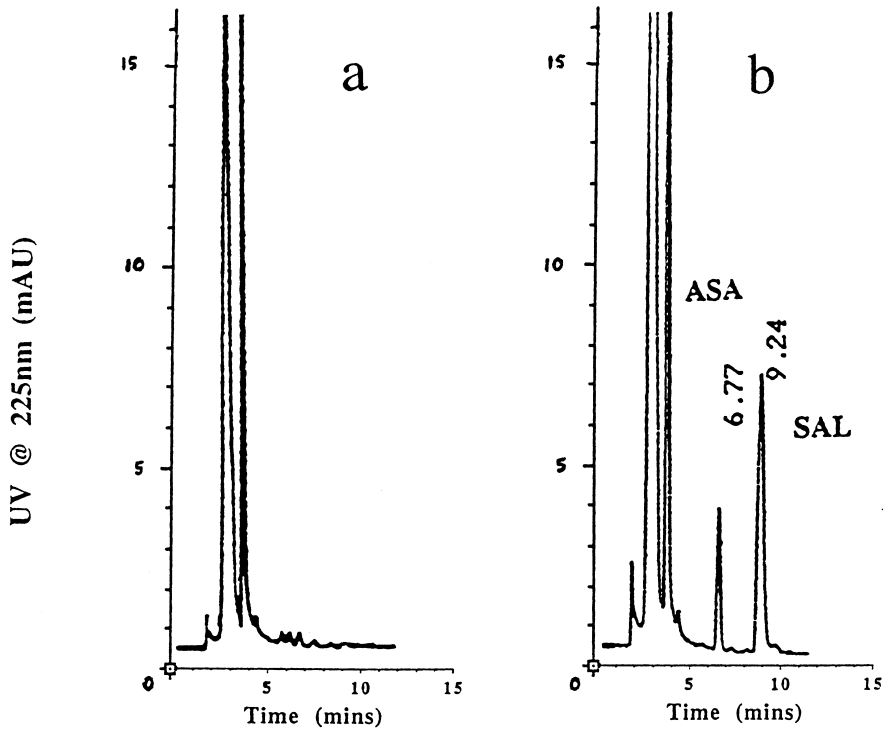

Fig. 2. Chromatograms of perfusate samples.

shows a chromatogram of (a) a blank perfusate and (b) a perfusate sample taken two hours after application of prodrug **I**. Fig. 3a,b show the transdermal results for both ASA and SAL in terms of their flux, i.e. micrograms of compound that traversed the skin per square centimetre of skin onto which the prodrug was applied. It can be seen that prodrug **I** delivered double the flux of ASA (Fig. 3a) and less SAL (Fig. 3b) when compared to the case where ASA was applied to the skin alone. The reason that prodrug **I** breaks down more readily than the other prodrugs could be explained by the fact that anhydride structures undergo hydrolysis more readily than ester structures.

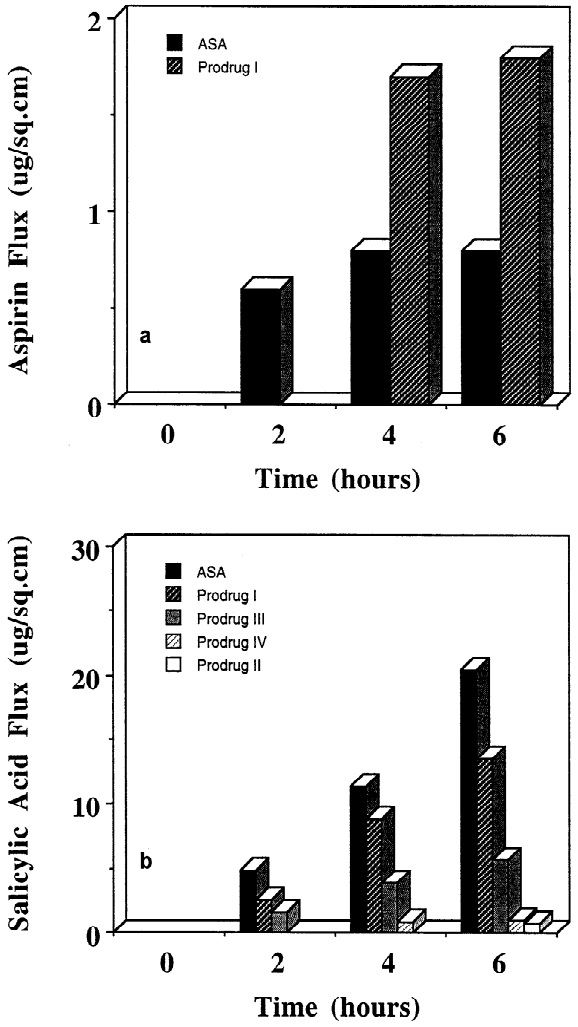

Fig. 3. (a) Transdermal fluxes ($\mu g/cm^2$) of ASA due to prodrug **I** and ASA itself. (b) Transdermal fluxes ($\mu g/cm^2$) of SAL due to prodrugs and ASA itself.

3.4. Qualitative analyses of prodrug transport and breakdown on skin surface

The retention times for the prodrugs using the gradient were as follows: prodrug **I** 18.4±0.2 min, prodrug **II** 19.1±0.3 min, prodrug **III** 20.2±0.3 min and prodrug **IV** 20.6±0.3 min. At no time did any of the prodrugs leak across the mouse skins intact. This was confirmed by gradient HPLC analysis of the perfusates by comparing the samples to external standards of the prodrugs down to a concentration of 2 μg/ml. This concentration would correspond to a transmembrane leakage of less than 0.25% of the prodrug. The top-of-skin samples for prodrugs **II**, **III** and **IV** contained only the corresponding parent compound. This indicated that these derivatives were not hydrolysed on the surface of the skin. Only prodrug **I** was hydrolysed on the surface of the skin to yield ASA.

4. Conclusion

The HPLC method presented was optimised and validated for the determination of ASA and SAL produced by prodrugs in transdermal samples. Samples could be directly injected and the analysis time was only ten minutes for determination of ASA and SAL. The method was easily adapted to allow determination of the nonpolar parent compounds using gradient elution. Using the method, four prodrugs were compared in terms of their ability to deliver ASA percutaneously. Evidence of hydrolysis of the ester compounds to ASA was seen, but it was not at a level sufficient to warrant further investigation of these compounds as aspirin prodrugs. The anhydride, prodrug **I**, did release significant amounts of ASA over time. In comparison to ASA alone, this compound produced more ASA and less SAL over the six hour time frame. Prodrug **I** is considered a suitable candidate for further investigation, and is currently undergoing more transdermal studies.

Acknowledgements

The study was supported by a grant from the Royal College of Surgeons, Dublin, Ireland and the Gundersen Foundation, LaCrosse, WI, USA.

References

[1] Anonymous, Drug Ther. Bull., 32 (1994) 1–3.
[2] The RISC Group, Lancet, 336 (1990) 827–830.
[3] Antiplatelet Trialists' Collaboration, Br. Med. J., 308 (1994) 81–106.
[4] M.C. Allison, A.G. Howatson, C.J. Torrance, F.D. Lee, R.I. Russell, N. Engl. J. Med. 327 (1992) 749–754.
[5] R. Peto, R. Gray, R. Collins, K. Wheatley, C.H. Hennekens, K. Jamrozik, C. Warlow, B. Hafner, E. Thompson, S. Norton, Br. Med. J. Clin. Res. Ed. 296 (1988) 313–316.
[6] R.M. Keimowitz, G. Pulvermacher, G. Mayo, D.J. Fitzgerald, Circulation 88 (1993) 556–561.
[7] B. McAdam, R.M. Keimowitz, M. Maher, D.J. Fitzgerald, J. Pharm. Exper. Ther. 277 (1996) 559–564.
[8] Z. Krivosikova, V. Spustova, R. Dzurik, Meth. Find. Exp. Clin. Pharmacol. 18 (1996) 527–532.
[9] F. Kees, D. Jehnich, H. Grobecker, J. Chromatogr. B 677 (1996) 172–177.
[10] F. Tagliaro, L. Cuzzolin, A. Adami, D. Scarcella, F. Crivellente, G. Benoni, Life Sci. 60 (1997) 101–106.
[11] K.P.L. Janssen, M.B. Katan, W.A. van Staveren, P.C. Hollman, D.P. Venema, Cancer Lett. 114 (1997) 163–164.
[12] D.P. Venema, P.C. Hollman, K.P.L. Janssen, M.B. Katan, J. Agric. Food Chem. 44 (1996) 1762–1767.
[13] S. Torrado, S. Torrado, R. Cadorniga, J. Pharm. Biomed. Anal. 12 (1994) 383–387.
[14] E.R. Montgomery, S. Taylor, J. Segretario, E. Engler, D. Sebastian, J. Pharm. Biomed. Anal. 15 (1996) 73–82.
[15] L.J. Edwards, Trans. Faraday Soc. 46 (1950) 723–735.